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Adam R. Karpf *Editor*

Epigenetic Alterations in Oncogenesis

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Preface

Epigenetics refers to heritable changes in gene expression or genome function encoded by marks other than DNA base sequence; information literally "above" the level of genetics. Epigenetic marks include cytosine methylation and cytosine hydroxymethylation, histone tail modifications, histone variants, and nucleosome positional information, all of which are resident along the DNA duplex. Epigenetic marks frequently show interdependent relationships, for example, the close association of DNA methylation states with particular histone tail modifications and histone variants. From the standpoint of cell physiology, epigenetics provides a mechanism for cells to integrate environmental or intrinsic stimuli into heritable changes in genome function. From the standpoint of development, epigenetics provides a platform for cell differentiation and cell specialization, which in principle cannot simply be the consequence of DNA sequence. Most relevant to this book is the fact that changes in epigenetic states are now recognized to play a fundamental role in cancer development and progression. Cancer, almost uniquely among common human diseases, is characterized by natural selection for cellular variants with improved fitness, e.g., proliferative capacity and rate, evasion of cell death, invasive growth, migration to and proliferation at secondary sites, chemotherapy resistance, and a myriad of other naturally or artificially selected phenotypes. Epigenetic changes play a key role in this phenotypic selection, possibly to an equal to or even greater extent than do genetic mutations.

As a field, cancer epigenetics has now reached young adulthood. The observations that started the field were of DNA hypomethylation changes in cancer in the 1980s, followed by the discovery of DNA hypermethylation in cancer in the 1990s. In the last decade, additional alterations at other levels of epigenetic control (e.g., histone modifications) have also been discovered and characterized in cancer. Also, over the past few years rapid progress has been made in translating the findings of epigenetic alterations into new cancer biomarkers and therapeutic targets. One clear highlight in the field has been the FDA-approval of DNA methyltransferase (DNMT) inhibitors and histone deacetylase (HDAC) inhibitors to treat a select number of human malignancies.

The early work in cancer epigenetics was largely hypothesis or "candidate-gene" driven. More recent work using unbiased and global approaches (i.e., epigenomics)

have validated and greatly extended the early observations. Evidence now suggests that DNA hypomethylation is linked to oncogenic gene activation and genomic instability, and that DNA hypermethylation leads to tumor suppressor gene inactivation, including inactivation of DNA repair genes that also may promote genomic instability. Thus, epigenetic mutations (epimutations) appear to promote genetic mutations and genomic rearrangements in cancer. Intriguingly, a number of recent findings largely from cancer genome sequencing data suggest that genes involved in epigenetic control processes are commonly mutated in a variety of cancers, thus demonstrating that genetic changes can also promote epigenetic alterations in cancer. Taken together, the data now indicate that the roles of genetics and epigenetics in cancer development are highly intertwined.

Epigenetic Alterations in Oncogenesis comprises 15 chapters contributed by leading active researchers in the field. The book is divided into three sections that run the gamut from a description of the basic epigenetic mechanisms that regulate gene expression in human cancer, to how alterations in epigenetic marks contribute to cancer biology, and concluding with an account of the uses for epigenetic-targeted drugs to treat human cancer, as well as the analysis methods to decipher cancer epigenomes.

Part I, *Epigenetic Marks and Mechanisms*, provides an introduction to the major epigenetic marks and how these are altered during oncogenesis. The part begins with a discussion by Jin and Robertson in Chap. 1 on cytosine DNMTs and DNA hypermethylation in cancer, and focuses particularly on the silencing of genes involved in DNA repair, which are a frequent target of hypermethylation. In addition, the authors summarize important recent work showing that DNMTs themselves participate in DNA repair processes. In Chap. 2, Ehrlich and Lacey turn attention to the flip side of the coin, DNA hypomethylation, which was the original epigenetic alteration observed in cancer. The authors discuss the diverse genomic contexts in which DNA hypomethylation can occur and present possible mechanisms to explain DNA hypomethylation in cancer. An exciting recent development in epigenetics is the discovery of 5-hydroxymethylcytosine (5-hmC) as a novel epigenetic mark, which itself appears to be linked to DNA hypomethylation. The biological significance of 5-hmC as well as the enzymes that catalyze its formation (ten–eleven translocation or TET proteins, which can be mutated in cancer) is discussed by Kinney and Pradhan in Chap. 3. In Chap. 4, attention turns to altered histone modifications in cancer with a detailed discussion by Campbell and Turner on how posttranslational histone modifications are controlled under normal circumstances and the mechanisms driving their alteration in malignancy. A critical concept in epigenetics is that DNA methylation and histone modifications ultimately impact gene expression and genome function via their effects on nucleosomes; the important topic of altered nucleosome occupancy in cancer is covered by Andreu-Vieyra and Liang in Chap. 5.

Part III, *The Impact of Epigenetic Alterations on Cancer Biology*, discusses how epigenetic changes contribute to critically important cancer phenotypes. The section begins in Chap. 6, where Fabbri and colleagues discuss miRNA expression alterations in cancer caused by epigenetic changes, including DNA methylation, histone modifications, and Polycomb proteins. The importance of this concept is illustrated by the inherent capacity of altered miRNA expression to derange entire

transcriptional programs in cancer cells. A large family of genes known as cancer-testis or cancer-germ line genes encodes antigens that are a major target of cancer vaccines. Additionally, a number of these genes have emerging oncogenic functions. In Chap. 7, De Smet and Loriot discuss how epigenetic mechanisms, most prominently DNA hypomethylation, lead to the activation of these genes in many human malignancies. Andersen and Jones follow this with a discussion in Chap. 8 of how DNA methylation controls cell fate in the intestine and how, when the tumor suppressor gene adenomatous polyposis coli (APC) is lost, this promotes DNA hypomethylation and intestinal tumorigenesis. In Chap. 9, Futscher describes how tractable cell model systems are being used to discern the temporal epigenetic alterations that are linked to cell immortalization and transformation. It is now recognized that epigenetic regulation lies at the heart of stem cell maintenance and differentiation. In Chap. 10, Huang and colleagues discuss epigenetic regulation of mesenchymal stem cells (MSC) during tumorigenesis, and highlight recent work showing that targeted DNA methylation of tumor suppressor genes provides a model system to study MSC-driven tumorigenesis.

Part III, *Clinical Implications and Analysis Methods*, provides an overview of important topics related to the utility of epigenetic alterations as cancer biomarkers and therapeutic targets, and provides a detailed overview of the methods used to decipher cancer epigenomes. In the past few years, a major link between environmental toxicants, epigenetic changes, and cancer has become apparent. In Chap. 11, Pogribny and Rusyn discuss these developments as they pertain to chemical carcinogens such as arsenic, as well as other pharmaceutical and biological agents. While epigenetic alterations in cancer cells and tumor tissues is well established, emerging data suggest that systemic epigenetic changes (i.e., those affecting normal tissues) can also occur in cancer patients, as well as in individuals with elevated risk for cancer. Marsit and Christensen highlight the current research in this exciting and potentially high impact area in Chap. 12. Epigenetic therapies have entered the clinic and received their first widespread use in the context of myeloid malignancies, particularly myelodysplastic syndrome (MDS) and acute myelogenous leukemia (AML). In Chap. 13, Griffiths and Gore discuss the clinical work in this arena, with a focus on the FDA-approved azanucleosides 5-azacytidine (vidaza) and decitabine (dacogen), but also touching on HDAC inhibitors. In Chap. 14, Balch and Nephew discuss how epigenetic therapies may be particularly well suited for chemotherapy sensitization to overcome drug resistance, and review the extensive preclinical work and rapidly accumulating clinical knowledge in this area. Finally, in Chap. 15, Costello and colleagues review the approaches used for the analysis of cancer epigenomes. In particular, they discuss the methods appropriate for the analysis of cytosine methylation and hydroxymethylation, discuss next-generation sequencing approaches, and touch on the computational methods now being used to explore cancer epigenomes.

Omaha, Nebraska, USA Adam R. Karpf

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Part I
Epigenetic Marks and Mechanisms

Chapter 1
DNA Methyltransferases, DNA Damage Repair, and Cancer

Bilian Jin and Keith D. Robertson

Abstract The maintenance DNA methyltransferase (DNMT) 1 and the de novo methyltransferases DNMT3A and DNMT3B are all essential for mammalian development. DNA methylation, catalyzed by the DNMTs, plays an important role in maintaining genome stability. Aberrant expression of DNMTs and disruption of DNA methylation patterns are closely associated with many forms of cancer, although the exact mechanisms underlying this link remain elusive. DNA damage repair systems have evolved to act as a genome-wide surveillance mechanism to maintain chromosome integrity by recognizing and repairing both exogenous and endogenous DNA insults. Impairment of these systems gives rise to mutations and directly contributes to tumorigenesis. Evidence is mounting for a direct link between DNMTs, DNA methylation, and DNA damage repair systems, which provide new insight into the development of cancer. Like tumor suppressor genes, an array of DNA repair genes frequently sustain promoter hypermethylation in a variety of tumors. In addition, DNMT1, but not the DNMT3s, appear to function coordinately with DNA damage repair pathways to protect cells from sustaining mutagenic events, which is very likely through a DNA methylation-independent mechanism. This chapter is focused on reviewing the links between DNA methylation and the DNA damage response.

1.1 Introduction

DNA methyltransferases (DNMTs), responsible for the transfer of a methyl group from the universal methyl donor, *S*-adenosyl-L-methionine (SAM), to the 5-position of cytosine residues in DNA, are essential for mammalian development [1].

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There are four members of the DNMT family, including DNMT1, DNMT3A, DNMT3B, and DNMT3L. DNMT3L, unlike the other DNMTs, does not possess any inherent enzymatic activity [2]. The other three family members are active on DNA. *DNMT1* encodes the maintenance methyltransferase and *DNMT3A/DNMT3B* encode the de novo methyltransferases [3, 4], required to establish and maintain genomic methylation. While this maintenance vs. de novo division has been convenient, there is clear evidence for functional overlap between the maintenance and the de novo methyltransferases [5, 6]. Gene knockout analysis in mice has shown that *Dnmt1* and *Dnmt3a/Dnmt3b* genes are all essential for viability. *Dnmt1* inactivation leads to very early lethality at embryonic day (E) 9.5, shortly after gastrulation [7–9], whereas *Dnmt3b* knockout induces embryo death at E14.5–18.5, due to multiple developmental defects including growth impairment and rostral neural tube defects [3, 8, 9]. *Dnmt3a*$^{-/-}$ mice become runted and die at about 4 weeks of age, although they appear to be relatively normal at birth [3].

DNMTs play an important role in genomic integrity, disruption of which may result in chromosome instability and tumor progression. It is well established that DNMTs are required for transcriptional silencing of a number of sequence classes, including imprinted genes, genes on the inactive X chromosome and transposable elements [1, 10], and silencing of these sequences is essential for maintaining chromosome stability. Much compelling evidence has come from targeted deletion experiments showing that all three DNMTs are involved in stabilization of the genome, particularly repetitive sequences [3, 11, 12]. For example, either single knockout of *Dnmt1* or double knockout of *Dnmt3a* and *Dnmt3b* enhances telomere recombination [11]. DNMT3B is specifically required for stabilization of pericentromeric satellite repeats. DNMT3B deficiency results in expansion and rearrangements of pericentromeric repeats [3, 12]. Immunodeficiency, centromere instability, and facial anomalies (ICF) syndrome is the only human genetic disorder known to involve biallelic mutations in *DNMT3B*. It is characterized by chromosomal instability arising due to destabilization of pericentromeric repeats, particularly those at juxtacentromeric regions of chromosomes 1, 9, and 16 [3, 12]. Of note, cells null for *DNMT1* or with hypomorphic mutations in *DNMT1* that partially reduce its levels to 30% of WT DNMT1 display significantly greater microsatellite instability (MSI) [13–17], a greater frequency of chromosomal translocations [18] and much higher sensitivity to genotoxic agents [17], which may promote the development of cancer.

The DNA damage response (DDR) is a genome-wide surveillance system that protects cells from potentially mutagenic DNA insults derived from either endogenous or exogenous sources. The DDR usually functions through the coordinated actions of DNA repair and checkpoint systems to promote DNA damage repair before replication or to activate cell death pathways if excessive damage exists [19]. Like the cellular DNA methylation machinery, an intact DDR is crucial for preventing cancer. Evidence is mounting to support a link between the DNA methylation and DNA damage repair systems, as first suggested by promoter hypermethylation and silencing of DNA repair genes in multiple types of cancer [20]. More importantly, DNMT1 may be directly involved in DNA damage repair in a DNA methylation-independent manner [14, 17, 21–23]. Strong support for this latter notion

comes from recent observations that DNMT1 is rapidly and transiently recruited to regions of DNA double-strand breaks (DSBs) via its interaction with proliferating cell nuclear antigen (PCNA) [21, 24], as well the PCNA-like DNA damage sliding clamp component RAD9 (of the 9-1-1 complex) [21]. In this chapter, we examine and outline the links between DNMTs and DNA repair systems and discuss the possible mechanisms of how they are orchestrated, with a focus on cancer.

1.2 Epigenetic Silencing of DNA Repair Pathways Through Aberrant Promoter Hypermethylation

DNA repair systems have evolved to maintain genomic integrity by countering threats posed by DNA lesions [19]. Deficiency in the DNA repair pathways may leave these lesions unrepaired or cause them to be repaired incorrectly, eventually leading to genome instability or mutations that contribute directly to a large array of human diseases including cancer. Carcinogenesis is believed to originate from and be driven by the acquisition of abnormal genetic and/or epigenetic changes. Aberrant DNA hypermethylation, when it occurs at promoter CpG islands (CGIs), leads to potent and heritable transcriptional silencing that inactivates key cellular pathways much like genetic changes (e.g., mutation/deletion) do. In addition to genetic mutations, promoter hypermethylation in DNA repair genes is closely linked to a variety of human tumor types including colorectal, breast, lung cancers, and glioma [20] (Table 1.1), suggesting that epigenetic silencing of DNA repair pathways is an important contributor to the development of cancer.

1.2.1 Epigenetic Inactivation of the DNA Mismatch Repair Pathway

Mismatch repair (MMR) is a genome surveillance system to maintain genomic integrity through recognizing and correcting mismatched nucleotides arising during DNA replication, homologous recombination (HR), or other forms of DNA damage. Impairment of this system gives rise to MSI [25, 26], which has now been recognized as a hallmark of MMR gene-deficient cancers. Microsatellite loci, widely dispersed in the genome, are repetitive sequences consisting of short runs of nucleotides, typically one to four bases in length. Repetitive regions may give rise to the formation of secondary structures, which are subject to expansion or contraction. The secondary structures, if incorrectly resolved, lead to slippage of DNA polymerases along repetitive sequences during replication. Microsatellites are particularly susceptible to length change mutations during replication and transcription, resulting in frameshift mutations if they are located within a gene [25, 26]. MMR deals with these changes to maintain microsatellite stability. MMR comprises

Table 1.1 Genes in DNA damage repair pathways that are hypermethylated in cancer

Repair pathway	Methylated gene	Cancer type	Samples studied	Samples methylated	Methylation frequency (%)	References
MMR	MLH1	Sporadic CRC (MSI+)	110	67	61	[41–44]
		Sporadic CRC (MSI−)	128	38	30	[42, 43]
		Sporadic early-onset CRC	110	55	50	[45]
		NSCLC	77	43	56	[32]
		Acute myeloid leukemia	177	11	6	[34–36]
		Ovarian cancer	672	72	11	[33]
		Oral squamous cell carcinoma	99	8	8	[29]
		HNPCC	179	2	1	[39, 40]
		Gastric cancer	306	58	19	[30, 31]
		HNSCC	49	14	29	[37]
	MSH2	NSCLC	14	4	29	[32]
		Gastric cancer	200	27	14	[30]
		Ovarian cancer	56	29	52	[46]
		Sporadic CRC	36	1	3	[47]
		HNPCC	46	11	24	[48]
	MSH3	Gastric cancer	200	25	13	[30]
	MSH6	Breast cancer	33		92–95[a]/20©	[50]
BER	TDG	Multiple myeloma	KAS-6/1 cell line			[52]
	MBD4	CRC	39		24[a]/14©	[53]
	OGG1	Thyroid cancer	38	2	5	[54]
NER	XPC	Bladder cancer	37	12	32	[56]
	ERCC1	Glioma	32	Unknown		[57]
	XRCC1	Gastric cancer	25	Unknown		[60]
	RAD23B	Multiple myeloma	KAS-6/1 cell line			[61]

HR	BRCA1	NSCLC	98	29	30	[69]
		Sporadic ovarian cancer	81	12	15	[66]
		Sporadic breast cancer	190	24	13	[64, 70]
		Hereditary breast cancer	162	18	11	[70]
		Early onset gastric cancer	104	0.6	1	[67]
		Bladder cancer	96	0.71	1	[68]
	BRCA2	Breast cancer	33		59–64[a]/10©	[50]
		NSCLC	98	41	42	[69]
	FANCC	Acute lymphoblastic leukemia	97	3	3	[74]
		Acute myeloid leukemia	143	1	1	[74]
	FANCF	HNSCC	89	13	15	[75]
		NSCLC	158	22	14	[75]
		Cervical cancer	91	27	30	[76]
		Ovarian cancer	53	7	13	[77]
	FANCL	Acute lymphoblastic leukemia	97	1	1	[74]
NHEJ	XRCC5	NSCLC	98	19	19	[69]
ATM/ATR	ATM	HNSCC	100	25	25	[101]
		Breast cancer	23	18	78	[100]
		CRC	HCT116 cell line			[99]
	CHK2	NSCLC	139	39	28	[106]
		Glioma	5	5	100	[107]

(continued)

Table 1.1 (continued)

Repair pathway	Methylated gene	Cancer type	Samples studied	Samples methylated	Methylation frequency (%)	References
Others	MGMT	Oral squamous cell carcinoma	99	40	40	[29]
		Gastric cancer	200	50	25	[30]
		CRC	36	14	39	[80]
		HNSCC	21	6	29	[80]
		NSCLC	34	10	29	[80]
		Lymphomas	61	15	25	[80]
		Glioma	140	54	39	[80]
	WRN	Gastric cancer	38	10	26	[91]
		CRC	182	69	38	[91]
		NSCLC	56	21	38	[91]
		Prostate cancer	20	4	20	[91]
		Breast cancer	58	10	17	[91]
		Thyroid cancer	32	4	13	[91]
		Non-Hodgkin lymphoma	118	28	24	[91]
		Acute lymphoblastic leukemia	21	2	10	[91]
		Acute myeloblastic leukemia	36	3	8	[91]
		Chondrosarcomas	15	5	33	[91]
		Osteosarcomas	27	3	11	[91]

CRC colorectal cancer; *NSCLC* non-small cell lung cancer; *HNPCC* hereditary non-polyposis colorectal cancer; *HNSCC* head and neck squamous cell carcinoma

[a]Mean methylation level (%) in cancer vs. © mean methylation level (%) in control

©Indicates references from which methylation data derived from similar samples was pooled for this summary

the MutS complex and the MutL complex. MutS recognizes the mismatched base, while MutL recruits repair enzymes to damage sites via its binding with MutS [27]. There are two main MutS complexes in humans, MutSα and MutSβ. MutSα, consisting of the MutS homologue 2 (MSH2) protein bound to MSH6, recognizes single-base mismatches or small insertion/deletion loops (indels), whereas MutSβ, consisting of MSH2 and MSH3, repairs only indels [28]. The main complex for MutL in humans is MutLα, consisting of a heterodimer of MLH1 and PMS2 [26]. Mutations in or epigenetic silencing of MMR genes like *MLH1* and *MSH2* is closely associated with a variety of human cancers such as hereditary non-polyposis colon cancer (HNPCC), sporadic colon cancer, and ovarian cancer [29].

MLH1 plays a central role in coordinating various steps in MMR via interacting with other MMR proteins and modulating their activities. Hypermethylation of the *MLH1* promoter is observed in a variety of cancers including oral squamous cell carcinoma [30], gastric cancer [31, 32], non-small cell lung cancer (NSCLC) [33], ovarian cancer [34], acute myeloid leukemia [35–37], head and neck squamous cell carcinoma (HNSCC) [38], HNPCC [39–41], and particularly in colorectal cancer (CRC) [42–45] (Table 1.1). The reduced MLH1 protein expression is correlated with high-level methylation detected in human CRC samples, whereas samples with low-level methylation display expression levels similar to those observed in methylation-negative samples [46], strongly suggesting that the *MLH1* gene is inactivated via promoter hypermethylation in a dose-dependent manner. Nonetheless, it is not clear whether a moderate degree of methylation affects MLH1 gene expression or not. On the basis of observations made in germ line cells, it has long been believed that *MLH1* promoter methylation involves only one allele of maternal origin. However, more recent findings demonstrate that there is biallelic involvement of *MLH1* promoter hypermethylation in many cancers [46]. The causal link between MSI and epigenetic inactivation of *MLH1* is further highlighted by the observation that 90% of MSI+ HNPCC have *MLH1* hypermethylation, while 95% of MSI samples do not [20].

MSH2 is also hypermethylated in multiple tumor types, including gastric cancer [31], NSCLC [33], ovarian cancer [47], sporadic CRC [48], and HNPCC [49] (Table 1.1). Interestingly, promoter methylation of *MSH2* in HNPCC occurs primarily in patients with germ line mutations in *MSH2* rather than in germ line mutation-negative cases [49]. Seventy percent of patients with *MSH2* methylation also present germ line mutations in this gene, clearly indicating that methylation is the second inactivating hit in these tumors [49]. DNA hypermethylation can be caused by transcription across a CGI within a promoter region. Recent studies have revealed that deletions of the last exons of the *EpCAM* gene, located immediately upstream of *MSH2*, give rise to somatic hypermethylation of the *MSH2* promoter [50]. Deletions at the most 3′-end of the *EpCAM* gene result in loss of its polyadenylation signal, which abolishes transcription termination. Transcription of *EpCAM* then continues downstream into the *MSH2* promoter and induces promoter hypermethylation of *MSH2*. DNA methylation triggered by transcriptional read-through of a neighboring gene, in either sense or antisense, direction may represent a general mutational mechanism that promotes aberrant epigenetic changes. Like *MLH2*, other MutS

homologues, including *MSH3* and *MSH6*, are also inactivated by hypermethylation in tumors such as breast [51] and gastric cancers [31] (Table 1.1).

1.2.2 Epigenetic Inactivation of the Base Excision Repair and Nucleotide Excision Repair Pathways

The specific pairing of DNA bases in the genome is constantly challenged by endogenous metabolic by-products and environmental insults. Base excision repair (BER) is responsible for the removal of damaged DNA bases and their backbones to prevent mutations that could give rise to cancer [19, 52]. In BER, abnormal DNA bases are recognized and removed by specific glycosylases, followed by recruitment of other enzymes including nuclease, polymerase, and ligase proteins, to complete the repair process via excising the remaining sugar fragments and reinstalling an intact correctly based-paired nucleotide [19].

Either thymine DNA glycosylase (TDG) or methyl-CpG-binding domain 4 (MBD4) mediate a specific BER pathway for the correction of G/T mismatches arising due to 5-methylcytosine deamination leading to C to T transitions. DNA hypermethylation-mediated silencing of *TDG* and *MBD4* may contribute to the frequent genomic instability that occurs in cancer cells [53] (Table 1.1). *TDG* promoter hypermethylation negatively correlates with its expression. TDG down-regulation leads to less efficient DNA repair activity in response to hydrogen peroxide-induced DNA damage. Ectopic expression of TDG, however, functionally compensates for lower repair activities of damaged DNA in the KAS-6/1 myeloma cell line with extensive endogenous *TDG* gene hypermethylation [53]. *MBD4*, like *TDG*, is also subject to promoter hypermethylation and gene silencing in tumors like sporadic CRC and ovarian cancer [54]. Another DNA glycosylase, OGG1, which mediates removal of 8-oxoguanine induced by oxidative damage, is also subject to inactivation via promoter methylation in cancer cells [55] (Table 1.1).

Of all the repair systems, nucleotide excision repair (NER) recognizes the most varied types of DNA lesions, contending with the diverse class of helix-distorting damage that interferes with base pairing and obstructs replication and transcription. In NER, there exist two sub-pathways that differ in the mechanism of lesion recognition: global genome-NER (GG-NER) that surveys the entire genome for distortions, and transcription-coupled repair (TCR), which targets damage that blocks elongating RNA polymerases [19, 56]. NER, therefore, plays a particularly important role in preventing mutations. Thus far, three syndromes, xeroderma pigmentosum, Cockayne syndrome, and trichothiodystrophy (TTD), are closely associated with NER defects [56]. Of these, patients with xeroderma pigmentosum, attributable to mutations in one of the seven xeroderma pigmentosum (XP) group genes (*XPA*–*XPG*), show a dramatically increased incidence of UV light-induced skin cancer [19, 56].

It was reported recently that the *XPC* promoter is epigenetically inactivated in bladder cancer [57] (Table 1.1). *XPC* promoter methylation is significantly elevated

in cancerous bladder compared to normal tissue, leading to reduced mRNA levels in the tumor [57]. Epigenetic defects in the *XPC* gene may also influence malignant behavior and prognosis. ERCC1 is a crucial protein in the NER pathway primarily involved in the repair of platinum-DNA adducts. Aberrant CGI methylation in the *ERCC1* promoter region has been observed in human glioma cell lines and primary tumors, which is associated with cisplatin chemosensitivity [58]. In a rat lung cancer model, however, *ERCC1* methylation is detected in only a very small proportion of samples [59]. Deficiency in XRCC1, a scaffolding protein for BER and single-strand break repair (SSBR), is associated with enhanced risk of lung cancer [60]. *XRCC1* is subject to aberrant promoter methylation in human gastric cancer tissues [61]. In lung cancer, infiltrating carcinomas exhibit statistically higher levels of methylation at the *XRCC1* promoter compared to normal, hyperplastic, and squamous metaplastic tissues [59]. RAD23B, a key component for damage recognition in NER, is also hypermethylated in multiple myeloma [62].

1.2.3 Epigenetic Inactivation of HR and Nonhomologous End-Joining DNA Repair Pathway Components

HR not only provides an important mechanism to repair several types of DNA lesions that pose a threat to genome integrity, including DNA DSBs, DNA damage encountered during DNA replication, and DNA interstrand cross-links (ICLs), but is also required to restart stalled replication forks during the late S and G2 phases of the cell cycle [63, 64]. HR promotes precise repair of DNA damage using the intact sister chromatid as a template. Deficiency of HR leads to more error-prone repair, which is associated with mutagenesis and predisposition to cancer [63].

The *BRCA1* and *BRCA2* genes are both essential for HR-mediated DNA repair. BRCA1 appears to act as a signal integrator that links DNA damage sensors with response mechanisms. BRCA2, however, is more directly involved in homology-directed DSB repair, as it mediates formation of a RAD51-DNA nucleoprotein filament that catalyzes strand invasion during HR. *BRCA1* and *BRCA2* are frequently mutated in hereditary breast and ovarian cancers, but seldom in sporadic cases of these tumor types. Epigenetic inactivation of *BRCA1* via promoter hypermethylation, however, plays an important role in tumorigenesis in a wide array of cancers including breast [65, 66], ovarian [67], gastric [68], bladder [69], and NSCLCs [70], both hereditary [71] and sporadic forms [20, 39] (Table 1.1). It is believed that epigenetic silencing of BRCA1 creates a new mutator pathway that generates mutations and gross chromosomal rearrangements via p53 signaling. This idea is supported by several observations including one demonstrating that p53 inactivation rescues the impact of BRCA1 deficiency on cell survival [20, 72]. Although much less frequently than *BRCA1*, *BRCA2* also acquires promoter region hypermethylation that is closely associated with its reduced expression in breast cancer [51] and NSCLC [70] (Table 1.1).

The primary function of the Fanconi anemia (FA) pathway is to repair interstrand DNA cross-links, which promotes HR via coordinating other DNA damage-responsive events to stabilize stalled replication forks, to convey signals to DNA checkpoint pathways, and to facilitate recovery of replication forks [73]. FA is a genomic instability syndrome characterized by bone marrow failure, developmental abnormalities, and increased cancer incidence, which is caused by mutations in one of thirteen distinct genes (*FANCA*, *FANCB*, *FANCC*, *FANCD1*, *FANCD2*, *FANCE*, *FANCF*, *FANCG*, *FANCI*, *FANCJ*, *FANCL*, *FANCM*, and *FANCN*) [73]. Eight of them (FANCA, B, C, E, F, G, L, and M) form the FA core complex. This group of genes contains a high GC content and CGIs at their promoter regions, making them potential targets for aberrant hypermethylation-mediated silencing [74]. This idea has received support from observations that *FANCC*, *FANCF,* and *FANCL* acquire promoter methylation during human carcinogenesis [39, 75]. Of these, *FANCF* displays hypermethylation the most frequently, occurring in 14–28% of different cancers including NSCLC [76], HNSCC [76], cervical [77], and ovarian [39, 78] (Table 1.1).

Unlike HR, which performs error-free repair, nonhomologous end-joining (NHEJ) simply restores DNA integrity by joining the two DNA ends. This type of repair is error-prone and frequently results in the loss or addition of several nucleotides at the break site. Despite its mutagenic consequences, NHEJ is the major DSB repair pathway in mammalian cells. Defects in NHEJ lead to chromosomal translocations and genomic instability. In NHEJ, DSBs are detected by the KU70/KU80 heterodimer; the KU complex then activates the protein kinase DNA-PKcs (DNA-dependent protein kinase catalytic subunit), leading to recruitment and activation of end-processing enzymes, polymerases, and finally ligation of the breaks by the XRCC4/DNA ligase IV complex. In the NHEJ pathway, only the *XRCC5* gene, encoding the KU80 protein, has been reported to be inactivated via epigenetic mechanisms [70] (Table 1.1). Low expression of XRCC5 in squamous cell carcinoma and NSCLC is significantly associated with promoter region hypermethylation. Treatment of NSCLC cells with the DNA demethylating agent 5-aza-2′-deoxycytidine (5-aza-CdR), however, does not result in increased KU80 expression [70]. Thus, the underlying mechanisms promoting and maintaining XRCC5 silencing await further investigation, particularly in more samples and more types of cancer.

1.2.4 Epigenetic Silencing of O⁶-Methylguanine-DNA Methyltransferase

O^6-methylguanine, which arises due to alkylation reactions, pairs with thymine rather than cytosine, resulting in G:C to A:T mutations during DNA replication. O^6-methylguanine-DNA methyltransferase (MGMT), also known as O^6-alkylguanine-DNA alkyltransferase (AGT), repairs DNA damage by transferring the methyl groups on the O^6 position of guanine to an active site cysteine residue to protect cells from sustaining mutagenic events, which has been demonstrated by gain- or loss-of-function experiments in vitro and in vivo [79]. The MGMT protein is unique among DNA-repair components because it acts alone to remove DNA adducts.

Although MGMT is ubiquitously expressed in normal human tissues, mean enzymatic activity in malignant tissues is usually higher than in their normal counterparts. However, there is a variety of tumors such as glioma, CRC, NSCLC, and HNSCC that lack MGMT expression [20, 39] (Table 1.1). It has been well documented that MGMT deficiency often arises due to abnormal promoter methylation [20, 39, 80]. For example, 29% of NSCLCs and 38% of CRCs display aberrant *MGMT* methylation, in which the presence of hypermethylation is highly associated with loss of MGMT protein [81]. *MGMT* is the most frequently methylated gene in central nervous system tumors. Epigenetic silencing of *MGMT* via promoter hypermethylation occurs in approximately 40% of primary glioblastomas and over 70% of secondary glioblastomas. It is also detected in 50% of the diffuse and anaplastic astrocytomas and approximately two-thirds of oligodendroglial and mixed tumors [82]. These results, together with a causal relationship between DNA methylation of the *MGMT* CGI and decreased transcription of the gene in cell culture-based studies, demonstrate that DNA methylation is an important mechanism for silencing the *MGMT* gene in human cancers.

Epigenetic silencing of *MGMT* may initiate an important mutator signaling cascade in human cancers since MGMT loss causes G:C to A:T transitions, which lead to downstream gene mutations. This proposal is strongly supported by an analysis of point mutations in *KRAS* and *p53*. KRAS, the most commonly altered oncogene in cancer, is an early key player in multiple signal pathways. Loss of MGMT is associated with increased *KRAS* mutations possessing G:C to A:T transitions in colon [83] and gastric cancer [84]. *p53* is the most frequently mutated tumor suppressor gene (TSG) in human cancer, and the majority of known *p53* mutations are G:C to A:T transitions [66, 85]. Epigenetic inactivation of *MGMT* may lead to G:C to A:T transition mutations in *p53*, which has been observed in several types of cancer including colorectal [66], liver [86], lung [87], esophageal squamous cell carcinomas [88], and glioma [89]. Interestingly, *MGMT* promoter methylation is associated with improved disease chemosensitivity and prolonged survival time in patients treated with alkylating agent-based therapies [90]. However, it is unclear whether the improved survival is specifically due to loss of MGMT expression or accompanying drug sensitivity.

1.2.5 Epigenetic Silencing of WRN

Werner syndrome (WS) is a rare autosomal recessive disease, characterized by premature onset of aging, genomic instability, and increased cancer incidence. WS is caused by null mutations at the *WRN* locus at 8p11.2–p12, which codes for a DNA helicase belonging to the RecQ family. Deficiency in WRN function causes defects in DNA replication and recombination, as well as DNA repair.

WRN is a 180-kd nuclear protein that has a unique interaction with its DNA substrates through its C terminal RQC domain during base separation [91]. In addition to two C-terminal ATPase domains encoding for helicase activity, the WRN protein contains an N-terminal domain coding for exonuclease activity. Its helicase

and exonuclease activities function in a coordinated manner, suggestive of roles in DNA repair, recombination, and replication. Recently, the WRN protein was also shown to be involved in telomere maintenance based on the discovery that its deficiency leads to accelerated telomere shortening in WS cells [92]. These multiple roles of the WRN protein highlight its importance in aging and cancer.

The evidence suggesting that WRN acts as a TSG is derived primarily from WS, which is characterized by the early onset development of a variety of cancers due to germ line *WRN* mutation; somatic mutations in the *WRN* gene have not been reported. Epigenetic inactivation of *WRN* provides additional support for its TSG role in sporadic cancer. The *WRN* promoter undergoes hypermethylation in a wide array of tumors including colorectal, gastric, prostate, non-small cell lung, and breast cancers [93, 94] (Table 1.1). Epigenetic silencing of *WRN* via methylation not only leads to the loss of protein and enzyme activity, but also to chromosomal instability. Furthermore, the above phenotype is reversed by DNA-demethylating agents. Most importantly, restoration of WRN expression induces its tumor-suppressor effects, such as inhibition of colony formation and tumor growth [93]. Taken together, aberrant epigenetic silencing of *WRN*, a candidate TSG, may play an important role in human cancers. Interestingly, WRN was recently shown to be associated with promoter methylation of the *OCT4* gene [95], which encodes a crucial transcription factor for the maintenance of cell pluripotency. During differentiation of human pluripotent NCCIT embryonic carcinoma cells, WRN localizes to the *OCT4* promoter region with *de novo* DNA methyltransferase DNMT3B and promotes differentiation-dependent *OCT4* silencing and promoter methylation [95]. Deficiency in WRN blocks DNMT3B recruitment to the promoter and leads to decreased promoter methylation of *OCT4* [95]. Therefore, WRN may also contribute to the control of stem cell differentiation via epigenetic silencing of the key pluripotency transcription factor OCT4.

1.2.6 Epigenetic Inactivation of ATM/ATR Signaling

DNA damage signaling requires the coordinated action of a large array of molecules that can be categorized as DNA damage sensors, transducers, mediators, and effectors according to their functions. Upon damage of DNA, the MRE11–RAD50–NBS1 (MRN) sensor complex recognizes DSBs and the replication protein A (RPA) complex processes accumulated single-stranded DNA (ssDNA). The transducer ataxia-telangiectasia mutated (ATM) and ATR kinases are recruited to and activated by DSBs and RPA-coated ssDNA, respectively. With the help of mediators (including 53BP1, MDC1, BRCA1, MCPH1, and PTIP in ATM signaling, and TopBP1 and Claspin in ATR signaling), ATM and ATR activate the effector kinases CHK2 and CHK1, respectively, which then spread the signal throughout the nucleus [96–98]. CHK1 and CHK2 decrease cyclin-dependent kinase (CDK) activity, which slows down or arrests cell cycle progression. Meanwhile, ATM/ATR

signaling promotes DNA repair through various mechanisms. Through ATM/ATR signaling, DNA repair and cell cycle progression are closely coordinated. The coordinated action of DNA repair and cell cycle controls either promotes the resumption of normal cell functioning before replication or triggers apoptosis/cell death when normal cell functioning cannot be restored; both mechanisms act as barriers to tumorigenesis [19].

Ataxia-telangiectasia (AT) is a rare autosomal recessive disorder, characterized by progressive cerebellar ataxia, oculocutaneous telangiectasia, susceptibility to bronchopulmonary disease, and lymphoid tumors. AT is caused by deficiency in the *ATM* gene, localized on chromosome 11q22–23. ATM is an Ser/Thr protein kinase of the phosphoinositide 3-kinase (PI3K)-related protein kinase (PIKK) family, which also includes ATR, DNA-PKcs, and SMG1. ATM may have as many as 700 substrates [99, 100], highlighting its multiple functions in various biological processes including cancer. Loss of heterozygosity in *ATM* results in reduced protein expression; however, this mechanism explains only a small proportion of cancers where ATM down-regulation is observed. In sporadic cancer, which accounts for 90–95% of tumors, the probability of *ATM* gene mutations is low, whereas altered expression of ATM is frequently observed. It is therefore likely that epigenetic modifications have an impact on ATM expression in these cases (Table 1.1). Initial proof for this idea came from studies using the human colon cancer cell line HCT116 [101]. In this cell line, *ATM* displays aberrant promoter methylation, which inversely correlates with its low expression and low radiosensitivity. The significance of this finding is underscored by further observations that treatment of HCT116 cells with 5-azacytidine (a DNA demethylating agent) restores expression of ATM and radiosensitivity [101]. *ATM* is also epigenetically silenced in primary cancers. For example, 78% of surgically removed breast tumors [102] and 25% of HNSCC [103] display aberrant methylation in the *ATM* promoter region accompanied by reduced ATM.

CHK2, the mammalian homologue of the yeast Rad53 and Cds1, is located at chromosome 22q12.1, spans approximately 50 kb, and consists of 14 exons [104]. CHK2, activated by ATM, responds primarily to DSBs. Its fundamental role is to coordinate cell cycle progression with DNA repair and cell survival or death. Germ line mutations in the *CHK2* gene predispose to Li–Fraumeni syndrome (LFS), characterized by multiple tumors at early age with a predominance of breast cancer and sarcomas [105]. Somatic mutations in *CHK2* exist also, although they occur in only a small subset of sporadic human malignancies, including carcinomas of the breast, lung, colon, and ovary, osteosarcomas, and lymphomas [106]. The finding of both germ line and somatic mutations suggests that *CHK2* acts as a TSG. This is further supported by the observation that down-regulation of CHK2 is associated with promoter methylation in sporadic cancers including lung cancer, glioma, and Hodgkin's lymphoma [107–109]. For example, DNA hypermethylation of the distal *CHK2* CGIs occurs in 28.1% of NSCLCs and 40.0% of squamous cell carcinomas, which inversely correlates with *CHK2* mRNA levels. It should be noted, however, that observations in breast, colon, and

ovarian cancers do not support a causative link between DNA methylation and gene expression of CHK2 [110, 111].

1.3 DNA Methyltransferase 1 and Mismatch Repair

The function of the MMR pathway is to correct base substitution mismatches and insertion–deletion mismatches generated in newly replicated DNA [112]. Deficiencies in or inactivation of this pathway has profound biological consequences. Loss of MMR activity is attributed to the initiation and promotion of multistage carcinogenesis [113]. A growing number of reports have demonstrated that loss of DNMT1 function has a significant impact on MSI—a hallmark of MMR efficiency, suggesting it has a role in the MMR pathway (Fig. 1.1). Using genetic screens in *Blm*-deficient embryonic stem (ES) cells, *Dnmt1* was identified as an MMR modifier gene. *Dnmt1* deficiency in murine ES cells results in a fourfold increase in the MSI rate [13]. Further support for this finding comes from several other laboratories [14–17, 114]. DNMT1 deficiency enhances microsatellite mutations for both integrated reporter genes [13, 14, 16, 17] and endogenous repeats [15]. This finding holds true for both ES cells and somatic cells. In a murine ES cell line with homologous deletion of *Dnmt1*, the stability of five endogenous microsatellite repeats (two mononucleotides and three dinucleotides), exhibiting instabilities in MMR-deficient cells was analyzed. A significantly higher frequency of instability was detected at three of the five markers in *Dnmt1*$^{-/-}$ ES cells compared to the wild-type ES cells [15]. The slippage rate of a stable reporter gene was also monitored. Dnmt1 deficiency led to a sevenfold higher rate of microsatellite slippage in *Dnmt1*$^{-/-}$ ES cells compared to wild-type cells [14]. Notably, no DNA methylation in the region flanking the reporter gene was discovered, regardless of Dnmt1 status, suggesting that the effect of Dnmt1 on MMR was not at the level of DNA methylation [14]. Enhanced MSI is associated with higher levels of histone H3 acetylation and lower MeCP2 binding at regions near the assayed microsatellite, suggesting that Dnmt1 loss decreases MMR efficiency by modifying chromatin structure. CAG repeat expansions are closely associated with human age-related diseases including 12 neurodegenerative disorders. Repeat instability induced by CAG repeat expansion requires the MMR components [16, 115]. DNMT1 deficiency induces destabilization and intergenerational expansion of CAG triplet repeats [16]. Double knockdown of MLH1 and DNMT1, however, additively increases the frequency of CAG contraction [114]. Specific targeting of DNMT1 in hTERT-immortalized normal human fibroblasts by siRNA induces both resistance to MSI and the drug 6-thioguanine (which induces cytotoxic DNA damage due to its misincorporation opposite thymine [116]) at a CA17 reporter gene; two hallmarks of MMR deficiency. Mutation rates correspond well with DNMT1 levels, ranging from 4.1-fold in cells with 31% of the normal DNMT1 protein level to tenfold in cells with 12% of the normal DNMT1 protein level [17]. This suggests that DNMT1 regulates microsatellite stability in a dose-dependent manner. The exact underlying mechanism of how

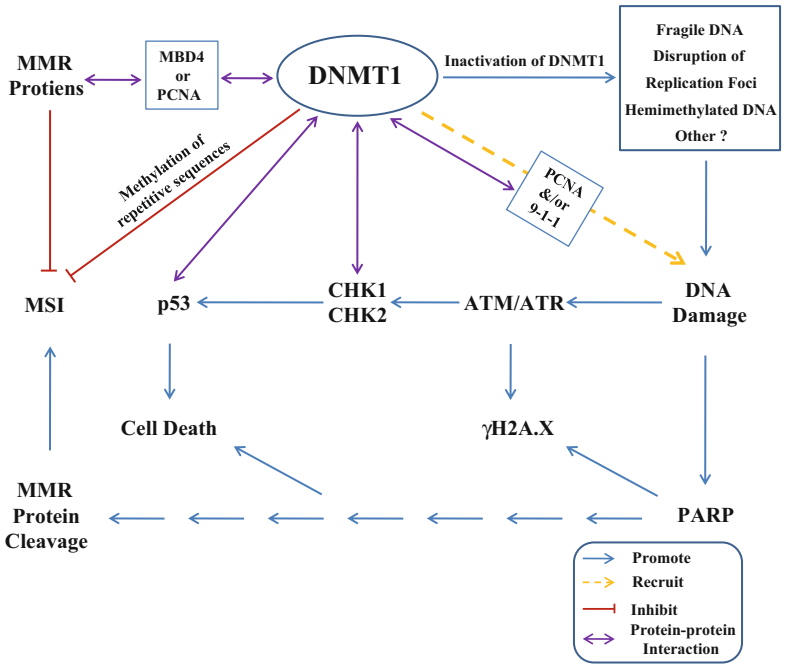

Fig. 1.1 Impact of DNMT1 on MMR and DDR. DNMT1 may promote stabilization of microsatellites via methylation of CpG repeats and it also interacts with DNA repair proteins via third-party mediators (e.g., MBD4 and PCNA). Moreover, deficiency in DNMT1 leads to activation of PARP signaling, eventually resulting in MMR protein cleavage. DNMT1 is also closely associated with DDR. Inactivation of DNMT1 may induce several changes to DNA and/or chromatin including increased DNA fragility, disruption of replication foci, and accumulation of hemimethylated DNA, which may be recognized as "damage" and activate the DDR. Strong support for a direct link between DNMT1 and DDR comes from the identification of several protein-protein interactions involving DNMT1 and DDR proteins. DNMT1 is recruited to sites of DNA damage via its interaction with PCNA and 9-1-1. DNMT1 is also capable of binding CHK1 and p53, which promote cell cycle arrest and apoptosis, respectively

DNMT1 is involved in MSI appears complex and remains elusive. Microsatellite methylation probably provides a mechanism for length stabilization by subsequent transcriptional repression of genes containing or proximal to microsatellites with methylated CpG repeats. However, increased mutations usually occur at microsatellite repeats that do not contain any CpG sites in the repeat itself [13, 15, 16, 114] or nearby [14], indicating that DNA methylation changes around microsatellite repeats, at least in some cases, are not the primary cause of the instability. Alternatively, DNMT1 might influence transcriptional repression and MSI through chromatin remodeling [14].

The impact of DNMT1 on the MMR pathway is further highlighted by the observation that DNMT1 and the MMR proteins probably interact with each other through a third-party mediator (Fig. 1.1). The methyl CpG-binding protein MBD4/MED1 may provide a functional link between MMR and DNMT1 through protein–protein

interaction. MBD4, which possesses glycosylase repair activity for G:T mismatches, is involved in NER as well as MMR. MBD4 binds MLH1 via its C-terminal glycosylase domain [117, 118]. Deletion of *Mbd4* in MEFs induced destabilization of MMR proteins and conferred resistance to antitumor drugs including 5-FU and platinum [119]. MBD4 and TDG have functional overlap and they interact with the de novo methyltransferases DNMT3A and DNMT3B [120, 121]. MBD4 also interacts with maintenance methyltransferase DNMT1 via its N-terminal MBD domain [118]. Based on a combination of immunoprecipitation and GST-pull down experiments in mouse, rat, and *Xenopus*, a minimal domain of approximately 70 amino acids in the N-terminal targeting sequence region of DNMT1 was shown to be required for MBD4 to bind to DNMT1 [118], which overlaps with a region in rat DNMT1 that interacts with MECP2 [122]. Through interacting directly with both DNMT1 and MLH1, MBD4 recruits MLH1 to heterochromatic sites that are coincident with DNMT1 localization [118]. Similarly, MBD4/MLH1 accumulates at DNA damage sites where DNMT1 is recruited after laser microirradiation [118]. Loss of DNMT1 induces p53-dependent apoptosis, which can be rescued by inactivation of p53 [123]. The MBD4/MLH1 complex also mediates the apoptotic response to DNMT1 depletion [118]. Colocalization of these proteins at damaged regions implies that they function coordinately in the cellular decision to repair the lesion or activate apoptosis. Like MBD4, PCNA may act as a mediator between MMR and DNMT1 because of its direct interaction with both systems. PCNA interacts with multiple components of the MMR pathway including MSH6, MSH3, and MLH1. Disruption of this interaction confers an MMR defect in vivo and in vitro [124–126]. Both MSH6 and MSH3 colocalize with PCNA at replication foci during S-phase [127]. MLH1 is recruited to damage sites where PCNA and DNMT1 also accumulate, although with slower kinetics than DNMT1 [118, 128]. The recruitment of DNMT1 to both the replication fork and DNA damage sites is through a direct interaction with PCNA and possibly CHK1 and the 9-1-1 complex as well [21, 24]. However, there is no report showing that PCNA, MLH1, and DNMT1 colocalize together, implying that PCNA might interact with each protein at a different time. Nonetheless, the functional mechanisms of whether and how these factors are orchestrated in response to DNA damage requires further investigation.

Most recently, DNMT1 deficiency has been shown to induce the depletion of multiple repair factors at the protein level (Fig. 1.1) [17], highlighting its importance not only in MMR efficiency, but also in DDR signaling. In normal human fibroblasts and CRC cell lines, DNMT1 knockdown leads to a matching decrease in MLH1 at the protein, but not the mRNA level [17]. Loss of MLH1, however, does not lead to expression changes in DNMT1 [17]. Promoter hypermethylation of *MLH1*, although frequently observed in sporadic colon cancers [39], does not appear to be the cause leading to gene inactivation in the context of DNMT1 deficiency. *MLH1* hypermethylation in DNMT1-deficient cells was further ruled out using a bisulfite pyrosequencing assay [17]. Further observations suggest that DNMT1 deficiency affects the steady-state levels of a number of repair proteins, including MSH2, MSH6, and PMS2, as well as MBD4 [17]. Loss of multiple MMR components in DNMT1 hypomorphic cells indicates that DNMT1 might play an

indirect role in the stabilization or proteolytic cleavage of these proteins, rather than directly interacting with each of them. It is documented that DNMT1 deficiency activates the DDR, which leads to cell cycle arrest [21, 123] and the triggering of cell death pathways [123] that may result in cleavage of proteins including MLH1 [129], which might account for MMR protein depletion after DNMT1 knockdown. Loss of DNMT1 activates ATM/ATR, which normally phosphorylate H2A.X leading to focal accumulation of γH2A.X, a hallmark of DDR [21]. If excessive damage exists, p53-dependent [123] and other cell death pathways are activated to maintain genomic integrity. Elevated γH2A.X levels in DNMT1 hypomorphic cells can be partially reduced through inhibition of ATM/ATR signaling [17]. However, the PAR polymerase (PARP) inhibitor DPQ also reduces the level of γH2A.X, to an extent exceeding that observed with the ATM/ATR inhibitor caffeine. In keeping with these observations, the viability of DNMT1-depleted cells treated with DPQ is enhanced to a greater extent than treatment of cells with agents that inhibit caspases or p53 [17]. These findings, together with the observation that PARylation increases after DNMT1 loss, clearly demonstrate that PARP is involved in the DDR and cell death process in cells deficient in DNMT1 (Fig. 1.1). PARP catalyzes the polymerization of ADP-ribose (PAR) units on target proteins using nicotinamide adenine dinucleotide (NAD^+) molecules as a donor [130]. NAD^+ depletion, induced by severe DNA damage, gives rise to mitochondrial membrane depolarization and apoptosis initiation factor (AIF) translocation. It eventually results in an activation of caspases that lead to protein cleavage and cell death. DNA repair protein MLH1 [129], along with BLM1 [131] and ATM [132], are preferred targets of caspases. Treatment with the PARP inhibitor DPQ, as expected, leads to an increase in full-length MLH1 protein levels in DNMT1-depleted cells [17]. Taken together, DDR signaling, particularly the cell death pathway mediated by PARP, may play a substantial role in regulating cleavage of MMR repair proteins in cells deficient for DNMT1 (Fig. 1.1).

1.4 DNMT1 and the DNA Damage Response

Reduction of DNMT1 levels activates a DDR usually initiated by the most lethal form of DNA damage-DSBs (Fig. 1.1). DNMT1 deficiency also inhibits DNA replication [22, 23, 133]. It was reported that DNMT1 knockdown triggers an intra-S-phase arrest of DNA replication, independent of DNA demethylation [22]. Similar to the observations for DNA damage checkpoints [134], the intra-S-phase arrest is transient, disappearing after 10 days of treatment with *DNMT1* siRNA. The S-phase cells induced by DNMT1 knockdown exist in two distinct populations: 70% incorporate BrdUr, while 30% do not, consistent with the presence of an intra-S-phase checkpoint triggering cell cycle arrest [134]. Cells are arrested at different positions throughout S-phase, suggesting that this response is not specific to distinct classes of origins of DNA replication. 5-aza-CdR, a nucleoside analogue, is a well-characterized and widely used inhibitor of DNA methylation, which inhibits

DNA methylation by trapping DNMT1 at the replication fork after being incorporated into DNA. 5-aza-CdR does not inhibit the de novo synthesis of DNMT1 protein or its presence in the nucleus. S-phase cells treated with 5-aza-CdR, which causes genome-wide demethylation, do not exhibit two distinct population distributions as observed in cells deficient in DNMT1. These results suggest that the intra-S-phase arrest is not correlated with the degree of DNA methylation, consistent with observations that DNA replication arrest following DNMT1 inhibition is probably due to a reduction in the physical presence of DNMT1 at the replication fork, rather than DNA demethylation [133]. As discussed above, the cell cycle distribution in DNMT1 knockdown cells resembles the transient intra-S-phase arrest in DNA replication that is evoked by genotoxic insults [135–137]. In addition, DNMT1 inhibition also leads to the induction of a set of genes that are implicated in the genotoxic stress response including *p21* [133], *p53* [123], and the growth arrest DNA damage inducible 45β gene (GADD45*β*) [22]. These results imply that DNMT1 is linked to DNA damage repair machineries to maintain chromosome integrity via blocking DNA replication, a notion further strengthened by observations that DNMT1 knockdown activates the checkpoint pathways in an ATR-dependent manner [23]. Upon DNMT1 depletion, CHK1 and CHK2, key proteins in ATM/ATR signaling, are phosphorylated, which in turn induce phosphorylation and degradation of cell division control protein 25 A (CDC25A) as well as CDC25B [23]. As a consequence, the capacity for loading CDC45, an essential factor for DNA replication [138], onto replication forks is decreased, resulting in replication arrest. DNMT1 knockdown also induces the formation of histone γH2A.X foci, a hallmark of the DNA DSB response. The response elicited by DNMT1 knockdown is blocked by siRNA-mediated depletion of ATR, suggestive of its ATR dependency. Further support for the importance of ATR came from the finding that the cellular response to DNMT1 depletion is markedly attenuated in cells derived from a patient with Seckel syndrome, a disorder due to ATR deficiency [23]. However, it is not clear whether ATM, another key transducer like ATR in the checkpoint pathway, is involved in the process or not. DNA demethylating agents do not trigger the stress response like genetic DNMT1 depletion does [23]. Moreover, this response is abolished by ectopic expression of either wild-type DNMT1 or a mutant form of DNMT1 lacking the catalytic domain [23], suggesting that loss of catalytic activity of DNMT1 is not driving this response. Also of importance, DNMT1 knockdown leads to very limited genomic demethylation [22, 23], consistent with observations made in cells containing hypomorphic mutations in *DNMT1* [139, 140]. One explanation for this limited demethylation is that de novo DNMTs compensate for the reduction of DNMT1 activity [139]. Another possibility is that DNMT1 loss triggers a checkpoint pathway (Fig. 1.1) to block DNA replication, preventing loss of DNA methylation in an attempt to maintain genome stability. Double knockdown of DNMT1 and ATR does indeed induce global DNA demethylation, whereas single knockdowns of either DNMT1 or ATR do not, implying that the arrest of DNA replication activated by ATR signaling following DNMT1 depletion prevents loss of DNA methylation and that blocking this

response results in global loss of DNA methylation [23]. Taken together, it appears that reduction of DNMT1 levels activates ATR signaling to block DNA replication in a DNA methylation-independent manner (Fig. 1.1). How this response to DNMT1 reduction is initiated, however, is still uncertain. It is possible that removal of DNMT1 from replication forks disrupts fork progression and eventually results in DSBs that elicit checkpoint signaling (Fig. 1.1). Alternatively, the presence of low levels of hemimethylated DNA due to the absence of DNMT1 may trigger this response (Fig. 1.1).

Complete inactivation of DNMT1 via genetic mechanisms also activates the DDR and causes genomic demethylation. The degree of demethylation, however, varies greatly depending on cellular context, ranging from 20% loss in human cancer cells [141] to 90% loss of genomic methylation in murine ES cells [7, 8]. As the principal enzyme responsible for maintaining DNA methylation, DNMT1 is essential for embryonic development and cell survival. Disruption of *Dnmt1* in mice results in loss of 90% of genomic methylation and embryonic lethality [7, 8]. Murine ES cells deficient for *Dnmt1* die when introduced to differentiate [7], mouse fibroblasts die within 2–4 cell divisions after conditional deletion in *Dnmt1* [123], and the human colon cancer cell line HCT116 undergoes marked apoptosis and cell death within one cell division if *DNMT1* is completely inactivated by cre-mediated conditional knockout [141, 142]. Notably, complete inactivation of DNMT1 triggers the DDR before cells die [141]. Deletion of *DNMT1* activates p53 [123, 141], a target of ATM whose phosphorylation correlates with accumulation of p53 in response to DNA damage [143]. Disruption of both alleles of *DNMT1* leads to activation of the G2/M checkpoint and G2 arrest, as verified by the presence of phosphorylated ATM and γH2A.X at discrete nuclear DNA damage foci [141]. Further support for checkpoint activation comes from the finding that treatment of cells with an ATM/ATR inhibitor, caffeine, facilitates mitotic entry and cell death in *DNMT1* null cells [141]. Most of these cells, however, eventually escape G2 arrest and reenter interphase with their unrepaired DNA, resulting in severe chromosomal and mitotic abnormalities (mitotic catastrophe) [141]. Thus far, the mechanisms by which DNMT1 inactivation leads to activation of DNA damage repair remains elusive. In the complete absence of DNMT1, DNA may become more fragile owing to reduced methylation and/or defective chromatin structure in critical regions of the genome, leading to activation of DNA damage signaling (Fig. 1.1) [142]. Alternatively, the accumulation of hemimethylated DNA in *DNMT1* mutant cells may be recognized as damage and trigger the damage response (Fig. 1.1). Both of these possibilities are consistent with the observation that agents that affect overall chromatin structure without damaging DNA also activate ATM [144]. Nonetheless, it cannot be excluded that oncogene activation or gene mutations initiate the DDR, as Dnmt1-deficient ES cells exhibit significantly increased mutation rates, particularly in the form of deletions and mutations [145].

Recruitment of DNMT1 to sites of DNA damage has been observed by our laboratory [21, 146] and others [24], providing compelling evidence to support the notion that DNMT1 is directly involved in DNA damage repair (Fig. 1.1).

Immediately after laser microirradiation-induced DSBs, an accumulation of DNMT1 and PCNA occurs at the damage sites in S and non-S phase cells, colocalizing with γH2A.X—a marker of DSBs. Recruitment of DNMT1 to damage sites is dependent on its interaction with PCNA through its PCNA-binding domain (PBD) [21, 24], but is independent of its catalytic activity [21]. In addition to PCNA, DNMT1 also interacts with other components of the DNA damage machinery including CHK1 [21, 146] and the 9-1-1 complex [21]. PCNA, along with CHK1 and 9-1-1, is essential for DNMT1's recruitment to DNA damage sites. After recruitment to damaged regions, DNMT1 modulates the rate of ATR signaling and is essential for suppressing abnormal activation of the DDR in the absence of exogenous damage [21]. Taken together, these data have revealed a direct link between DNMT1 and the DNA damage repair process.

PCNA mediates recruitment of DNMT1, not only to DNA replication sites, but also to DNA damage sites. The DNMT1–PCNA interaction implies that the role of DNMT might be to restore epigenetic information after damage repair. However, recent studies demonstrate that this interaction is not essential for maintaining DNA methylation [5, 147]. Furthermore, the observation [21] that DNMT1 is very rapidly recruited and retained only transiently, likely before resynthesis is completed, suggest that genomic methylation is not the main function of DNMT1 at these sites, at least in the early part of the DDR. The recruitment kinetics of WT *DNMT1* and *DNMT1* with a point mutation in the catalytic domain are almost identical [21]. CHK1/CHK2 activation and γH2A.X foci formation induced by DNMT1 deficiency are rescued by expression of a catalytically inactive form of DNMT1 [23]. Therefore, although the possibility that DNMT1 participates in the restoration of DNA methylation patterns during damage repair cannot be excluded, it seems more likely that DNMT1 functions in sensing and/or mobilizing the response to certain forms of DNA damage (Fig. 1.1).

In summary, both DNMTs and DNA damage repair systems have evolved to maintain genomic integrity and disruption of these pathways contributes to the development of cancer [19]. Therefore, we have examined and outlined the interaction of DNMTs and DNA methylation with DNA damage repair systems and have discussed possible mechanisms for how the two systems may function coordinately to deal with DNA damage. Promoter methylation, catalyzed by DNMTs, plays an established role in silencing key genes in multiple DNA damage repair pathways; inactivation of these pathways may predispose to a large array of tumors [20]. These findings are consistent with observations that TSGs are frequently silenced via epigenetic mechanisms in cancer cells. Unexpectedly perhaps, more recent observations strongly suggest that DNMTs, particular DNMT1, are directly involved in DNA damage repair systems via what is likely to be a DNA-methylation-independent mechanism [17, 21–23, 141]. The exact nature of the links between the DNMTs, DNA methylation, and DNA damage repair systems is complex and remains to be further investigated. A more thorough understanding of these links will not only help dissect the mechanisms of tumor development, but also identify new antitumor targets and therapeutic strategies.

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References

1. Robertson KD (2005) DNA methylation and human disease. Nat Rev Genet 6(8):597–610
2. Kareta MS, Botello ZM, Ennis JJ, Chou C, Chedin F (2006) Reconstitution and mechanism of the stimulation of de novo methylation by human DNMT3L. J Biol Chem 281(36): 25893–25902
3. Okano M, Bell DW, Haber DA, Li E (1999) DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell 99(3):247–257
4. Okano M, Xie S, Li E (1998) Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases. Nat Genet 19(3):219–220
5. Egger G, Jeong S, Escobar SG et al (2006) Identification of DNMT1 (DNA methyltransferase 1) hypomorphs in somatic knockouts suggests an essential role for DNMT1 in cell survival. Proc Natl Acad Sci USA 103(38):14080–14085
6. Riggs AD, Xiong Z (2004) Methylation and epigenetic fidelity. Proc Natl Acad Sci USA 101(1):4–5
7. Li E, Bestor TH, Jaenisch R (1992) Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. Cell 69(6):915–926
8. Lei H, Oh SP, Okano M et al (1996) De novo DNA cytosine methyltransferase activities in mouse embryonic stem cells. Development 122(10):3195–3205
9. Li E (2002) Chromatin modification and epigenetic reprogramming in mammalian development. Nat Rev Genet 3(9):662–673
10. Yoder JA, Walsh CP, Bestor TH (1997) Cytosine methylation and the ecology of intragenomic parasites. Trends Genet 13(8):335–340
11. Gonzalo S, Jaco I, Fraga MF et al (2006) DNA methyltransferases control telomere length and telomere recombination in mammalian cells. Nat Cell Biol 8(4):416–424
12. Xu GL, Bestor TH, Bourc'his D et al (1999) Chromosome instability and immunodeficiency syndrome caused by mutations in a DNA methyltransferase gene. Nature 402(6758): 187–191
13. Guo G, Wang W, Bradley A (2004) Mismatch repair genes identified using genetic screens in Blm-deficient embryonic stem cells. Nature 429(6994):891–895
14. Kim M, Trinh BN, Long TI, Oghamian S, Laird PW (2004) Dnmt1 deficiency leads to enhanced microsatellite instability in mouse embryonic stem cells. Nucleic Acids Res 32(19): 5742–5749
15. Wang KY, James Shen CK (2004) DNA methyltransferase Dnmt1 and mismatch repair. Oncogene 23(47):7898–7902
16. Dion V, Lin Y, Hubert L Jr, Waterland RA, Wilson JH (2008) Dnmt1 deficiency promotes CAG repeat expansion in the mouse germline. Hum Mol Genet 17(9):1306–1317
17. Loughery JE, Dunne PD, O'Neill KM, Meehan RR, McDaid JR, Walsh CP (2011) DNMT1 deficiency triggers mismatch repair defects in human cells through depletion of repair protein levels in a process involving the DNA damage response. Hum Mol Genet 20(16): 3241–3255
18. Karpf AR, Matsui S (2005) Genetic disruption of cytosine DNA methyltransferase enzymes induces chromosomal instability in human cancer cells. Cancer Res 65(19):8635–8639
19. Jackson SP, Bartek J (2009) The DNA-damage response in human biology and disease. Nature 461(7267):1071–1078

20. Jacinto FV, Esteller M (2007) Mutator pathways unleashed by epigenetic silencing in human cancer. Mutagenesis 22(4):247–253
21. Ha K, Lee GE, Palii SS et al (2011) Rapid and transient recruitment of DNMT1 to DNA double-strand breaks is mediated by its interaction with multiple components of the DNA damage response machinery. Hum Mol Genet 20(1):126–140
22. Milutinovic S, Zhuang Q, Niveleau A, Szyf M (2003) Epigenomic stress response. Knockdown of DNA methyltransferase 1 triggers an intra-S-phase arrest of DNA replication and induction of stress response genes. J Biol Chem 278(17):14985–14995
23. Unterberger A, Andrews SD, Weaver IC, Szyf M (2006) DNA methyltransferase 1 knockdown activates a replication stress checkpoint. Mol Cell Biol 26(20):7575–7586
24. Mortusewicz O, Schermelleh L, Walter J, Cardoso MC, Leonhardt H (2005) Recruitment of DNA methyltransferase I to DNA repair sites. Proc Natl Acad Sci USA 102(25):8905–8909
25. Laghi L, Bianchi P, Malesci A (2008) Differences and evolution of the methods for the assessment of microsatellite instability. Oncogene 27(49):6313–6321
26. Kunkel TA, Erie DA (2005) DNA mismatch repair. Annu Rev Biochem 74:681–710
27. Raschle M, Dufner P, Marra G, Jiricny J (2002) Mutations within the hMLH1 and hPMS2 subunits of the human MutLalpha mismatch repair factor affect its ATPase activity, but not its ability to interact with hMutSalpha. J Biol Chem 277(24):21810–21820
28. Kantelinen J, Kansikas M, Korhonen MK et al (2010) MutSbeta exceeds MutSalpha in dinucleotide loop repair. Br J Cancer 102(6):1068–1073
29. Thibodeau SN, French AJ, Cunningham JM et al (1998) Microsatellite instability in colorectal cancer: different mutator phenotypes and the principal involvement of hMLH1. Cancer Res 58(8):1713–1718
30. Viswanathan M, Tsuchida N, Shanmugam G (2003) Promoter hypermethylation profile of tumor-associated genes p16, p15, hMLH1, MGMT and E-cadherin in oral squamous cell carcinoma. Int J Cancer 105(1):41–46
31. Kim HG, Lee S, Kim DY et al (2010) Aberrant methylation of DNA mismatch repair genes in elderly patients with sporadic gastric carcinoma: A comparison with younger patients. J Surg Oncol 101(1):28–35
32. Brucher BL, Geddert H, Langner C et al (2006) Hypermethylation of hMLH1, HPP1, p14(ARF), p16(INK4A) and APC in primary adenocarcinomas of the small bowel. Int J Cancer 119(6):1298–1302
33. Wang YC, Lu YP, Tseng RC et al (2003) Inactivation of hMLH1 and hMSH2 by promoter methylation in primary non-small cell lung tumors and matched sputum samples. J Clin Invest 111(6):887–895
34. Murphy MA, Wentzensen N (2011) Frequency of mismatch repair deficiency in ovarian cancer: a systematic review. Int J Cancer 129:1914–1922
35. Seedhouse CH, Das-Gupta EP, Russell NH (2003) Methylation of the hMLH1 promoter and its association with microsatellite instability in acute myeloid leukemia. Leukemia 17(1):83–88
36. Lenz G, Hutter G, Hiddemann W, Dreyling M (2004) Promoter methylation and expression of DNA repair genes hMLH1 and MGMT in acute myeloid leukemia. Ann Hematol 83(10):628–633
37. Nomdedeu JF, Perea G, Estivill C et al (2005) Microsatellite instability is not an uncommon finding in adult de novo acute myeloid leukemia. Ann Hematol 84(6):368–375
38. Tawfik HM, El-Maqsoud NM, Hak BH, El-Sherbiny YM (2011) Head and neck squamous cell carcinoma: mismatch repair immunohistochemistry and promoter hypermethylation of hMLH1 gene. Am J Otolaryngol 32(6):528–36
39. Lahtz C, Pfeifer GP (2011) Epigenetic changes of DNA repair genes in cancer. J Mol Cell Biol 3(1):51–58
40. Valle L, Carbonell P, Fernandez V et al (2007) MLH1 germline epimutations in selected patients with early-onset non-polyposis colorectal cancer. Clin Genet 71(3):232–237
41. Gazzoli I, Loda M, Garber J, Syngal S, Kolodner RD (2002) A hereditary nonpolyposis colorectal carcinoma case associated with hypermethylation of the MLH1 gene in normal tissue

and loss of heterozygosity of the unmethylated allele in the resulting microsatellite instability-high tumor. Cancer Res 62(14):3925–3928
42. Herman JG, Umar A, Polyak K et al (1998) Incidence and functional consequences of hMLH1 promoter hypermethylation in colorectal carcinoma. Proc Natl Acad Sci USA 95(12):6870–6875
43. Nakagawa H, Nuovo GJ, Zervos EE et al (2001) Age-related hypermethylation of the 5′ region of MLH1 in normal colonic mucosa is associated with microsatellite-unstable colorectal cancer development. Cancer Res 61(19):6991–6995
44. Kuismanen SA, Holmberg MT, Salovaara R et al (1999) Epigenetic phenotypes distinguish microsatellite-stable and -unstable colorectal cancers. Proc Natl Acad Sci USA 96(22): 12661–12666
45. Wheeler JM, Beck NE, Kim HC, Tomlinson IP, Mortensen NJ, Bodmer WF (1999) Mechanisms of inactivation of mismatch repair genes in human colorectal cancer cell lines: the predominant role of hMLH1. Proc Natl Acad Sci USA 96(18):10296–10301
46. Auclair J, Vaissiere T, Desseigne F et al (2011) Intensity-dependent constitutional MLH1 promoter methylation leads to early onset of colorectal cancer by affecting both alleles. Genes Chromosomes Cancer 50(3):178–185
47. Zhang H, Zhang S, Cui J, Zhang A, Shen L, Yu H (2008) Expression and promoter methylation status of mismatch repair gene hMLH1 and hMSH2 in epithelial ovarian cancer. Aust N Z J Obstet Gynaecol 48(5):505–509
48. Vlaykova T, Mitkova A, Stancheva G et al (2011) Microsatellite instability and promoter hypermethylation of MLH1 and MSH2 in patients with sporadic colorectal cancer. J BUON 16(2):265–273
49. Nagasaka T, Rhees J, Kloor M et al (2010) Somatic hypermethylation of MSH2 is a frequent event in Lynch Syndrome colorectal cancers. Cancer Res 70(8):3098–3108
50. Ligtenberg MJ, Kuiper RP, Chan TL et al (2009) Heritable somatic methylation and inactivation of MSH2 in families with Lynch syndrome due to deletion of the 3′ exons of TACSTD1. Nat Genet 41(1):112–117
51. Moelans CB, Verschuur-Maes AH, van Diest PJ (2011) Frequent promoter hypermethylation of BRCA2, CDH13, MSH6, PAX5, PAX6 and WT1 in ductal carcinoma in situ and invasive breast cancer. J Pathol 225(2):222–231
52. David SS, O'Shea VL, Kundu S (2007) Base-excision repair of oxidative DNA damage. Nature 447(7147):941–950
53. Peng B, Hurt EM, Hodge DR, Thomas SB, Farrar WL (2006) DNA hypermethylation and partial gene silencing of human thymine- DNA glycosylase in multiple myeloma cell lines. Epigenetics 1(3):138–145
54. Howard JH, Frolov A, Tzeng CW et al (2009) Epigenetic downregulation of the DNA repair gene MED1/MBD4 in colorectal and ovarian cancer. Cancer Biol Ther 8(1):94–100
55. Guan H, Ji M, Hou P et al (2008) Hypermethylation of the DNA mismatch repair gene hMLH1 and its association with lymph node metastasis and T1799A BRAF mutation in patients with papillary thyroid cancer. Cancer 113(2):247–255
56. Hoeijmakers JH (2001) Genome maintenance mechanisms for preventing cancer. Nature 411(6835):366–374
57. Yang J, Xu Z, Li J et al (2010) XPC epigenetic silence coupled with p53 alteration has a significant impact on bladder cancer outcome. J Urol 184(1):336–343
58. Chen HY, Shao CJ, Chen FR, Kwan AL, Chen ZP (2010) Role of ERCC1 promoter hypermethylation in drug resistance to cisplatin in human gliomas. Int J Cancer 126(8):1944–1954
59. Liu WB, Ao L, Cui ZH et al (2011) Molecular analysis of DNA repair gene methylation and protein expression during chemical-induced rat lung carcinogenesis. Biochem Biophys Res Commun 408(4):595–601
60. Jiang J, Liang X, Zhou X et al (2010) DNA repair gene X-ray repair cross complementing group 1 Arg194Trp polymorphism on the risk of lung cancer: a meta-analysis on 22 studies. J Thorac Oncol 5(11):1741–1747

61. Wang P, Tang JT, Peng YS, Chen XY, Zhang YJ, Fang JY (2010) XRCC1 downregulated through promoter hypermethylation is involved in human gastric carcinogenesis. J Dig Dis 11(6):343–351
62. Peng B, Hodge DR, Thomas SB et al (2005) Epigenetic silencing of the human nucleotide excision repair gene, hHR23B, in interleukin-6-responsive multiple myeloma KAS-6/1 cells. J Biol Chem 280(6):4182–4187
63. Moynahan ME, Jasin M (2010) Mitotic homologous recombination maintains genomic stability and suppresses tumorigenesis. Nat Rev Mol Cell Biol 11(3):196–207
64. Mazon G, Mimitou EP, Symington LS. SnapShot: homologous recombination in DNA double-strand break repair. Cell. 2010;142(4):646, 646.e1.
65. Esteller M, Silva JM, Dominguez G et al (2000) Promoter hypermethylation and BRCA1 inactivation in sporadic breast and ovarian tumors. J Natl Cancer Inst 92(7):564–569
66. Esteller M, Risques RA, Toyota M et al (2001) Promoter hypermethylation of the DNA repair gene O(6)-methylguanine-DNA methyltransferase is associated with the presence of G:C to A:T transition mutations in p53 in human colorectal tumorigenesis. Cancer Res 61(12): 4689–4692
67. Baldwin RL, Nemeth E, Tran H et al (2000) BRCA1 promoter region hypermethylation in ovarian carcinoma: a population-based study. Cancer Res 60(19):5329–5333
68. Bernal C, Vargas M, Ossandon F et al (2008) DNA methylation profile in diffuse type gastric cancer: evidence for hypermethylation of the BRCA1 promoter region in early-onset gastric carcinogenesis. Biol Res 41(3):303–315
69. Cabello MJ, Grau L, Franco N et al (2011) Multiplexed methylation profiles of tumor suppressor genes in bladder cancer. J Mol Diagn 13(1):29–40
70. Lee MN, Tseng RC, Hsu HS et al (2007) Epigenetic inactivation of the chromosomal stability control genes BRCA1, BRCA2, and XRCC5 in non-small cell lung cancer. Clin Cancer Res 13(3):832–838
71. Esteller M, Fraga MF, Guo M et al (2001) DNA methylation patterns in hereditary human cancers mimic sporadic tumorigenesis. Hum Mol Genet 10(26):3001–3007
72. Ludwig T, Chapman DL, Papaioannou VE, Efstratiadis A (1997) Targeted mutations of breast cancer susceptibility gene homologs in mice: lethal phenotypes of Brca1, Brca2, Brca1/Brca2, Brca1/p53, and Brca2/p53 nullizygous embryos. Genes Dev 11(10): 1226–1241
73. Kee Y, D'Andrea AD (2010) Expanded roles of the Fanconi anemia pathway in preserving genomic stability. Genes Dev 24(16):1680–1694
74. Meier D, Schindler D (2011) Fanconi anemia core complex gene promoters harbor conserved transcription regulatory elements. PLoS One 6(8):e22911
75. Hess CJ, Ameziane N, Schuurhuis GJ et al (2008) Hypermethylation of the FANCC and FANCL promoter regions in sporadic acute leukaemia. Cell Oncol 30(4):299–306
76. Marsit CJ, Liu M, Nelson HH, Posner M, Suzuki M, Kelsey KT (2004) Inactivation of the Fanconi anemia/BRCA pathway in lung and oral cancers: implications for treatment and survival. Oncogene 23(4):1000–1004
77. Narayan G, Arias-Pulido H, Nandula SV et al (2004) Promoter hypermethylation of FANCF: disruption of Fanconi anemia-BRCA pathway in cervical cancer. Cancer Res 64(9): 2994–2997
78. Lim SL, Smith P, Syed N et al (2008) Promoter hypermethylation of FANCF and outcome in advanced ovarian cancer. Br J Cancer 98(8):1452–1456
79. Pegg AE, Dolan ME, Moschel RC (1995) Structure, function, and inhibition of O6-alkylguanine-DNA alkyltransferase. Prog Nucleic Acid Res Mol Biol 51:167–223
80. Gerson SL (2004) MGMT: its role in cancer aetiology and cancer therapeutics. Nat Rev Cancer 4(4):296–307
81. Esteller M, Hamilton SR, Burger PC, Baylin SB, Herman JG (1999) Inactivation of the DNA repair gene O6-methylguanine-DNA methyltransferase by promoter hypermethylation is a common event in primary human neoplasia. Cancer Res 59(4):793–797

82. Weller M, Stupp R, Reifenberger G et al (2010) MGMT promoter methylation in malignant gliomas: ready for personalized medicine? Nat Rev Neurol 6(1):39–51
83. Whitehall VL, Walsh MD, Young J, Leggett BA, Jass JR (2001) Methylation of O-6-methylguanine DNA methyltransferase characterizes a subset of colorectal cancer with low-level DNA microsatellite instability. Cancer Res 61(3):827–830
84. Park TJ, Han SU, Cho YK, Paik WK, Kim YB, Lim IK (2001) Methylation of O(6)-methylguanine-DNA methyltransferase gene is associated significantly with K-ras mutation, lymph node invasion, tumor staging, and disease free survival in patients with gastric carcinoma. Cancer 92(11):2760–2768
85. Greenblatt MS, Bennett WP, Hollstein M, Harris CC (1994) Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. Cancer Res 54(18): 4855–4878
86. Zhang YJ, Chen Y, Ahsan H et al (2003) Inactivation of the DNA repair gene O6-methylguanine-DNA methyltransferase by promoter hypermethylation and its relationship to aflatoxin B1-DNA adducts and p53 mutation in hepatocellular carcinoma. Int J Cancer 103(4): 440–444
87. Wolf P, Hu YC, Doffek K, Sidransky D, Ahrendt SA (2001) O(6)-Methylguanine-DNA methyltransferase promoter hypermethylation shifts the p53 mutational spectrum in non-small cell lung cancer. Cancer Res 61(22):8113–8117
88. Zhang L, Lu W, Miao X, Xing D, Tan W, Lin D (2003) Inactivation of DNA repair gene O6-methylguanine-DNA methyltransferase by promoter hypermethylation and its relation to p53 mutations in esophageal squamous cell carcinoma. Carcinogenesis 24(6):1039–1044
89. Nakamura M, Watanabe T, Yonekawa Y, Kleihues P, Ohgaki H (2001) Promoter methylation of the DNA repair gene MGMT in astrocytomas is frequently associated with G:C –> A:T mutations of the TP53 tumor suppressor gene. Carcinogenesis 22(10):1715–1719
90. Sabharwal A, Middleton MR (2006) Exploiting the role of O6-methylguanine-DNA-methyltransferase (MGMT) in cancer therapy. Curr Opin Pharmacol 6(4):355–363
91. Kitano K, Kim SY, Hakoshima T (2010) Structural basis for DNA strand separation by the unconventional winged-helix domain of RecQ helicase WRN. Structure 18(2):177–187
92. Opresko PL (2008) Telomere ResQue and preservation—roles for the Werner syndrome protein and other RecQ helicases. Mech Ageing Dev 129(1–2):79–90
93. Agrelo R, Cheng WH, Setien F et al (2006) Epigenetic inactivation of the premature aging Werner syndrome gene in human cancer. Proc Natl Acad Sci USA 103(23):8822–8827
94. Kawasaki T, Ohnishi M, Suemoto Y et al (2008) WRN promoter methylation possibly connects mucinous differentiation, microsatellite instability and CpG island methylator phenotype in colorectal cancer. Mod Pathol 21(2):150–158
95. Smith JA, Ndoye AM, Geary K, Lisanti MP, Igoucheva O, Daniel R (2010) A role for the Werner syndrome protein in epigenetic inactivation of the pluripotency factor Oct4. Aging Cell 9(4):580–591
96. Polo SE, Jackson SP (2011) Dynamics of DNA damage response proteins at DNA breaks: a focus on protein modifications. Genes Dev 25(5):409–433
97. Harrison JC, Haber JE (2006) Surviving the breakup: the DNA damage checkpoint. Annu Rev Genet 40:209–235
98. Lazzaro F, Giannattasio M, Puddu F et al (2009) Checkpoint mechanisms at the intersection between DNA damage and repair. DNA Repair (Amst) 8(9):1055–1067
99. Linding R, Jensen LJ, Ostheimer GJ et al (2007) Systematic discovery of in vivo phosphorylation networks. Cell 129(7):1415–1426
100. Matsuoka S, Ballif BA, Smogorzewska A et al (2007) ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage. Science 316(5828): 1160–1166
101. Kim WJ, Vo QN, Shrivastav M, Lataxes TA, Brown KD (2002) Aberrant methylation of the ATM promoter correlates with increased radiosensitivity in a human colorectal tumor cell line. Oncogene 21(24):3864–3871

102. Vo QN, Kim WJ, Cvitanovic L, Boudreau DA, Ginzinger DG, Brown KD (2004) The ATM gene is a target for epigenetic silencing in locally advanced breast cancer. Oncogene 23(58): 9432–9437
103. Ai L, Vo QN, Zuo C et al (2004) Ataxia-telangiectasia-mutated (ATM) gene in head and neck squamous cell carcinoma: promoter hypermethylation with clinical correlation in 100 cases. Cancer Epidemiol Biomarkers Prev 13(1):150–156
104. Bartek J, Falck J, Lukas J (2001) CHK2 kinase—a busy messenger. Nat Rev Mol Cell Biol 2(12):877–886
105. Bell DW, Varley JM, Szydlo TE et al (1999) Heterozygous germ line hCHK2 mutations in Li-Fraumeni syndrome. Science 286(5449):2528–2531
106. Bartek J, Lukas J (2003) Chk1 and Chk2 kinases in checkpoint control and cancer. Cancer Cell 3(5):421–429
107. Kato N, Fujimoto H, Yoda A et al (2004) Regulation of Chk2 gene expression in lymphoid malignancies: involvement of epigenetic mechanisms in Hodgkin's lymphoma cell lines. Cell Death Differ 11(Suppl 2):S153–161
108. Kim DS, Kim MJ, Lee JY et al (2009) Epigenetic inactivation of checkpoint kinase 2 gene in non-small cell lung cancer and its relationship with clinicopathological features. Lung Cancer 65(2):247–250
109. Wang H, Wang S, Shen L et al (2010) Chk2 down-regulation by promoter hypermethylation in human bulk gliomas. Life Sci 86(5–6):185–191
110. Sullivan A, Yuille M, Repellin C et al (2002) Concomitant inactivation of p53 and Chk2 in breast cancer. Oncogene 21(9):1316–1324
111. Williams LH, Choong D, Johnson SA, Campbell IG (2006) Genetic and epigenetic analysis of CHEK2 in sporadic breast, colon, and ovarian cancers. Clin Cancer Res 12(23):6967–6972
112. Jascur T, Boland CR (2006) Structure and function of the components of the human DNA mismatch repair system. Int J Cancer 119(9):2030–2035
113. Loeb LA, Loeb KR, Anderson JP (2003) Multiple mutations and cancer. Proc Natl Acad Sci USA 100(3):776–781
114. Lin Y, Wilson JH (2009) Diverse effects of individual mismatch repair components on transcription-induced CAG repeat instability in human cells. DNA Repair (Amst) 8(8):878–885
115. Lin Y, Dion V, Wilson JH (2006) Transcription promotes contraction of CAG repeat tracts in human cells. Nat Struct Mol Biol 13(2):179–180
116. Karran P (2006) Thiopurines, DNA damage, DNA repair and therapy-related cancer. Br Med Bull 79–80:153–170
117. Bellacosa A, Cicchillitti L, Schepis F et al (1999) MED1, a novel human methyl-CpG-binding endonuclease, interacts with DNA mismatch repair protein MLH1. Proc Natl Acad Sci USA 96(7):3969–3974
118. Ruzov A, Shorning B, Mortusewicz O, Dunican DS, Leonhardt H, Meehan RR (2009) MBD4 and MLH1 are required for apoptotic induction in xDNMT1-depleted embryos. Development 136(13):2277–2286
119. Cortellino S, Turner D, Masciullo V et al (2003) The base excision repair enzyme MED1 mediates DNA damage response to antitumor drugs and is associated with mismatch repair system integrity. Proc Natl Acad Sci USA 100(25):15071–15076
120. Boland MJ, Christman JK (2008) Characterization of Dnmt3b:thymine-DNA glycosylase interaction and stimulation of thymine glycosylase-mediated repair by DNA methyltransferase(s) and RNA. J Mol Biol 379(3):492–504
121. Li YQ, Zhou PZ, Zheng XD, Walsh CP, Xu GL (2007) Association of Dnmt3a and thymine DNA glycosylase links DNA methylation with base-excision repair. Nucleic Acids Res 35(2): 390–400
122. Kimura H, Shiota K (2003) Methyl-CpG-binding protein, MeCP2, is a target molecule for maintenance DNA methyltransferase, Dnmt1. J Biol Chem 278(7):4806–4812
123. Jackson-Grusby L, Beard C, Possemato R et al (2001) Loss of genomic methylation causes p53-dependent apoptosis and epigenetic deregulation. Nat Genet 27(1):31–39

124. Flores-Rozas H, Clark D, Kolodner RD (2000) Proliferating cell nuclear antigen and Msh2p-Msh6p interact to form an active mispair recognition complex. Nat Genet 26(3):375–378
125. Iyer RR, Pohlhaus TJ, Chen S et al (2008) The MutSalpha-proliferating cell nuclear antigen interaction in human DNA mismatch repair. J Biol Chem 283(19):13310–13319
126. Plotz G, Welsch C, Giron-Monzon L et al (2006) Mutations in the MutSalpha interaction interface of MLH1 can abolish DNA mismatch repair. Nucleic Acids Res 34(22):6574–6586
127. Kleczkowska HE, Marra G, Lettieri T, Jiricny J (2001) hMSH3 and hMSH6 interact with PCNA and colocalize with it to replication foci. Genes Dev 15(6):724–736
128. Umar A, Buermeyer AB, Simon JA et al (1996) Requirement for PCNA in DNA mismatch repair at a step preceding DNA resynthesis. Cell 87(1):65–73
129. Chen F, Arseven OK, Cryns VL (2004) Proteolysis of the mismatch repair protein MLH1 by caspase-3 promotes DNA damage-induced apoptosis. J Biol Chem 279(26):27542–27548
130. Kim MY, Zhang T, Kraus WL (2005) Poly(ADP-ribosyl)ation by PARP-1: 'PAR-laying' NAD+ into a nuclear signal. Genes Dev 19(17):1951–1967
131. Bischof O, Galande S, Farzaneh F, Kohwi-Shigematsu T, Campisi J (2001) Selective cleavage of BLM, the bloom syndrome protein, during apoptotic cell death. J Biol Chem 276(15): 12068–12075
132. Wang J, Pabla N, Wang CY, Wang W, Schoenlein PV, Dong Z (2006) Caspase-mediated cleavage of ATM during cisplatin-induced tubular cell apoptosis: inactivation of its kinase activity toward p53. Am J Physiol Renal Physiol 291(6):F1300–1307
133. Knox JD, Araujo FD, Bigey P et al (2000) Inhibition of DNA methyltransferase inhibits DNA replication. J Biol Chem 275(24):17986–17990
134. Bartek J, Lukas J (2001) Mammalian G1- and S-phase checkpoints in response to DNA damage. Curr Opin Cell Biol 13(6):738–747
135. Kastan MB, Lim DS (2000) The many substrates and functions of ATM. Nat Rev Mol Cell Biol 1(3):179–186
136. Falck J, Mailand N, Syljuasen RG, Bartek J, Lukas J (2001) The ATM-Chk2-Cdc25A checkpoint pathway guards against radioresistant DNA synthesis. Nature 410(6830):842–847
137. Maser RS, Mirzoeva OK, Wells J et al (2001) Mre11 complex and DNA replication: linkage to E2F and sites of DNA synthesis. Mol Cell Biol 21(17):6006–6016
138. Hardy CF (1997) Identification of Cdc45p, an essential factor required for DNA replication. Gene 187(2):239–246
139. Rhee I, Bachman KE, Park BH et al (2002) DNMT1 and DNMT3b cooperate to silence genes in human cancer cells. Nature 416(6880):552–556
140. Ting AH, Jair KW, Suzuki H, Yen RW, Baylin SB, Schuebel KE (2004) CpG island hypermethylation is maintained in human colorectal cancer cells after RNAi-mediated depletion of DNMT1. Nat Genet 36(6):582–584
141. Chen T, Hevi S, Gay F et al (2007) Complete inactivation of DNMT1 leads to mitotic catastrophe in human cancer cells. Nat Genet 39(3):391–396
142. Brown KD, Robertson KD (2007) DNMT1 knockout delivers a strong blow to genome stability and cell viability. Nat Genet 39(3):289–290
143. Canman CE, Lim DS, Cimprich KA et al (1998) Activation of the ATM kinase by ionizing radiation and phosphorylation of p53. Science 281(5383):1677–1679
144. Bakkenist CJ, Kastan MB (2003) DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. Nature 421(6922):499–506
145. Chen RZ, Pettersson U, Beard C, Jackson-Grusby L, Jaenisch R (1998) DNA hypomethylation leads to elevated mutation rates. Nature 395(6697):89–93
146. Palii SS, Van Emburgh BO, Sankpal UT, Brown KD, Robertson KD (2008) DNA methylation inhibitor 5-Aza-2′-deoxycytidine induces reversible genome-wide DNA damage that is distinctly influenced by DNA methyltransferases 1 and 3B. Mol Cell Biol 28(2):752–771
147. Spada F, Haemmer A, Kuch D et al (2007) DNMT1 but not its interaction with the replication machinery is required for maintenance of DNA methylation in human cells. J Cell Biol 176(5):565–571

Chapter 2
DNA Hypomethylation and Hemimethylation in Cancer

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Abstract In contrast to earlier views that there was much compartmentalization of the types of sequences subject to cancer-linked changes in DNA epigenetics, it is now clear that both cancer-associated DNA hypomethylation and hypermethylation are found throughout the genome. The hypermethylation includes promoters of tumor suppressor genes whose expression becomes repressed, thereby facilitating cancer formation. How hypomethylation contributes to carcinogenesis has been less clear. Recent insights into tissue-specific intra- and intergenic methylation and into cancer methylomes suggest that some of the DNA hypomethylation associated with cancers is likely to aid in tumor formation and progression by many different pathways, including effects on transcription in *cis*. Cancer-associated loss of DNA methylation from intergenic enhancers, promoter regions, silencers, and chromatin boundary elements may alter transcription rates. In addition, cancer-associated intragenic DNA hypomethylation might modulate alternative promoter usage,

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production of intragenic noncoding RNA transcripts, cotranscriptional splicing, and transcription initiation or elongation. Initial studies of hemimethylation of DNA in cancer and many new studies of DNA demethylation in normal tissues suggest that active demethylation with spreading of hypomethylation can explain much of the cancer-associated DNA hypomethylation. The new discoveries that genomic 5-hydroxymethylcytosine is an intermediate in DNA demethylation, a base with its own functionality, and a modified base that, like 5-methylcytosine, exhibits cancer-associated losses, suggest that both decreased hydroxymethylation and decreased methylation of DNA play important roles in carcinogenesis.

2.1 Introduction

Altered methylation of DNA in human cancers was first described as overall genomic hypomethylation in various cancers vs. a wide variety of normal tissues [1] and as hypomethylation of a few gene regions in colon adenocarcinomas vs. normal colonic epithelium [2]. Almost all types of cancers exhibit both hypermethylation of some DNA sequences and hypomethylation of others relative to appropriate controls that account for the tissue specificity of DNA methylation [3]. The cancer-associated hypermethylation and hypomethylation of the genome are generally independent of each other [4, 5]. Until recently, it appeared that cancer-specific changes in DNA methylation were usually hypermethylation of unique gene regions and hypomethylation of DNA repeats, albeit with many notable exceptions [6–11]. Deep sequencing of the genome has revealed far greater size and complexity to the transcriptome than previously appreciated [12]. Similarly, recent whole-genome analysis of the cancer methylome demonstrates that there is much more cancer-linked hypomethylation of unique gene sequences and hypermethylation of repeated sequences than previously found, although there are differences in the frequency with which subsets of sequences undergo hypo- or hypermethylation [13–18].

This chapter reviews new insights into genome-wide DNA and chromatin epigenetics in normal cell populations as well as in cancers [19–29]. Recent studies are drawing attention to previously unsuspected roles of epigenetic marks in the body of genes as well as at promoters and intergenic transcription control regions. These findings are likely to be relevant to the biological impact of cancer-associated DNA hypomethylation. In addition to effects on normal gene expression, cancer-associated DNA hypomethylation probably favors oncogenesis by enhancing recombination [30–33]; occasionally activating a small number of endogenous retroviral elements [34, 35]; altering the intranuclear positioning of chromatin; and modulating the sequestration transcription factors at tandem DNA repeats, as reviewed previously [3, 6]. In addition, the little-studied area of DNA hemimethylation in cancer is discussed in this chapter in the context of our growing understanding of pathways for the conversion of genomic 5-methylcytosine (5mC) residues to C residues.

2.2 Genomic Hypomethylation Profiles in Cancer and Their Relevance at Promoters and Enhancers

Until recent high-resolution genome-wide analyses of DNA methylation, cancer-specific portions of methylomes were considered to consist predominantly of hypomethylated DNA repeats and hypermethylated gene regions [3, 7, 36]. DNA repeats are often used as a surrogate for average genomic methylation changes (usually losses of 5mC), with DNA epigenetic changes in some classes of repeats more closely associated with certain tumor types [6, 18, 35, 37–39]. In our 1983 analysis of global DNA hypomethylation in human cancers by high performance liquid chromatography analysis of enzymatic DNA digests [1], we fractionated one adenocarcinoma DNA into highly repetitive, moderately repetitive, and unique sequence classes. Because we found that each of these cancer DNA fractions had similar ratios of mol% 5mC to those from normal human tissues, we concluded that cancer-linked hypomethylation was not confined to repeated DNA. Indeed, cancer-linked DNA hypomethylation often occurs in unique sequences in and around genes, including metastasis-associated genes, as originally revealed in studies using CpG methylation-sensitive restriction endonucleases or sodium sulfite-based methods to study individual gene regions [2, 6, 40].

Recent genome-wide studies of DNA methylation in various normal and cancer cell populations indicate much tissue specificity throughout the genome in normal samples and pervasive cancer-linked DNA hypomethylation and hypermethylation [13, 15, 16, 41–45]. Regions of cancer-associated changes in DNA methylation are found in short interspersed or clustered regions as well as in long blocks [7, 42, 44, 46, 47]. There is increasing evidence for cause-and-effect relationships between normal tissue-specific DNA hypomethylation and increased transcription as well as many associations between cancer-linked hypomethylation and cancer-linked increases in gene expression [16, 17, 19, 21, 24, 48–55]. The inverse relationships between expression and DNA methylation include imprinted genes implicated in carcinogenesis [56].

A small percentage of annotated gene promoters overlap tissue-specific (T-DMR) or cancer-specific (C-DMR) differentially methylated DNA regions [49, 57]. However, most of the non-imprinted, autosomal T-DMR promoters are not the main type of vertebrate DNA promoters, which are part of CpG islands (CGIs, a class of CpG-rich regions surrounded by CpG-poor DNA). Among the genes with T-DMR promoters are some that become activated upon experimentally induced demethylation with a low dose of 5-deoxyazacytidine but not upon treatment with a histone deacetylase inhibitor, trichostatin A [49].

Enhancers too sometimes show a correlation between upregulation of expression of the associated gene and DNA demethylation in normal cells. For example, the binding of FoxA1/FOXA1 to enhancers is inhibited by site-specific DNA methylation at the corresponding binding site [58]. This differentiation-associated transcription regulatory factor can open up DNA compacted in chromatin of inactive enhancers (as a “pioneer” factor) and then recruit effector transcription factors to

make the enhancer active [59–61]. A window of DNA demethylation provided by previous binding of FoxD3, another pioneer factor, allows recruitment of FoxA1 and conversion of the enhancer to a state that is poised for activity. Moreover, in embryonal stem cells, local DNA demethylation per se, rather than any changes in histone H3K27 or H3K9 methylation, is associated with the binding of pioneer factors to certain tissue-specific non-CGI promoters [58]. Pioneer factors, including FOXA1, are implicated in various types of carcinogenesis [62]. Given the extensive hypomethylation of DNA in cancers, many known and yet more unknown enhancer regions are likely to become demethylated specifically in tumors. However, specific losses of DNA methylation from transcription regulatory regions might facilitate, but not independently cause, changes in expression [63].

Broad DNA regions enriched in hypomethylation are sometimes also associated with increases in copy number of DNA regions and can, thereby, synergistically increase expression of some of the affected genes [13, 33, 42]. Such broad regional hypomethylation (which can encompass occasional sites of persistent methylation) might reflect higher order chromatin structure. The latter is influenced, in turn, by the type, frequency, and spacing of DNA repeats; the G+C and CpG contents of subregions; the gene density; the nucleosome density; broad regions of distinct histone composition modification; and the presence of clusters of co-regulated genes. Nonetheless, a long region of cancer-linked DNA hypermethylation can be adjacent to a region of cancer-linked DNA hypomethylation with a sharp border between them, as demonstrated for a tandem repeat array (D4Z4) and its border sequences [9]. Despite evidence for functionality, DNA demethylation in cancer probably involves frequent overshooting of targeted sequences. These are referred to as *passenger* DNA methylation changes [64]. The hypomethylation in cancers of many more sites than are biologically relevant is probably due to a relaxed specificity of the demethylation apparatus during carcinogenesis and tumor progression and to the spreading of DNA demethylation patterns.

2.3 Genomic Hypomethylation in Cancer Within Gene Bodies

Recent findings implicate intragenic epigenetic marks in the regulation of normal gene expression. T-DMRs have been found inside many genes, and increased methylation in the central gene body or downstream promoter-flanking region of certain subsets of genes is associated with increased transcription [23, 65–68]. Moreover, there are nonrandom associations between positions of CpG methylation within genes and exon–intron boundaries, distance from the transcription start site, and distance from the 3′ end of the gene [66, 69]. Besides first exons, T-DMRs are present in various exonic and intronic sequences, including internal CGIs, sequences adjacent to internal CGIs (“CGI shores”), insulators, intragenic ncRNA genes, and 3′ terminal regions [17, 19, 28, 59, 70, 71]. They are present in both repeated and unique sequences. These findings are consistent with the many interrelationships between DNA and chromatin epigenetics and tissue-specific chromatin epigenetic marks inside genes [65, 68, 72, 73].

Differentiation-related DNA and/or chromatin epigenetic marks within genes may help determine alternative promoter usage, modulate the rate of transcription initiation or elongation, and possibly help direct the choice of alternative splice sites [19, 21, 24, 26, 27, 29, 34, 52, 74, 75]. The average DNA methylation level in the central portion of moderately expressed genes is associated with higher average transcription levels, possibly by being related to nucleosome positioning [76]. For example, immediately downstream of proximal CpG-poor promoters, it was unexpectedly found that methylation of sequences antagonizes binding of Polycomb repressor complexes [68]. Methylation of gene-body CGIs appears to be associated with repression of intragenic promoters [28]. However, for some sets of genes under certain conditions, lower expression was correlated with increases in gene-body methylation [69].

With respect to alternative splicing, evidence implicates certain histone modifications in helping to regulate the choice of splice junctions by altering rates of transcription, nucleosome positioning, or direct interactions with proteins that mark exon–intron junctions of pre-mRNA [77, 78]. Changes in physiological conditions can alter the chromatin modifications at these junctions and concomitantly modulate exon skipping [78]. DNA methylation may also be involved in regulating alternative splicing because of the many DNA methylation/chromatin epigenetic interrelationships and the finding that intron–exon junctions are enriched in sharp transitions in DNA methylation levels [66]. A recent report that malignant prostate cancer cells have enrichment of DNA hypermethylation at exon–intron junctions [45] is consistent with the cancer-linked involvement of DNA methylation levels in determining alternative splicing.

Programmed changes in DNA methylation in intra- and intergenic regions are not restricted to differentiation-related events. For example, electroconvulsive stimulation of mouse neuronal cells in vivo was recently demonstrated to cause rapid decreases and increases in DNA methylation in a substantial minority of CpG sites, especially at CpG-poor regions [69]. The physiologically linked DNA demethylation included rapid demethylation of exons and introns in various positions of the genes. Importantly, there was enrichment in these DNA epigenetic changes in the vicinity of brain-related genes. Thus, there is ample precedent from studies of normal cell functioning to suggest that cancer-associated DNA hypomethylation in intronic and exonic sequences can modulate the amount and type of gene products and thereby contribute to tumor formation or progression.

Cancer-linked DNA hypomethylation in the gene body is illustrated in Fig. 2.1 for three genes whose expression has been reported to be altered in certain cancers [79–81]. *TGFB2* has an intronic Alu repeat that was hypomethylated in some cancer cell lines relative to a wide variety of normal tissues (Fig. 2.1a) and untransformed cell cultures. The only exceptions to this intronic region being highly methylated in normal tissues and cell strains were found in skeletal muscle (Fig. 2.1a), myoblasts, and myotubes (data not shown). Their hypomethylation at this site might be related to the significant upregulation of *TGFB2* in myoblasts and myotubes vs. 19 types of non-muscle cell cultures [82] and is an example of the frequent relationship between targets for cancer-associated hypo- or hypermethylation and targets for differentiation-associated epigenetic changes [17, 83]. Like *TGFB2, PRDM16* (Fig. 2.1b) exhibited gene-body hypomethylation in

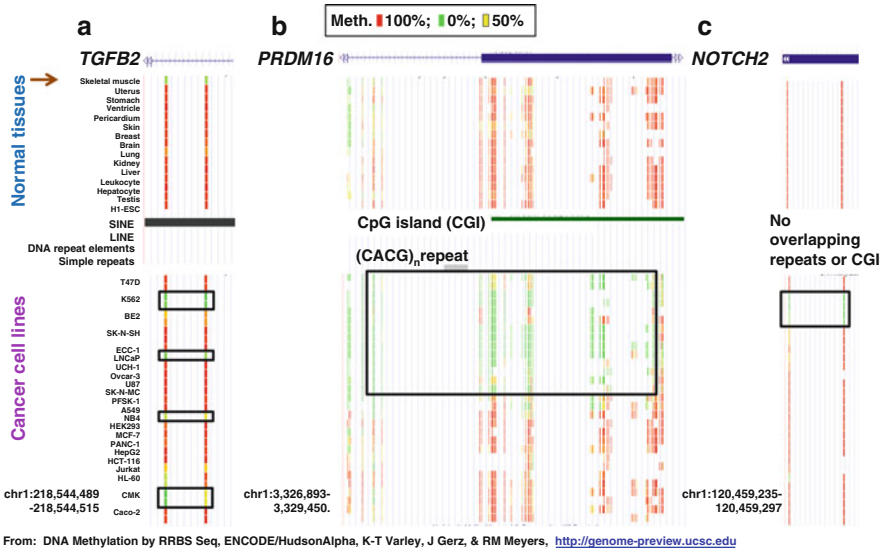

Fig. 2.1 Examples of cancer cell-associated hypomethylation (*boxed*) within gene bodies and overlapping a DNA repeat (**a**), a CGI (**b**), or neither (**c**) as determined by whole-genome analysis using reduced representation bisulfite sequencing (RRBS). (**a**), *TGFB2* , intron 1; the cancer hypomethylation overlaps an Alu repeat that is also hypomethylated in skeletal muscle (see *arrow*). (**b**), *PRDM16,* exon 9 and intron 8; the cancer hypomethylation overlaps a CGI and CGI shore. (**c**), *NOTCH2*, exon 34; no overlapping repeats or CGI. In contrast to the cancer-derived cell lines, non-immortalized cell strains (not shown) showed the same hypermethylation seen in normal tissues with the exception of myoblasts and myotubes for *TGFB2.* Myoblasts and myotubes overexpress *TGFB2* relative to 19 other types of cultured cell popula*tions*. All analyses were done in duplicate, and representative duplicates are shown

some of the cancer cell lines; however, this hypomethylation was in a region largely overlapping a CGI in an exon. *NOTCH2* (Fig. 2.1c) also showed gene-body hypomethylation in several cancer cell lines, but this hypomethylation was neither in a subregion with a CGI nor a DNA repeat. We note that some of the cancer cell lines with *TGFB2* or *PRDM16* gene hypomethylation also displayed cancer cell-linked promoter hypermethylation (data not shown).

Recently, the presence of 5-hydroxymethylcytosine (5hmC) as the sixth naturally programmed base in vertebrate DNA has been established [84]. It is generated from 5mC by hydroxylation via the enzymes TET1, TET2, or TET3 and is even more highly tissue specific in its relative levels in DNA than is 5mC [84–86]. It is implicated in stem cell renewal and distinct types of differentiation [87–89], as described further in an accompanying chapter by Pradhan and Kinney. Like 5mC, 5hmC is enriched in certain intragenic regions and exhibits major decreases in its genomic levels in cancer [84–86]. However, unlike 5mC, exons, intragenic CGIs, and enhancers have significantly elevated 5hmC levels relative to other portions of the genome [87, 90, 91]. These findings further highlight the need for studies of the functional significance of decreases in intragenic DNA epigenetic marks in cancer. In addition, they introduce a complication into almost all studies to date of 5mC that use either bisulfite or conventional CpG methylation-sensitive

restriction analysis to distinguish 5mC from unmethylated C, as these methods cannot resolve 5hmC and 5mC [69, 92, 93]. Therefore, a caveat to conclusions about 5mC distribution is that 5hmC might have been monitored instead, especially in exonic or enhancer regions in more 5hmC-rich tissues like brain [84, 85]. However, in some other cell types, like breast, heart, cell lines, and cancers, 5hmC is very much lower [84–86, 93], and 5hmC levels are also low in intronic and intergenic regions [90, 94].

2.4 Hypomethylation of DNA Repeats in Cancer

Global losses of DNA methylation with less numerous increases in methylation in other portions of the genome are typical of cancer [5, 6] although there are exceptions [18]. A major contributor toward the overall DNA hypomethylation is hypomethylation of tandem and interspersed DNA repeats, which is observed in most examined cancers [6, 95–97]. Most hypomethylation of DNA repeats in cancers is apparently the result of demethylation and not preexisting hypomethylation in a cancer stem cell [3], with the exception of seminomas as discussed below. Besides the effects on transcription and possible effects on alternative splicing described in the previous section, hypomethylation of a minor portion of interspersed DNA repeats may occasionally cause induction of retroviral element transcription [35]. In addition, hypomethylation of certain promoter-containing interspersed DNA repeats may affect chromatin boundaries resulting in effects on transcription of nearby genes [98, 99].

In a study of mononuclear cells from a few patients with chronic lymphocytic leukemia vs. the analogous cells from controls, Dante et al. described hypomethylation of LINE-1, a highly repeated interspersed repeat [100]. Hypomethylation of LINE-1 and Alu repeats was subsequently observed in many other types of cancers [38, 101–104]. Similarly, we found that tandem repeats in centromeric and juxtacentromeric satellite DNA are frequently hypomethylated in breast adenocarcinomas, ovarian epithelial cancers, and Wilms tumors [30, 105, 106], as confirmed for many other types of cancers [3, 107]. Additional classes of tandem repeats (including macrosatellite DNAs) and segmental duplications are also susceptible to DNA hypomethylation in malignancies [9, 18, 39, 43, 83, 108–110], although different subclasses of DNA repeat families can vary in their susceptibility to loss of DNA methylation in cancer [38, 39, 102, 111–113]. In some cancers, satellite DNA repeats showed the strongest DNA hypomethylation of all types of sequences analyzed [18, 33].

The frequency of cancer-associated hypomethylation of DNA repeats depends on the grade, the stage, and the individual tumor specimen [46, 114]. This hypomethylation is seen sometimes in non-tumor tissue adjacent to the cancer and in benign neoplasms and tissue lesions such as breast fibroadenomas and ovarian cystadenomas, although often to a lesser extent than in cancers [13, 51, 95, 105, 106, 112, 115]. In a mouse model of prostate tumor progression, repeat DNA hypomethylation was observed at the stage of prostatic intraepithelial neoplasia and prior to promoter hypermethylation [116]. However, depending on the tumor type

or specimen, repeat DNA hypomethylation may increase with tumor progression, a relationship inferred since the 1980s [1, 117]. In many types of cancer, repeat DNA hypomethylation is a highly informative prognostic marker and/or predictor of survival [46, 107, 118–122].

2.5 DNA Hypomethylation and Germ Cells: Comparison to Cancer Hypomethylation

Differential methylation of testes-specific genes has some similarities to cancer-associated DNA hypomethylation. Most genes that are specifically expressed in testis (like the cancer-testis genes) have little or no methylation in their promoter regions in testis and sperm although they are highly methylated, and transcriptionally repressed, in somatic tissues [123]. In sperm, as well as in many cancers, tandem DNA repeats and certain subclasses of interspersed DNA repeats display low methylation levels compared with normal postnatal somatic tissues [38, 112, 124–126]. Reminiscent of the tendency (with many exceptions, as described above) towards DNA repeats and unique sequences having opposite methylation changes in cancer, single-copy genes become demethylated but tandem and interspersed repeats retain their methylation in murine primordial germ cells at 12.5–13.5 dpc [123].

Another interface between the germ line epigenome and cancer is seen in the exceptionally strong global DNA hypomethylation in seminomatous testicular germ cell tumors. In our 1982 study of 62 tumors representing 23 different types, we found that a testicular seminoma had only 1.4% of its genomic C present as 5mC, while the next lowest 5mC level for a cancer was 2.4% [1]. The range of genomic 5mC levels among the normal tissues that we studied was 3.5–4.1% of C residues methylated. Smiraglia et al. confirmed the extraordinary depletion of 5mC in the genomes of many seminomas [127]. This finding has been ascribed to the origin of seminomas from primordial germ cells that had undergone massive demethylation before oncogenic transformation without subsequent de novo methylation thereafter [127, 128]. Importantly, seminomas generally show none of the CGI hypermethylation so prevalent in other types of cancer, but rather display extreme overall DNA hypomethylation [127]. Therefore, cancers can develop without gene region hypermethylation but with extreme overall genomic hypomethylation.

2.6 Opposite Cancer-Linked Changes in DNA Methylation in DNA Repeats: Hypo- and Hypermethylation

Opposite types of cancer-linked DNA methylation changes can occur in the same DNA sequence, as we found in a Southern blot study of methylation of NBL2, a 1.4-kb sequence repeated in tandem mostly near the centromeres of acrocentric chromosomes [39]. NBL2 was hypomethylated at HhaI sites (5′-CGCG-3′ sites) in

17% of ovarian carcinomas and hypermethylated in >70% of ovarian carcinomas and Wilms tumors at the same sites [39]. Various normal postnatal somatic tissues exhibited partial methylation at HhaI sites in NBL2 and were similar to each other in their methylation patterns at this tandem repeat. Using NotI (5′-GCGGCCGC-3′) for Southern blotting, only the cancer-linked hypomethylation of NBL2 was previously observed [108, 110] because NotI cleaves control somatic DNA too infrequently to reveal hypermethylation in cancers. This is an example of the importance of considering the technique used in evaluating results on DNA methylation [92] as well as the appropriate control DNA for comparison to the cancer. A few cancer DNAs digested with HhaI displayed two distinct fractions of NBL2 sequences, one with overall hypermethylation and the other with overall hypomethylation relative to all the somatic controls, which suggests that the repeats at one chromosomal location underwent de novo methylation and at another underwent demethylation during carcinogenesis. Hairpin genomic sequencing [129] (see below) at two ~0.3-kb subregions of the 1.4-kb *NBL2* ([8] and Nishiyama and Ehrlich, unpublished data) confirmed that hypomethylation at NBL2 predominated in some cancers and hypermethylation in others in comparison to normal somatic tissues, which displayed much site specificity in the methylation status of individual CpG sites. Therefore, a small region of DNA can be made unstable epigenetically during carcinogenesis so that CpG sites that are very near to each other undergo opposite changes in DNA methylation. The plasticity of the directionality of methylation changes at DNA repeats in cancers has also been seen in recent genome-wide studies [15, 18].

D4Z4, a heterologous tandem array (macrosatellite) located at subtelomeric 4q and 10q, also exhibited strong hypomethylation in the bulk of the array in some cancers and hypermethylation in others of the same type [9]. Several of the cancers had extremely high levels of methylation in more than three consecutive 3.3-kb repeat units of D4Z4, indicative of the spreading of de novo methylation. This methylation spreading seems to have limits to its processivity and to be prone to stop at certain subregions of the repeat unit.

2.7 Tagging Classes of DNA Sequences for Demethylation

Because NBL2 and D4Z4 tandem repeats displayed overall hypomethylation in some cancers and hypermethylation in others, it was highly informative to compare their methylation changes in a given cancer. Among 17 ovarian carcinomas and 44 Wilms tumors, there was a significant correlation ($p<0.001$) between the direction (either hypo- or hypermethylation) and degree of methylation change (strong, moderate, or weak) at D4Z4 and the dissimilar NBL2 [9]. This suggests that diverse sequences on different chromosomes may be similarly tagged for demethylation or de novo methylation (methylation of symmetrically unmethylated CpG dyads) during carcinogenesis. However, many cancers with extensive hypermethylation of D4Z4 and NBL2 repeats displayed hypomethylation of another, heterologous tandem repeat, juxtacentromeric satellite 2 on chromosome 1 (Sat2) [39].

NBL2 (mostly in the short arm of the acrocentric chromosomes) and D4Z4 (in the subtelomeric region of chromosomes 4 and 10) are both rich in G+C and look like very long CGIs. However, they differ appreciably in their G+C composition (61% and 73%, respectively) and their CpG content (5.7% and 9.9%, respectively). Analysis of histone modification and DNaseI sensitivity has been done for D4Z4 and indicates that its chromatin has properties midway between constitutive heterochromatin and unexpressed euchromatin [130, 131]. In contrast, Sat2, which is in the pericentromeric region, is constitutively heterochromatic and highly condensed in interphase. It has only 38% G+C but, nonetheless, it has 5.1% CpG. Therefore, the CpG suppression seen in the overall genome is not evident in Sat2. Sometimes even Sat2, with its rather CpG-rich character, becomes hypermethylated in cancers at a CpG dyad that exhibits a low methylation level in normal somatic tissues [132].

That the G+C content and chromatin structure is important for recruiting machinery for either demethylation or de novo methylation is consistent with our findings on the HpaII site immediately proximal to the D4Z4 array. It is located in a 0.2-kb D4Z4-proximal subregion that has 43% G+C, while D4Z4 has 73% G+C in all of its essentially identical, tandem 3.3-kb repeats. This 0.2-kb sequence immediately adjacent to the array is prone to tumor-linked hypomethylation even in cancers displaying strong hypermethylation within the array [9]. Surprisingly, even the adjacent D4Z4 repeat unit at the proximal end of the array became hypomethylated in cancers with hypermethylation of the bulk of the array. Probably, the array-adjacent sequence with its much lower G+C content helps confer a different chromatin structure on the neighboring D4Z4 repeat unit, which, in turn, affects the directionality of cancer-linked methylation change. Interestingly, a study of tandem transgenic repeats in mice revealed that, in some animals, all of the (G+C)-rich transgene units became methylated except for one copy adjacent to cellular DNA [133]. Despite the regional properties of DNA and chromatin that may recruit cancer-associated DNA methylation or demethylation apparati, there are, as mentioned above, very local sequence-specific effects which allow individual CpG dyads to circumvent regional demethylation or de novo methylation [8, 9].

DNA demethylation both influences and is strongly influenced by histone modifications. For example, histone H3 trimethylation at lysine 4 (H3K4me3) correlates best with the lack of DNA methylation around the transcription start site [66]. This was found for both CGI promoters [134] and promoters that do not contain a CGI, and for CpG methylation as well as the appreciable amount of CpA methylation in embryonal stem cells [66]. A histone H3 unmethylated at lysine 4 has been implicated as necessary for de novo methylation by DNMT3A in conjunction with its interacting partner DNMT3L [135]. Increased activity of the histone lysine demethylase LSD1 (KDM1A), which, depending on its interacting partners, demethylates K4- or K9-methylated histone H3, has been found to correlate with an adverse outcome and a less differentiated phenotype in neuroblastomas [136]. Conversely, mutation of the *Lsd1* gene blocks murine gastrulation [137] and results in global DNA hypomethylation. This may be partially due to the need for Lsd1/LSD1 to demethylate the DNMT1 enzyme itself and thereby increase its stability

[137] but also could reflect the role of this enzyme in the demethylation of H3K9me3. There are many other players that could influence DNA methylation during carcinogenesis by their effects on chromatin structure, e.g., poly(ADP-ribosyl)ation, other types of histone modifications, histone variants, nonhistone chromatin proteins, specific interactions with DNMT proteins, and modulation of the set of DNA methyltransferase isoforms produced at the RNA or protein levels [138–143]. Nonetheless, multi-functionality of LSD1 in its ability to demethylate proteins and both activating and repressive histone methylation marks may serve as a paradigm for how, paradoxically, there can be both increases and decreases in DNA methylation in a given cancer cell.

2.8 Active Versus Passive DNA Demethylation

There are two broad classes of mechanisms by which 5mC residues can be replaced by C residues (DNA demethylation). During replicative or repair DNA synthesis there may be a failure to methylate the newly synthesized DNA strand at a symmetrically methylated CpG dyad (passive demethylation), which will initially result in a hemimethylated dyad (Fig. 2.2). If this failure occurs again at the same CpG dyad in the next round of replication, then a symmetrically unmethylated CpG dyad will be the result. Active demethylation involves 5mC residues being physically replaced with C residues (at the base or mononucleotide level) or, less likely, the methyl group being removed enzymatically. Accumulating evidence favors mainly active demethylation contributing to the naturally occurring DNA demethylation by the replacement of C residues [144, 145]. Active demethylation is consistent with

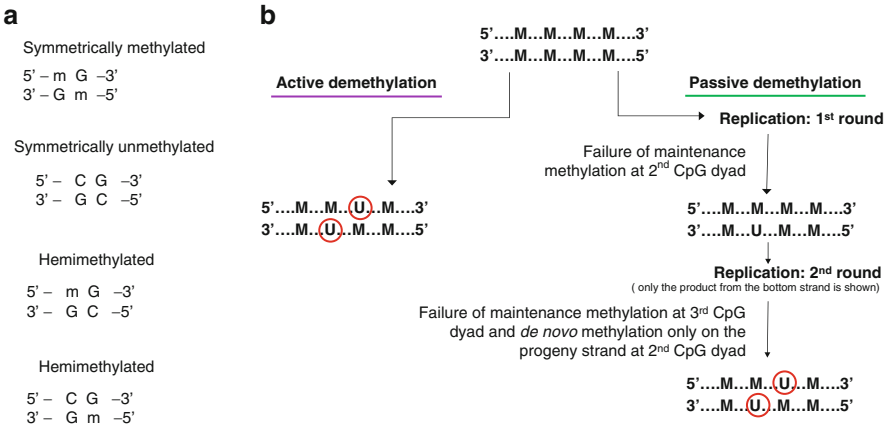

Fig. 2.2 Findings of consecutive hemimethylated dyads of opposite orientation in normal and cancer cells are best explained by active demethylation. (**a**) m, 5mC; C, unmethylated cytosine. (**b**) M, 5′-5mCpG-3′; U, 5′-CpG-3′. The generation of hemimethylated dyads of opposite orientation by passive demethylation would involve improbable changes in the second round of replication

the rapid and distributive loss of 5mC and the replication independence that has been demonstrated for many examples of naturally programmed demethylation of mammalian genomes [146, 147]. However, passive demethylation or a combination of active and passive demethylation due to inadequate maintenance methylation [148] is likely to also play a role in normal and pathological decreases in DNA methylation. Hemimethylated dyads (Fig. 2.2) can be intermediates in both active and passive demethylation of DNA as well as being intermediates in maintenance methylation.

2.9 Maintenance of DNA Methylation Patterns Through Hemimethylated Intermediates

The processes by which DNA methylation patterns are maintained are highly relevant to understanding how DNA demethylation occurs. Over 30 years ago, mechanisms for the inheritance of DNA methylation were initially proposed [149, 150]. In the traditional view, methylation at each site is assumed to be governed by the processes of de novo methylation and maintenance methylation, and these processes are independent of one another. The maintenance of methylation patterns has been attributed to the methyltransferase Dnmt1. As summarized in a 2009 review by Jones and Liang, "The basis of this model is that DNA methylation patterns are established in germ cells and in developing embryos by the activity of the de novo DNA methyltransferases Dnmt3a and Dnmt3B. Subsequently, methylation patterns are inherited after DNA replication primarily owing to the activity of Dnmt1, which has a preference for hemimethylated sites that are generated through DNA synthesis" [151]. The premise of independently acting mechanisms for de novo and maintenance methylation has led to the construction of stochastic models for methylation inheritance [152–157].

2.10 Alternative Mechanisms for Maintenance Methylation

The accepted dogma of de novo methylation catalyzed by DNMT3A/Dnmt3a, DNMT3B/Dnmt3b, and maintenance methylation through obligatory hemimethylated intermediates via DNMT1/Dnmt1 has recently been called into question. According to the original model for maintenance methylation, hemimethylated CpG dyads (Fig. 2.2) should be short-lived and difficult to detect. However, as early as 1986, demethylation with long-lived hemimethylated CpG dyads was observed at individual CpG sites in the avian vitellogenin II gene following treatment with estradiol, which suggested an active pathway through excision repair and/or enzymatic demethylation [158]. A later study of the rat alpha-actin gene promoter provided evidence for hemimethylated intermediates persisting more than 48 hours prior to becoming fully demethylated and suggested active demethylation involving

cis-acting DNA elements [159]. Subsequently, Liang et al. [160] developed an assay that allowed determination of hemimethylation at *Hpa*II sites (CCGG). In mouse embryonic stem cells, levels of hemimethylation in some repetitive sequence regions were significantly higher than the traditional model of maintenance methylation by Dnmt1 would predict. By looking at gene knockouts for *Dnmt1* and *Dnmt3a* and *Dnmt3b*, they deduced that ongoing de novo methylation by Dnmt3a or Dnmt3b in a highly cooperative manner with Dnmt1 in embryonal stem cells compensated for inefficient maintenance methylation by Dnmt1 in these regions. These results suggest a constant, rather than sporadic or only differentiation-associated, role for de novo methylation in vivo. They concluded that sequences would gradually become demethylated without this constant role for de novo methylation to compensate for inefficient replication-coupled maintenance methylation. Furthermore, in a study by Chen et al. [161], loss of Dnmt1 gave only a 10% decrease in methylation overall following one cell cycle of replication in human colorectal carcinoma cells. This conditional knockout resulted in hemimethylation of 18% of sites analyzed by hairpin genomic sequencing in the CGI of an L1 transposable element. The overall level of methylation at CpG dyads in these sequences in cells with normal Dnmt1 was around 85% with no detectable hemimethylation.

In the alternative model for maintaining DNA methylation patterns that was proposed by Jones and Liang [151], DNMT1, the most abundant DNA methyltransferase is still considered to be primarily a maintenance methylase and is responsible for most of the replication-associated DNA methylation. However, they propose that DNMT3A and DNMT3B enzymes remain bound to nucleosomes that contain high levels of DNA methylation. Following replication, CpG dyads whose methylation fails to be correctly maintained by DNMT1 would then be "corrected" by DNMT3A and DNMT3B, so that these enzymes would preserve highly methylated regions without strictly "reading" the patterns on the parental strand. In this way, the methylation state of a region is maintained rather than a site-specific methylation pattern. In addition, DNMT1 might participate in some of this correction of lingering hemimethylated sites that have left the vicinity of the replication fork, perhaps recruited by proteins such as UHRF1 which recognizes hemimethylated sites (see below). This concept of repair methylation is consistent with findings that methylation patterns in highly methylated regions tend to vary among molecules and higher rates of de novo methylation are observed in highly methylated sequences [129]. Moreover, non-CpG methylation at asymmetrical sites, which is found mostly in embryonal stem cells [70], should rely on de novo methyltransferase activity for perpetuating the DNA methylation patterns, as described below.

In cancers, the frequent presence of long blocks of hypomethylated DNA [7, 16, 42, 47, 105] and the usual predominance of overall decreases rather than increases in 5mC content of the genome suggest that passive demethylation contributes to cancer-associated genomic hypomethylation. Passive demethylation might involve either a lack of methylation of hemimethylated sites by DNMT1 or a failure of DNMT3A or DNMT3B to retain dense methylation of a normally highly methylated region. However, the current, more layered view of the maintenance of DNA methylation patterns suggests that while some of the demethyla-

tion of DNA in cancer occurs by a failure of maintenance methylation, most is due to an active mechanism. Recent studies of normal differentiation- or physiology-associated DNA demethylation support an active type of DNA methylation involving enzymatically catalyzed modification of 5mC residues to 5hmC residues (and subsequent oxidation products) or thymine residues followed by DNA repair [162–164]. Three-step processes for active DNA demethylation have been proposed in which 5mC is first enzymatically modified; then demethylated on one strand, most likely by excision repair; and later fully demethylated by a mechanism that avoids inducing double-strand breaks during removal of both 5mCs of a 5mCpG dyad [165]. The last step could involve a repair mechanism that preferentially acts on hemimethylated substrates [165] or passive demethylation of a hemimethylated or hemihydroxymethylated dyad. The latter could be due to the 5hmC residues on one strand of a hemihydroxymethylated dyad not being recognized for maintenance methylation [148].

UHRF1 (also known as NP95) is a cofactor that interacts specifically with hemimethylated DNA and may participate in demethylation as well as de novo methylation of cancer epigenomes. UHRF1 also interacts with DNMT1, and even more strongly with DNMT3A and DNMT3B [166], and thereby, may be involved in the recruitment of DNMT3A/3B to unmethylated regions during tumorigenesis leading to de novo methylation [167]. However, recent work on gliomas has identified the disruption of DNMT1, PCNA, and UHRF1 interactions as a crucial oncogenic event promoting DNA hypomethylation-induced tumorigenesis in the absence of DNMT1 deficiencies [168]. Thus, while upregulation of UHRF1 may contribute to the silencing of tumor suppressors through de novo methylation, the disruption of DNMT1/PCNA/UHRF1 interactions might result in cancer-associated DNA hypomethylation affecting transcription.

2.11 Insights into Cancer-Associated DNA Demethylation from Studies of DNA Hemimethylation

The introduction of hairpin-bisulfite PCR (hairpin genomic sequencing) by Laird et al. in 2004 [129] has enabled the observation of the methylation status on both strands of individual DNA molecules on a site-by-site basis. In bisulfite-based genomic sequencing, bisulfite causes deamination of unmethylated C residues, but not methylated C residues [169]. Hairpin genomic sequencing allows analysis of methylation at every CG dinucleotide pair in a given region on covalently linked DNA strands of a restriction fragment. A caveat about these studies of DNA hemimethylation is that bisulfite-based DNA methylation analysis cannot distinguish between 5hmC and 5mC, as described above, and 5hmC on one strand at a CpG dyad is not recognized for maintenance methylation [170]. Therefore, it is possible that the detected hemimethylation is actually a CpG dyad with one unmethylated C residue and one 5hmC residue. However, in the studies of tandem DNA repeats in cancers described below, this is unlikely because 5hmC is predominantly in gene

regions and all studied cancers and cancer cell lines have extremely low levels of 5hmC [84–86].

By sodium bisulfite-based whole-methylome analysis using next-generation sequencing, Lister et al. analyzed more than 90% of the cytosines in human H1 embryonic stem cells (H1 ES) and IMR90 fetal lung fibroblasts [70]. While nearly all of the methylcytosines detected in the IMR90 fibroblasts were in the CG dinucleotide context, considerable methylation in non-CG contexts (mCHG and mCHH, where H = A, C or T) was observed in the H1 stem cells, comprising almost 25% of the total methylation, in agreement with a recent study by Laurent et al. [66]. Methylation at mCHG sites in H1 ES was also highly asymmetrical, with 98% of such sites observed to be methylated on only one strand. Non-CpG methylation was also found to be significantly higher on the antisense strand of gene bodies, suggesting a nonrandom bias in the observed asymmetry. Non-CpG methylation disappeared upon differentiation of the H1 stem cells, but was restored in differentiated cells induced to form pluripotent stem cells. These findings suggest that asymmetrical methylation at non-CG dinucleotide sites may contribute to maintenance of the pluripotent state. They are reminiscent of the less frequent, hemimethylated CG dinucleotide sites that we and Laird et al. have seen in various DNA repeats [8, 132, 171] or single-copy sequences [129] in normal or cancer tissues.

2.12 Hemimethylated CpG Dyads in Cancer

Although reports of DNA hemimethylation in cancer are few, our studies of hemimethylated DNA in cancers support the involvement of active demethylation in generating cancer-linked genomic hypomethylation. We analyzed DNA methylation changes in depth at the above-mentioned tandem repeats NBL2 and at Sat2 in ovarian epithelial tumors and Wilms tumors by hairpin genomic sequencing [8, 132]. In a study of 13 CpGs in a 0.2-kb subregion of Sat2 in ovarian carcinomas and somatic control tissues, hairpin genomic sequencing not only revealed significantly greater clonal variability in methylation patterns in the cancers than in diverse control tissues but also provided statistically significant evidence of clustering among both hemimethylated and fully demethylated sites [132]. Runs of hemimethylated sites with identical orientation were seen at higher than expected rates in the cancers. Similarly, an analysis of 14 CpGs in the NBL2 repeat unit identified both hypomethylation and hypermethylation in ovarian carcinomas and Wilms tumors, again with a high degree of clonal variation in methylation patterns within each sample [8].

Diverse control and cancer samples contained some DNA clones derived from unusual, consecutive hemimethylated CpG dyads of opposite polarity. Figure 2.2b illustrates how an M/U (5′-5mCpG-3′/3′-GpC-5′) dyad near a U/M dyad (5′-CpG-3′/3′-Gp5mC-5′) could be generated by active vs. passive demethylation. Passive demethylation would require inhibition of maintenance methylation (by DNMT1 alone or in conjunction with DNMT3A and DNMT3B, as discussed above) at a single CpG dyad in one round of replicative DNA synthesis. The next round of replica-

tion would then have to involve both asymmetrical de novo methylation of only the opposite strand of this dyad and inhibition, once again, of maintenance methylation at a neighboring CpG dyad. In contrast to this highly unlikely sequence of events, active demethylation can easily explain the generation of various patterns of hemimethylation in contiguous CpG dyads with either identical or opposite orientation.

In a simulation study jointly analyzing the Sat2 and NBL2 regions, we found that the observed methylation patterns in the carcinomas were best explained by a mechanism that accounted for site-to-site correlation [157]. Prior studies have produced evidence of spreading of methylation in cancer [172–176]. Our analysis suggests that demethylation may progress by spreading as well.

We propose that during carcinogenesis a highly methylated DNA sequence becomes partially demethylated by active demethylation. The sequence may then attain a density of 5mC residues in an atypical intermediate range. This intermediate level of methylation might confer less stability during successive cell divisions for maintenance of the methylation pattern or methylation density. The stability of a given partially methylated sequence could be determined, in part, by the efficiency with which DNMT3A and DNMT3B recognize unmethylated CpG sites in the sequence for repair methylation. Abnormally low methylation levels may favor the generation of yet lower levels, with some site-specific effects superimposed on the regional ones. Thus, active demethylation might start cancer-associated demethylation and a failure of maintenance methylation (including repair methylation) might continue it. The result could explain the observation that tumor progression is frequently linked to a progressive decrease in methylation.

2.13 Conclusions

Recently, there has been a burst of studies increasing our understanding of the importance of changes in DNA methylation in intragenic, promoter, and intergenic regions during differentiation and in response to some types of physiological change. These findings suggest that much more of the cancer-associated DNA hypomethylation contributes to tumor formation and progression than previously recognized. Similarly, high-resolution analysis of cancer methylomes in comparison to appropriate controls indicates that the extent of cancer-linked hypomethylation is larger than previously appreciated and affects a greater variety of DNA sequences. We propose that the pathways for normal DNA demethylation that operate during differentiation or induction of certain physiological changes become hijacked during carcinogenesis and tumor progression, leading to the initiation of cancer-associated DNA demethylation. This demethylation then may spread in *cis* by both additional rounds of active demethylation and by passive demethylation involving failures in classical maintenance methylation and replication-associated repair methylation. The net result of some of this cancer-associated DNA demethylation could be abnormal modulation of transcription and even some aberrant posttranscriptional processing of transcripts as well as increases in DNA recombination, thereby contributing to tumor formation and progression.

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References

1. Gama-Sosa MA, Slagel VA, Trewyn RW, Oxenhandler R, Kuo KC, Gehrke CW, Ehrlich M (1983) The 5-methylcytosine content of DNA from human tumors. Nucleic Acids Res 11:6883–6894
2. Feinberg AP, Vogelstein B (1983) Hypomethylation distinguishes genes of some human cancers from their normal counterparts. Nature 301(5895):89–92
3. Ehrlich M (2009) DNA hypomethylation in cancer cells. Epigenomics 1(2):239–259
4. Ehrlich M, Jiang G, Fiala ES, Dome JS, Yu MS, Long TI, Youn B, Sohn O-S, Widschwendter M, Tomlinson GE, Chintagumpala M, Champagne M, Parham DM, Liang G, Malik K, Laird PW (2002) Hypomethylation and hypermethylation of DNA in Wilms tumors. Oncogene 21(43):6694–6702
5. Ehrlich M (2006) Cancer-linked DNA hypomethylation and its relationship to hypermethylation. Curr Top Microbiol Immunol 310:251–274
6. Ehrlich M (2002) DNA methylation in cancer: too much, but also too little. Oncogene 21(35):5400–5413
7. Pfeifer GP, Rauch TA (2009) DNA methylation patterns in lung carcinomas. Semin Cancer Biol 19(3):181–187
8. Nishiyama R, Qi L, Lacey M, Ehrlich M (2005) Both hypomethylation and hypermethylation in a 0.2-kb region of a DNA repeat in cancer. Molec Cancer Res 3:617–626
9. Tsumagari K, Qi L, Jackson K, Shao C, Lacey M, Sowden J, Tawil R, Vedanarayanan V, Ehrlich M (2008) Epigenetics of a tandem DNA repeat: chromatin DNaseI sensitivity and opposite methylation changes in cancers. Nucleic Acids Res 36:2196–2207
10. Lindsey JC, Lusher ME, Anderton JA, Gilbertson RJ, Ellison DW, Clifford SC (2007) Epigenetic deregulation of multiple S100 gene family members by differential hypomethylation and hypermethylation events in medulloblastoma. Br J Cancer 97(2):267–274
11. Grunau C, Brun ME, Rivals I, Selves J, Hindermann W, Favre-Mercuret M, Granier G, De Sario A (2008) BAGE hypomethylation, a new epigenetic biomarker for colon cancer detection. Cancer Epidemiol Biomarkers Prev 17(6):1374–1379
12. Sultan M, Schulz MH, Richard H, Magen A, Klingenhoff A, Scherf M, Seifert M, Borodina T, Soldatov A, Parkhomchuk D, Schmidt D, O'Keeffe S, Haas S, Vingron M, Lehrach H, Yaspo ML (2008) A global view of gene activity and alternative splicing by deep sequencing of the human transcriptome. Science 321(5891):956–960
13. Alvarez H, Opalinska J, Zhou L, Sohal D, Fazzari MJ, Yu Y, Montagna C, Montgomery EA, Canto M, Dunbar KB, Wang J, Roa JC, Mo Y, Bhagat T, Ramesh KH, Cannizzaro L, Mollenhauer J, Thompson RF, Suzuki M, Meltzer SJ, Melnick A, Greally JM, Maitra A, Verma A (2011) Widespread hypomethylation occurs early and synergizes with gene amplification during esophageal carcinogenesis. PLoS Genet 7(3):e1001356
14. Sandoval J, Heyn HA, Moran S, Serra-Musach J, Pujana MA, Bibikova M, Esteller M (2011) Validation of a DNA methylation microarray for 450,000 CpG sites in the human genome. Epigenetics 6(6):692–702
15. Ruike Y, Imanaka Y, Sato F, Shimizu K, Tsujimoto G (2010) Genome-wide analysis of aberrant methylation in human breast cancer cells using methyl-DNA immunoprecipitation combined with high-throughput sequencing. BMC Genomics 11:137
16. Hansen KD, Timp W, Bravo HC, Sabunciyan S, Langmead B, McDonald OG, Wen B, Wu H, Liu Y, Diep D, Briem E, Zhang K, Irizarry RA, Feinberg AP (2011) Increased methylation variation in epigenetic domains across cancer types. Nat Genet 43(8):768–775

17. Irizarry RA, Ladd-Acosta C, Wen B, Wu Z, Montano C, Onyango P, Cui H, Gabo K, Rongione M, Webster M, Ji H, Potash JB, Sabunciyan S, Feinberg AP (2009) The human colon cancer methylome shows similar hypo- and hypermethylation at conserved tissue-specific CpG island shores. Nat Genet 41(2):178–186
18. Feber A, Wilson GA, Zhang L, Presneau N, Idowu B, Down TA, Rakyan VK, Noon LA, Lloyd AC, Stupka E, Schiza V, Teschendorff AE, Schroth GP, Flanagan A, Beck S (2011) Comparative methylome analysis of benign and malignant peripheral nerve sheath tumors. Genome Res 21(4):515–524
19. Brenet F, Moh M, Funk P, Feierstein E, Viale AJ, Socci ND, Scandura JM (2011) DNA methylation of the first exon is tightly linked to transcriptional silencing. PLoS One 6(1):e14524
20. Song L, Zhang Z, Grasfeder LL, Boyle AP, Giresi PG, Lee BK, Sheffield NC, Graf S, Huss M, Keefe D, Liu Z, London D, McDaniell RM, Shibata Y, Showers KA, Simon JM, Vales T, Wang T, Winter D, Zhang Z, Clarke ND, Birney E, Iyer VR, Crawford GE, Lieb JD, Furey TS (2011) Open chromatin defined by DNaseI and FAIRE identifies regulatory elements that shape cell-type identity. Genome Res 21(10):1757–67
21. Tao Y, Xi S, Briones V, Muegge K (2010) Lsh mediated RNA polymerase II stalling at HoxC6 and HoxC8 involves DNA methylation. PLoS One 5(2):e9163
22. Bauer AP, Leikam D, Krinner S, Notka F, Ludwig C, Langst G, Wagner R (2010) The impact of intragenic CpG content on gene expression. Nucleic Acids Res 38(12):3891–3908
23. Schwartz S, Ast G (2010) Chromatin density and splicing destiny: on the cross-talk between chromatin structure and splicing. Embo J 29(10):1629–1636
24. Okitsu CY, Hsieh CL (2007) DNA methylation dictates histone H3K4 methylation. Mol Cell Biol 27(7):2746–2757
25. Okitsu CY, Hsieh JC, Hsieh CL (2010) Transcriptional activity affects the H3K4me3 level and distribution in the coding region. Mol Cell Biol 30(12):2933–2946
26. Lorincz MC, Dickerson DR, Schmitt M, Groudine M (2004) Intragenic DNA methylation alters chromatin structure and elongation efficiency in mammalian cells. Nat Struct Mol Biol 11(11):1068–1075
27. Deaton AM, Webb S, Kerr AR, Illingworth RS, Guy J, Andrews R, Bird A (2011) Cell type-specific DNA methylation at intragenic CpG islands in the immune system. Genome Res 21(7):1074–1086
28. Maunakea AK, Nagarajan RP, Bilenky M, Ballinger TJ, D'Souza C, Fouse SD, Johnson BE, Hong C, Nielsen C, Zhao Y, Turecki G, Delaney A, Varhol R, Thiessen N, Shchors K, Heine VM, Rowitch DH, Xing X, Fiore C, Schillebeeckx M, Jones SJ, Haussler D, Marra MA, Hirst M, Wang T, Costello JF (2010) Conserved role of intragenic DNA methylation in regulating alternative promoters. Nature 466(7303):253–257
29. Aporntewan C, Phokaew C, Piriyapongsa J, Ngamphiw C, Ittiwut C, Tongsima S, Mutirangura A (2011) Hypomethylation of intragenic LINE-1 represses transcription in cancer cells through AGO2. PLoS One 6(3):e17934
30. Qu G, Grundy PE, Narayan A, Ehrlich M (1999) Frequent hypomethylation in Wilms tumors of pericentromeric DNA in chromosomes 1 and 16. Cancer Genet Cytogenet 109:34–39
31. Eden A, Gaudet F, Waghmare A, Jaenisch R (2003) Chromosomal instability and tumors promoted by DNA hypomethylation. Science 300(5618):455
32. Yamada Y, Jackson-Grusby L, Linhart H, Meissner A, Eden A, Lin H, Jaenisch R (2005) Opposing effects of DNA hypomethylation on intestinal and liver carcinogenesis. Proc Natl Acad Sci USA 102(38):13580–13585
33. Cadieux B, Ching TT, Vandenberg SR, Costello JF (2006) Genome-wide hypomethylation in human glioblastomas associated with specific copy number alteration, methylenetetrahydrofolate reductase allele status, and increased proliferation. Cancer Res 66(17):8469–8476
34. Trejbalova K, Blazkova J, Matouskova M, Kucerova D, Pecnova L, Vernerova Z, Heracek J, Hirsch I, Hejnar J (2011) Epigenetic regulation of transcription and splicing of syncytins, fusogenic glycoproteins of retroviral origin. Nucleic Acids Res 39(20):8728–39

35. Goering W, Ribarska T, Schulz WA (2011) Selective changes of retroelement expression in human prostate cancer. Carcinogenesis 32(10):1484–92
36. Park SY, Yoo EJ, Cho NY, Kim N, Kang GH (2009) Comparison of CpG island hypermethylation and repetitive DNA hypomethylation in premalignant stages of gastric cancer, stratified for Helicobacter pylori infection. J Pathol 219(4):410–6
37. Yang AS, Estecio MR, Doshi K, Kondo Y, Tajara EH, Issa JP (2004) A simple method for estimating global DNA methylation using bisulfite PCR of repetitive DNA elements. Nucleic Acids Res 32(3):e38
38. Weisenberger DJ, Campan M, Long TI, Kim M, Woods C, Fiala E, Ehrlich M, Laird PW (2005) Analysis of repetitive element DNA methylation by MethyLight. Nucleic Acids Res 33(21):6823–6836
39. Nishiyama R, Qi L, Tsumagari K, Dubeau L, Weissbecker K, Champagne M, Sikka S, Nagai H, Ehrlich M (2005) A DNA repeat, NBL2, is hypermethylated in some cancers but hypomethylated in others. Cancer Biol Ther 4(4):440–448
40. Pulukuri SM, Estes N, Patel J, Rao JS (2007) Demethylation-linked activation of urokinase plasminogen activator is involved in progression of prostate cancer. Cancer Res 67(3): 930–939
41. Clark SJ (2007) Action at a distance: epigenetic silencing of large chromosomal regions in carcinogenesis. Hum Mol Genet 16 Spec No 1:R88–95
42. Andrews J, Kennette W, Pilon J, Hodgson A, Tuck AB, Chambers AF, Rodenhiser DI (2010) Multi-platform whole-genome microarray analyses refine the epigenetic signature of breast cancer metastasis with gene expression and copy number. PLoS One 5(1):e8665
43. Novak P, Jensen T, Oshiro MM, Watts GS, Kim CJ, Futscher BW (2008) Agglomerative epigenetic aberrations are a common event in human breast cancer. Cancer Res 68(20): 8616–8625
44. Coolen MW, Stirzaker C, Song JZ, Statham AL, Kassir Z, Moreno CS, Young AN, Varma V, Speed TP, Cowley M, Lacaze P, Kaplan W, Robinson MD, Clark SJ (2010) Consolidation of the cancer genome into domains of repressive chromatin by long-range epigenetic silencing (LRES) reduces transcriptional plasticity. Nat Cell Biol 12(3):235–246
45. Yegnasubramanian S, Wu Z, Haffner MC, Esopi D, Aryee MJ, Badrinath R, He TL, Morgan JD, Carvalho B, Zheng Q, De Marzo AM, Irizarry RA, Nelson WG (2011) Chromosome-wide mapping of DNA methylation patterns in normal and malignant prostate cells reveals pervasive methylation of gene-associated and conserved intergenic sequences. BMC Genomics 12:313
46. Widschwendter M, Jiang G, Woods C, Muller HM, Fiegl H, Goebel G, Marth C, Holzner EM, Zeimet AG, Laird PW, Ehrlich M (2004) DNA hypomethylation and ovarian cancer biology. Cancer Res 64(13):4472–4480
47. Weber M, Davies JJ, Wittig D, Oakeley EJ, Haase M, Lam WL, Schubeler D (2005) Chromosome-wide and promoter-specific analyses identify sites of differential DNA methylation in normal and transformed human cells. Nat Genet 37(8):853–862
48. Ehrlich M (2003) Expression of various genes is controlled by DNA methylation during mammalian development. J Cell Biochem 88:899–910
49. Shen L, Kondo Y, Guo Y, Zhang J, Zhang L, Ahmed S, Shu J, Chen X, Waterland RA, Issa JP (2007) Genome-wide profiling of DNA methylation reveals a class of normally methylated CpG island promoters. PLoS Genet 3(10):2023–2036
50. Ortmann CA, Eisele L, Nuckel H, Klein-Hitpass L, Fuhrer A, Duhrsen U, Zeschnigk M (2008) Aberrant hypomethylation of the cancer-testis antigen PRAME correlates with PRAME expression in acute myeloid leukemia. Ann Hematol 87(10):809–818
51. Milicic A, Harrison LA, Goodlad RA, Hardy RG, Nicholson AM, Presz M, Sieber O, Santander S, Pringle JH, Mandir N, East P, Obszynska J, Sanders S, Piazuelo E, Shaw J, Harrison R, Tomlinson IP, McDonald SA, Wright NA, Jankowski JA (2008) Ectopic expression of P-cadherin correlates with promoter hypomethylation early in colorectal carcinogenesis and enhanced intestinal crypt fission in vivo. Cancer Res 68(19):7760–7768

52. Cheung HH, Davis AJ, Lee TL, Pang AL, Nagrani S, Rennert OM, Chan WY (2011) Methylation of an intronic region regulates miR-199a in testicular tumor malignancy. Oncogene 30(31):3404–3415
53. Colaneri A, Staffa N, Fargo DC, Gao Y, Wang T, Peddada SD, Birnbaumer L (2011) Expanded methyl-sensitive cut counting reveals hypomethylation as an epigenetic state that highlights functional sequences of the genome. Proc Natl Acad Sci USA 108(23):9715–9720
54. Kwon MJ, Shin YK (2011) Epigenetic regulation of cancer-associated genes in ovarian cancer. Int J Mol Sci 12(2):983–1008
55. Laursen KB, Wong PM, Gudas LJ (2011) Epigenetic regulation by RAR{alpha} maintains ligand-independent transcriptional activity. Nucleic Acids Res 40(1):102–15
56. Baba Y, Nosho K, Shima K, Huttenhower C, Tanaka N, Hazra A, Giovannucci EL, Fuchs CS, Ogino S (2010) Hypomethylation of the IGF2 DMR in colorectal tumors, detected by bisulfite pyrosequencing, is associated with poor prognosis. Gastroenterology 139(6):1855–1864
57. Eckhardt F, Lewin J, Cortese R, Rakyan VK, Attwood J, Burger M, Burton J, Cox TV, Davies R, Down TA, Haefliger C, Horton R, Howe K, Jackson DK, Kunde J, Koenig C, Liddle J, Niblett D, Otto T, Pettett R, Seemann S, Thompson C, West T, Rogers J, Olek A, Berlin K, Beck S (2006) DNA methylation profiling of human chromosomes 6, 20 and 22. Nat Genet 38(12):1378–1385
58. Smale ST (2010) Pioneer factors in embryonic stem cells and differentiation. Curr Opin Genet Dev 20(5):519–526
59. Serandour AA, Avner S, Percevault F, Demay F, Bizot M, Lucchetti-Miganeh C, Barloy-Hubler F, Brown M, Lupien M, Metivier R, Salbert G, Eeckhoute J (2011) Epigenetic switch involved in activation of pioneer factor FOXA1-dependent enhancers. Genome Res 21(4): 555–565
60. Xu J, Pope SD, Jazirehi AR, Attema JL, Papathanasiou P, Watts JA, Zaret KS, Weissman IL, Smale ST (2007) Pioneer factor interactions and unmethylated CpG dinucleotides mark silent tissue-specific enhancers in embryonic stem cells. Proc Natl Acad Sci USA 104(30): 12377–12382
61. Taube JH, Allton K, Duncan SA, Shen L, Barton MC (2010) Foxa1 functions as a pioneer transcription factor at transposable elements to activate Afp during differentiation of embryonic stem cells. J Biol Chem 285(21):16135–16144
62. Magnani L, Eeckhoute J, Lupien M (2011) Pioneer factors: directing transcriptional regulators within the chromatin environment. Trends Genet 27(11):465–74
63. Hatada I, Namihira M, Morita S, Kimura M, Horii T, Nakashima K (2008) Astrocyte-specific genes are generally demethylated in neural precursor cells prior to astrocytic differentiation. PLoS One 3(9):e3189
64. Kalari S, Pfeifer GP (2010) Identification of driver and passenger DNA methylation in cancer by epigenomic analysis. Adv Genet 70:277–308
65. Meissner A, Mikkelsen TS, Gu H, Wernig M, Hanna J, Sivachenko A, Zhang X, Bernstein BE, Nusbaum C, Jaffe DB, Gnirke A, Jaenisch R, Lander ES (2008) Genome-scale DNA methylation maps of pluripotent and differentiated cells. Nature 454(7205):766–770
66. Laurent L, Wong E, Li G, Huynh T, Tsirigos A, Ong CT, Low HM, Kin Sung KW, Rigoutsos I, Loring J, Wei CL (2010) Dynamic changes in the human methylome during differentiation. Genome Res 20(3):320–331
67. Ball MP, Li JB, Gao Y, Lee JH, LeProust EM, Park IH, Xie B, Daley GQ, Church GM (2009) Targeted and genome-scale strategies reveal gene-body methylation signatures in human cells. Nat Biotechnol 27(4):361–368
68. Wu H, Coskun V, Tao J, Xie W, Ge W, Yoshikawa K, Li E, Zhang Y, Sun YE (2010) Dnmt3a-dependent nonpromoter DNA methylation facilitates transcription of neurogenic genes. Science 329(5990):444–448
69. Guo JU, Ma DK, Mo H, Ball MP, Jang MH, Bonaguidi MA, Balazer JA, Eaves HL, Xie B, Ford E, Zhang K, Ming GL, Gao Y, Song H (2011) Neuronal activity modifies the DNA methylation landscape in the adult brain. Nat Neurosci 14(10):1345–1351
70. Lister R, Pelizzola M, Dowen RH, Hawkins RD, Hon G, Tonti-Filippini J, Nery JR, Lee L, Ye Z, Ngo QM, Edsall L, Antosiewicz-Bourget J, Stewart R, Ruotti V, Millar AH, Thomson

JA, Ren B, Ecker JR (2009) Human DNA methylomes at base resolution show widespread epigenomic differences. Nature 462(7271):315–322
71. De Bustos C, Ramos E, Young JM, Tran RK, Menzel U, Langford CF, Eichler EE, Hsu L, Henikoff S, Dumanski JP, Trask BJ (2009) Tissue-specific variation in DNA methylation levels along human chromosome 1. Epigenetics Chromatin 2(1):7
72. Ke XS, Qu Y, Cheng Y, Li WC, Rotter V, Oyan AM, Kalland KH (2010) Global profiling of histone and DNA methylation reveals epigenetic-based regulation of gene expression during epithelial to mesenchymal transition in prostate cells. BMC Genomics 11:669
73. Cheng X, Blumenthal RM (2010) Coordinated chromatin control: structural and functional linkage of DNA and histone methylation. Biochemistry 49(14):2999–3008
74. Stengel S, Fiebig U, Kurth R, Denner J (2010) Regulation of human endogenous retrovirus-K expression in melanomas by CpG methylation. Genes Chromosomes Cancer 49(5):401–411
75. Appanah R, Dickerson DR, Goyal P, Groudine M, Lorincz MC (2007) An unmethylated 3' promoter-proximal region is required for efficient transcription initiation. PLoS Genet 3(2):e27
76. Chodavarapu RK, Feng S, Bernatavichute YV, Chen PY, Stroud H, Yu Y, Hetzel JA, Kuo F, Kim J, Cokus SJ, Casero D, Bernal M, Huijser P, Clark AT, Kramer U, Merchant SS, Zhang X, Jacobsen SE, Pellegrini M (2010) Relationship between nucleosome positioning and DNA methylation. Nature 466(7304):388–392
77. Hodges E, Smith AD, Kendall J, Xuan Z, Ravi K, Rooks M, Zhang MQ, Ye K, Bhattacharjee A, Brizuela L, McCombie WR, Wigler M, Hannon GJ, Hicks JB (2009) High definition profiling of mammalian DNA methylation by array capture and single molecule bisulfite sequencing. Genome Res 19(9):1593–1605
78. Luco RF, Allo M, Schor IE, Kornblihtt AR, Misteli T (2011) Epigenetics in alternative pre-mRNA splicing. Cell 144(1):16–26
79. Shing DC, Trubia M, Marchesi F, Radaelli E, Belloni E, Tapinassi C, Scanziani E, Mecucci C, Crescenzi B, Lahortiga I, Odero MD, Zardo G, Gruszka A, Minucci S, Di Fiore PP, Pelicci PG (2007) Overexpression of sPRDM16 coupled with loss of p53 induces myeloid leukemias in mice. J Clin Invest 117(12):3696–3707
80. Chu D, Zhang Z, Zhou Y, Wang W, Li Y, Zhang H, Dong G, Zhao Q, Ji G (2011) Notch1 and Notch2 have opposite prognostic effects on patients with colorectal cancer. Ann Oncol 22(11):2440–7
81. Figueroa JD, Flanders KC, Garcia-Closas M, Anderson WF, Yang XR, Matsuno RK, Duggan MA, Pfeiffer RM, Ooshima A, Cornelison R, Gierach GL, Brinton LA, Lissowska J, Peplonska B, Wakefield LM, Sherman ME (2010) Expression of TGF-beta signaling factors in invasive breast cancers: relationships with age at diagnosis and tumor characteristics. Breast Cancer Res Treat 121(3):727–735
82. Tsumagari K, Chang S-C, Lacey M, Baribault C, Chittur SV, Sowden J, Tawil R, Crawford GE, Ehrlich M (2011) Gene expression during normal and FSHD myogenesis. BMC Medical Genomics 4:67
83. Nagai H, Kim YS, Yasuda T, Ohmachi Y, Yokouchi H, Monden M, Emi M, Konishi N, Nogami M, Okumura K, Matsubara K (1999) A novel sperm-specific hypomethylation sequence is a demethylation hotspot in human hepatocellular carcinomas. Gene 237(1): 15–20
84. Kriaucionis S, Heintz N (2009) The nuclear DNA base 5-hydroxymethylcytosine is present in Purkinje neurons and the brain. Science 324(5929):929–930
85. Li W, Liu M (2011) Distribution of 5-hydroxymethylcytosine in different human tissues. J Nucleic Acids 2011:870726
86. Haffner MC, Chaux A, Meeker AK, Esopi DM, Gerber J, Pellakuru LG, Toubaji A, Argani P, Iacobuzio-Donahue C, Nelson WG, Netto GJ, De Marzo AM, Yegnasubramanian S (2011) Global 5-hydroxymethylcytosine content is significantly reduced in tissue stem/progenitor cell compartments and in human cancers. Oncotarget 2(8):627–37
87. Stroud H, Feng S, Morey Kinney S, Pradhan S, Jacobsen SE (2011) 5-Hydroxymethylcytosine is associated with enhancers and gene bodies in human embryonic stem cells. Genome Biol 12(6):R54

88. Ficz G, Branco MR, Seisenberger S, Santos F, Krueger F, Hore TA, Marques CJ, Andrews S, Reik W (2011) Dynamic regulation of 5-hydroxymethylcytosine in mouse ES cells and during differentiation. Nature 473(7347):398–402
89. Pastor WA, Pape UJ, Huang Y, Henderson HR, Lister R, Ko M, McLoughlin EM, Brudno Y, Mahapatra S, Kapranov P, Tahiliani M, Daley GQ, Liu XS, Ecker JR, Milos PM, Agarwal S, Rao A (2011) Genome-wide mapping of 5-hydroxymethylcytosine in embryonic stem cells. Nature 473(7347):394–397
90. Szulwach KE, Li X, Li Y, Song CX, Han JW, Kim S, Namburi S, Hermetz K, Kim JJ, Rudd MK, Yoon YS, Ren B, He C, Jin P (2011) Integrating 5-hydroxymethylcytosine into the epigenomic landscape of human embryonic stem cells. PLoS Genet 7(6):e1002154
91. Song CX, Szulwach KE, Fu Y, Dai Q, Yi C, Li X, Li Y, Chen CH, Zhang W, Jian X, Wang J, Zhang L, Looney TJ, Zhang B, Godley LA, Hicks LM, Lahn BT, Jin P, He C (2011) Selective chemical labeling reveals the genome-wide distribution of 5-hydroxymethylcytosine. Nat Biotechnol 29(1):68–72
92. Robinson MD, Statham AL, Speed TP, Clark SJ (2010) Protocol matters: which methylome are you actually studying? Epigenomics 2(4):587–598
93. Kinney SM, Chin HG, Vaisvila R, Bitinaite J, Zheng Y, Esteve PO, Feng S, Stroud H, Jacobsen SE, Pradhan S (2011) Tissue-specific distribution and dynamic changes of 5-hydroxymethylcytosine in mammalian genomes. J Biol Chem 286(28):24685–24693
94. Jin SG, Wu X, Li AX, Pfeifer GP (2011) Genomic mapping of 5-hydroxymethylcytosine in the human brain. Nucleic Acids Res 39(12):5015–5024
95. Ehrlich M, Woods C, Yu M, Dubeau L, Yang F, Campan M, Weisenberger D, Long TI, Youn B, Fiala E, Laird P (2006) Quantitative analysis of association between DNA hypermethylation, hypomethylation, and DNMT RNA levels in ovarian tumors. Oncogene 25:2636–2645
96. Rodriguez J, Vives L, Jorda M, Morales C, Munoz M, Vendrell E, Peinado MA (2008) Genome-wide tracking of unmethylated DNA Alu repeats in normal and cancer cells. Nucleic Acids Res 36(3):770–784
97. Kim MJ, White-Cross JA, Shen L, Issa JP, Rashid A (2009) Hypomethylation of long interspersed nuclear element-1 in hepatocellular carcinomas. Mod Pathol 22(3):442–449
98. Roman AC, Gonzalez-Rico FJ, Molto E, Hernando H, Neto A, Vicente-Garcia C, Ballestar E, Gomez-Skarmeta JL, Vavrova-Anderson J, White RJ, Montoliu L, Fernandez-Salguero PM (2011) Dioxin receptor and SLUG transcription factors regulate the insulator activity of B1 SINE retrotransposons via an RNA polymerase switch. Genome Res 21(3):422–432
99. Wang J, Lunyak VV, Jordan IK (2011) Genome-wide prediction and analysis of human chromatin boundary elements. Nucleic Acids Res 40(2):511–29
100. Dante R, Dante-Paire J, Rigal D, Roizes G (1992) Methylation patterns of long interspersed repeated DNA and alphoid repetitive DNA from human cell lines and tumors. Anticancer Res 12(2):559–563
101. Jurgens B, Schmitz-Drager BJ, Schulz WA (1996) Hypomethylation of L1 LINE sequences prevailing in human urothelial carcinoma. Cancer Res 56(24):5698–5703
102. Florl AR, Lower R, Schmitz-Drager BJ, Schulz WA (1999) DNA methylation and expression of LINE-1 and HERV-K provirus sequences in urothelial and renal cell carcinomas. Br J Cancer 80(9):1312–1321
103. Schulz WA, Steinhoff C, Florl AR (2006) Methylation of endogenous human retroelements in health and disease. Curr Top Microbiol Immunol 310:211–250
104. Chan SW, Zilberman D, Xie Z, Johansen LK, Carrington JC, Jacobsen SE (2004) RNA silencing genes control de novo DNA methylation. Science 303(5662):1336
105. Narayan A, Ji W, Zhang X-Y, Marrogi A, Graff JR, Baylin SB, Ehrlich M (1998) Hypomethylation of pericentromeric DNA in breast adenocarcinomas. Int J Cancer 77:833–838
106. Qu G, Dubeau L, Narayan A, Yu M, Ehrlich M (1999) Satellite DNA hypomethylation vs. overall genomic hypomethylation in ovarian epithelial tumors of different malignant potential. Mut Res 423:91–101
107. Bollati V, Fabris S, Pegoraro V, Ronchetti D, Mosca L, Deliliers GL, Motta V, Bertazzi PA, Baccarelli A, Neri A (2009) Differential repetitive DNA methylation in multiple myeloma molecular subgroups. Carcinogenesis 30(8):1330–1335

108. Thoraval D, Asakawa J, Wimmer K, Kuick R, Lamb B, Richardson B, Ambros P, Glover T, Hanash S (1996) Demethylation of repetitive DNA sequences in neuroblastoma. Genes Chromosomes Cancer 17(4):234–244
109. Nagai H, Baba M, Konishi N, Kim YS, Nogami M, Okumura K, Emi M, Matsubara K (1999) Isolation of NotI clusters hypomethylated in HBV-integrated hepatocellular carcinomas by two-dimensional electrophoresis. DNA Res 6(4):219–225
110. Itano O, Ueda M, Kikuchi K, Hashimoto O, Hayatsu S, Kawaguchi M, Seki H, Aiura K, Kitajima M (2002) Correlation of postoperative recurrence in hepatocellular carcinoma with demethylation of repetitive sequences. Oncogene 21(5):789–797
111. Katargin AN, Pavlova LS, Kisseljov FL, Kisseljova NP (2009) Hypermethylation of genomic 3.3-kb repeats is frequent event in HPV-positive cervical cancer. BMC Med Genomics 2:30
112. Szpakowski S, Sun X, Lage JM, Dyer A, Rubinstein J, Kowalski D, Sasaki C, Costa J, Lizardi PM (2009) Loss of epigenetic silencing in tumors preferentially affects primate-specific retroelements. Gene 448(2):151–167
113. Choi SH, Worswick S, Byun HM, Shear T, Soussa JC, Wolff EM, Douer D, Garcia-Manero G, Liang G, Yang AS (2009) Changes in DNA methylation of tandem DNA repeats are different from interspersed repeats in cancer. Int J Cancer 125(3):723–729
114. Ehrlich M, Hopkins N, Jiang G, Dome JS, Yu MS, Woods CB, Tomlinson GE, Chintagumpala M, Champagne M, Diller L, Parham DM, Sawyer J (2003) Satellite hypomethylation in karyotyped Wilms tumors. Cancer Genet Cytogenet 141:97–105
115. Jackson K, Yu M, Arakawa K, Fiala E, Youn B, Fiegl H, Muller-Holzner E, Widschwendter M, Ehrlich M (2004) DNA hypomethylation is prevalent even in low-grade breast cancers. Cancer Biol Ther 3(12):1225–1231
116. Morey Kinney SR, Smiraglia DJ, James SR, Moser MT, Foster BA, Karpf AR (2008) Stage-specific alterations of DNA methyltransferase expression, DNA hypermethylation, and DNA hypomethylation during prostate cancer progression in the transgenic adenocarcinoma of mouse prostate model. Mol Cancer Res 6(8):1365–1374
117. Kerbel RS, Frost P, Liteplo R, Carlow DA, Elliott BE (1984) Possible epigenetic mechanisms of tumor progression: induction of high-frequency heritable but phenotypically unstable changes in the tumorigenic and metastatic properties of tumor cell populations by 5-azacytidine treatment. J Cell Physiol Suppl 3:87–97
118. Santourlidis S, Florl A, Ackermann R, Wirtz HC, Schulz WA (1999) High frequency of alterations in DNA methylation in adenocarcinoma of the prostate. Prostate 39(3):166–174
119. Itano O, Ueda M, Kikuchi K, Shimazu M, Kitagawa Y, Aiura K, Kitajima M (2000) A new predictive factor for hepatocellular carcinoma based on two- dimensional electrophoresis of genomic DNA. Oncogene 19(13):1676–1683
120. Grunau C, Sanchez C, Ehrlich M, van der Bruggen P, Hindermann W, Rodriguez C, Krieger S, De Sario A (2005) Frequent DNA hypomethylation in the human juxtacentromeric BAGE loci in cancer. Genes Chrom Cancer 43(1):11–24
121. Roman-Gomez J, Jimenez-Velasco A, Agirre X, Castillejo JA, Navarro G, San Jose-Eneriz E, Garate L, Cordeu L, Cervantes F, Prosper F, Heiniger A, Torres A (2008) Repetitive DNA hypomethylation in the advanced phase of chronic myeloid leukemia. Leuk Res 32(3): 487–490
122. Yegnasubramanian S, Haffner MC, Zhang Y, Gurel B, Cornish TC, Wu Z, Irizarry RA, Morgan J, Hicks J, DeWeese TL, Isaacs WB, Bova GS, De Marzo AM, Nelson WG (2008) DNA hypomethylation arises later in prostate cancer progression than CpG island hypermethylation and contributes to metastatic tumor heterogeneity. Cancer Res 68(21):8954–8967
123. Marchal R, Chicheportiche A, Dutrillaux B, Bernardino-Sgherri J (2004) DNA methylation in mouse gametogenesis. Cytogenet Genome Res 105(2–4):316–324
124. Zhang X-Y, Loflin PT, Gehrke CW, Andrews PA, Ehrlich M (1987) Hypermethylation of human DNA sequences in embryonal carcinoma cells and somatic tissues but not sperm. Nucleic Acids Res 15:9429–9449
125. Rubin CM, VandeVoort CA, Teplitz RL, Schmid CW (1994) Alu repeated DNAs are differentially methylated in primate germ cells. Nucleic Acids Res 22(23):5121–5127
126. Dupressoir A, Heidmann T (1997) Expression of intracisternal A-particle retrotransposons in primary tumors of oncogene-expressing transgenic mice. Oncogene 14(24):2951–2958

127. Smiraglia DJ, Szymanska J, Kraggerud SM, Lothe RA, Peltomaki P, Plass C (2002) Distinct epigenetic phenotypes in seminomatous and nonseminomatous testicular germ cell tumors. Oncogene 21(24):3909–3916
128. Netto GJ, Nakai Y, Nakayama M, Jadallah S, Toubaji A, Nonomura N, Albadine R, Hicks JL, Epstein JI, Yegnasubramanian S, Nelson WG, De Marzo AM (2008) Global DNA hypomethylation in intratubular germ cell neoplasia and seminoma, but not in nonseminomatous male germ cell tumors. Mod Pathol 21(11):1337–1344
129. Laird CD, Pleasant ND, Clark AD, Sneeden JL, Hassan KM, Manley NC, Vary JC Jr, Morgan T, Hansen RS, Stoger R (2004) Hairpin-bisulfite PCR: assessing epigenetic methylation patterns on complementary strands of individual DNA molecules. Proc Natl Acad Sci USA 101(1):204–209
130. Jiang G, Yang F, van Overveld PG, Vedanarayanan V, van der Maarel S, Ehrlich M (2003) Testing the position-effect variegation hypothesis for facioscapulohumeral muscular dystrophy by analysis of histone modification and gene expression in subtelomeric 4q. Hum Mol Genet 12:2909–2921
131. Zeng W, de Greef JC, Chen YY, Chien R, Kong X, Gregson HC, Winokur ST, Pyle A, Robertson KD, Schmiesing JA, Kimonis VE, Balog J, Frants RR, Ball AR Jr, Lock LF, Donovan PJ, van der Maarel SM, Yokomori K (2009) Specific loss of histone H3 lysine 9 trimethylation and HP1gamma/cohesin binding at D4Z4 repeats is associated with facioscapulohumeral dystrophy (FSHD). PLoS Genet 5(7):e1000559
132. Shao C, Lacey M, Dubeau L, Ehrlich M (2009) Hemimethylation footprints of DNA demethylation in cancer. Epigenetics 4(3):165–175
133. Lau S, Jardine K, McBurney MW (1999) DNA methylation pattern of a tandemly repeated LacZ transgene indicates that most copies are silent. Dev Dyn 215(2):126–138
134. Takai D, Jones PA (2002) Comprehensive analysis of CpG islands in human chromosomes 21 and 22. Proc Natl Acad Sci USA 99(6):3740–3745
135. Hu JL, Zhou BO, Zhang RR, Zhang KL, Zhou JQ, Xu GL (2009) The N-terminus of histone H3 is required for de novo DNA methylation in chromatin. Proc Natl Acad Sci USA 106(52):22187–22192
136. Schulte JH, Lim S, Schramm A, Friedrichs N, Koster J, Versteeg R, Ora I, Pajtler K, Klein-Hitpass L, Kuhfittig-Kulle S, Metzger E, Schule R, Eggert A, Buettner R, Kirfel J (2009) Lysine-specific demethylase 1 is strongly expressed in poorly differentiated neuroblastoma: implications for therapy. Cancer Res 69(5):2065–2071
137. Wang J, Hevi S, Kurash JK, Lei H, Gay F, Bajko J, Su H, Sun W, Chang H, Xu G, Gaudet F, Li E, Chen T (2009) The lysine demethylase LSD1 (KDM1) is required for maintenance of global DNA methylation. Nat Genet 41(1):125–129
138. Zampieri M, Passananti C, Calabrese R, Perilli M, Corbi N, De Cave F, Guastafierro T, Bacalini MG, Reale A, Amicosante G, Calabrese L, Zlatanova J, Caiafa P (2009) Parp1 localizes within the Dnmt1 promoter and protects its unmethylated state by its enzymatic activity. PLoS One 4(3):e4717
139. Ostler KR, Davis EM, Payne SL, Gosalia BB, Exposito-Cespedes J, Le Beau MM, Godley LA (2007) Cancer cells express aberrant DNMT3B transcripts encoding truncated proteins. Oncogene 26(38):5553–5563
140. Lopez de Silanes I, Gorospe M, Taniguchi H, Abdelmohsen K, Srikantan S, Alaminos M, Berdasco M, Urdinguio RG, Fraga MF, Jacinto FV, Esteller M (2009) The RNA-binding protein HuR regulates DNA methylation through stabilization of DNMT3b mRNA. Nucleic Acids Res 37(8):2658–2671
141. Shukla V, Coumoul X, Lahusen T, Wang RH, Xu X, Vassilopoulos A, Xiao C, Lee MH, Man YG, Ouchi M, Ouchi T, Deng CX (2010) BRCA1 affects global DNA methylation through regulation of DNMT1. Cell Res 20(11):1201–1215
142. Felle M, Joppien S, Nemeth A, Diermeier S, Thalhammer V, Dobner T, Kremmer E, Kappler R, Langst G (2011) The USP7/Dnmt1 complex stimulates the DNA methylation activity of Dnmt1 and regulates the stability of UHRF1. Nucleic Acids Res 39(19):8355–65
143. Sharma S, De Carvalho DD, Jeong S, Jones PA, Liang G (2011) Nucleosomes containing methylated DNA stabilize DNA methyltransferases 3A/3B and ensure faithful epigenetic inheritance. PLoS Genet 7(2):e1001286

144. Chen ZX, Riggs AD (2011) DNA methylation and demethylation in mammals. J Biol Chem 286(21):18347–18353
145. He YF, Li BZ, Li Z, Liu P, Wang Y, Tang Q, Ding J, Jia Y, Chen Z, Li L, Sun Y, Li X, Dai Q, Song CX, Zhang K, He C, Xu GL (2011) Tet-mediated formation of 5-carboxylcytosine and its excision by TDG in mammalian DNA. Science 333(6047):1303–1307
146. Kress C, Thomassin H, Grange T (2006) Active cytosine demethylation triggered by a nuclear receptor involves DNA strand breaks. Proc Natl Acad Sci USA 103(30):11112–11117
147. Bhutani N, Burns DM, Blau HM (2011) DNA demethylation dynamics. Cell 146(6): 866–872
148. Inoue A, Zhang Y (2011) Replication-dependent loss of 5-hydroxymethylcytosine in mouse preimplantation embryos. Science 334(6053):194
149. Holliday R, Pugh JE (1975) DNA modification mechanisms and gene activity during development. Science 187:226
150. Riggs AD (1975) X chromosome inactivation, differentiation and DNA methylation. Cytogenet Cell Genet 14:9–25
151. Jones PA, Liang G (2009) Rethinking how DNA methylation patterns are maintained. Nat Rev Genet 10(11):805–811
152. Otto SP, Walbot V (1990) DNA methylation in eukaryotes: kinetics of demethylation and de novo methylation during the life cycle. Genetics 124(2):429–437
153. Pfeifer GP, Steigerwald SD, Hansen RS, Gartler SM, Riggs AD (1990) Polymerase chain reaction-aided genomic sequencing of an X chromosome-linked CpG island: methylation patterns suggest clonal inheritance, CpG site autonomy, and an explanation of activity state stability. Proc Natl Acad Sci USA 87(21):8252–8256
154. Nicolas P, Kim KM, Shibata D, Tavare S (2007) The stem cell population of the human colon crypt: analysis via methylation patterns. PLoS Comput Biol 3(3):e28
155. Sontag LB, Lorincz MC, Georg Luebeck E (2006) Dynamics, stability and inheritance of somatic DNA methylation imprints. J Theor Biol 242(4):890–899
156. Genereux DP, Miner BE, Bergstrom CT, Laird CD (2005) A population-epigenetic model to infer site-specific methylation rates from double-stranded DNA methylation patterns. Proc Natl Acad Sci USA 102(16):5802–5807
157. Lacey M, Ehrlich M (2009) Modeling dependence in methylation patterns with application to ovarian carcinomas. Stat Appl Genet Mol Biol 8(1):40
158. Saluz HP, Jiricny J, Jost JP (1986) Genomic sequencing reveals a positive correlation between the kinetics of strand-specific DNA demethylation of the overlapping estradiol/glucocorticoid-receptor binding sites and the rate of avian vitellogenin mRNA synthesis. Proc Natl Acad Sci USA 83(19):7167–7171
159. Paroush Z, Keshet I, Yisraeli J, Cedar H (1990) Dynamics of demethylation and activation of the alpha-actin gene in myoblasts. Cell 63(6):1229–1237
160. Liang G, Chan MF, Tomigahara Y, Tsai YC, Gonzales FA, Li E, Laird PW, Jones PA (2002) Cooperativity between DNA methyltransferases in the maintenance methylation of repetitive elements. Mol Cell Biol 22(2):480–491
161. Chen T, Hevi S, Gay F, Tsujimoto N, He T, Zhang B, Ueda Y, Li E (2007) Complete inactivation of DNMT1 leads to mitotic catastrophe in human cancer cells. Nat Genet 39(3): 391–396
162. Gehring M, Reik W, Henikoff S (2009) DNA demethylation by DNA repair. Trends Genet 25(2):82–90
163. Zhu JK (2009) Active DNA demethylation mediated by DNA glycosylases. Annu Rev Genet 43:143–166
164. Bhutani N, Brady JJ, Damian M, Sacco A, Corbel SY, Blau HM (2010) Reprogramming towards pluripotency requires AID-dependent DNA demethylation. Nature 463(7284): 1042–1047
165. Kress C, Thomassin H, Grange T (2001) Local DNA demethylation in vertebrates: how could it be performed and targeted? FEBS Lett 494(3):135–140
166. Meilinger D, Fellinger K, Bultmann S, Rothbauer U, Bonapace IM, Klinkert WE, Spada F, Leonhardt H (2009) Np95 interacts with de novo DNA methyltransferases, Dnmt3a and Dnmt3b, and mediates epigenetic silencing of the viral CMV promoter in embryonic stem cells. EMBO Rep 10(11):1259–1264

167. Jeong S, Liang G, Sharma S, Lin JC, Choi SH, Han H, Yoo CB, Egger G, Yang AS, Jones PA (2009) Selective anchoring of DNA methyltransferases 3A and 3B to nucleosomes containing methylated DNA. Mol Cell Biol 29(19):5366–5376
168. Hervouet E, Lalier L, Debien E, Cheray M, Geairon A, Rogniaux H, Loussouarn D, Martin SA, Vallette FM, Cartron PF (2010) Disruption of Dnmt1/PCNA/UHRF1 interactions promotes tumorigenesis from human and mice glial cells. PLoS One 5(6):e11333
169. Frommer M, McDonald LE, Millar DS, Collis CM, Watt F, Grigg GW, Molloy PL, Paul CL (1992) A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. Proc Natl Acad Sci USA 89:1827–1831
170. Valinluck V, Sowers LC (2007) Endogenous cytosine damage products alter the site selectivity of human DNA maintenance methyltransferase DNMT1. Cancer Res 67(3):946–950
171. Burden AF, Manley NC, Clark AD, Gartler SM, Laird CD, Hansen RS (2005) Hemimethylation and non-CpG methylation levels in a promoter region of human LINE-1 (L1) repeated elements. J Biol Chem 280(15):14413–14419
172. Turker MS (2002) Gene silencing in mammalian cells and the spread of DNA methylation. Oncogene 21(35):5388–5393
173. Yan PS, Shi H, Rahmatpanah F, Hsiau TH, Hsiau AH, Leu YW, Liu JC, Huang TH (2003) Differential distribution of DNA methylation within the RASSF1A CpG island in breast cancer. Cancer Res 63(19):6178–6186
174. Nguyen C, Liang G, Nguyen TT, Tsao-Wei D, Groshen S, Lubbert M, Zhou JH, Benedict WF, Jones PA (2001) Susceptibility of nonpromoter CpG islands to de novo methylation in normal and neoplastic cells. J Natl Cancer Inst 93(19):1465–1472
175. Stirzaker C, Song JZ, Davidson B, Clark SJ (2004) Transcriptional gene silencing promotes DNA hypermethylation through a sequential change in chromatin modifications in cancer cells. Cancer Res 64(11):3871–3877
176. Homma N, Tamura G, Honda T, Matsumoto Y, Nishizuka S, Kawata S, Motoyama T (2006) Spreading of methylation within RUNX3 CpG island in gastric cancer. Cancer Sci 97(1): 51–56

Chapter 3
Ten Eleven Translocation Enzymes and 5-Hydroxymethylation in Mammalian Development and Cancer

Shannon R. Morey Kinney and Sriharsa Pradhan

Abstract 5-Hydroxymethylcytosine (5hmC) is an oxidative product of 5-methylcytosine (5mC), catalyzed by the ten eleven translocation (TET) family of enzymes. Although 5hmC was discovered several decades ago, it was only after its recent identification in murine brain and stem cell DNA that it has become a major focus of epigenomic research. Part of the reason for this delay is due to the difficulty in detecting both global and locus-specific 5hmC levels. Several studies have addressed this issue with the development of novel techniques to locate and measure 5hmC, which led to multiple reports detailing 5hmC patterns in stem cells and global 5hmC levels during embryogenesis. Based on these studies of 5hmC levels and reports of tissue-specific TET expression, these enzymes are thought to play a role in mammalian development and differentiation. In addition, the TET enzymes are mutated in several types of cancer, affecting their activity and likely altering genomic 5hmC and 5mC patterns. Furthermore, oxidation of 5mC appears to be a step in several active DNA demethylation pathways, which may be important for normal processes, as well as global hypomethylation during cancer development and progression. Much has been revealed about this interesting DNA modification in recent years, but more research is needed for understanding the role of TET proteins and 5hmC in gene regulation and disease.

3.1 Discovery and History of 5-Hydroxymethylation

Methylation of cytosine residues at the 5-carbon position (5-methylcytosine, 5mC) has been studied as a stable epigenetic modification for decades [1]. However, oxidation of DNA has traditionally been considered a DNA damage event, which is readily removed by DNA repair pathways [2]. Recently, it was demonstrated that

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enzymatic oxidation of 5mC to 5hmC (5-hydroxymethylcytosine) may act as a stable modification of DNA and downstream removal of 5hmC may actually be part of a complex and intricate process of epigenetic gene regulation [3].

5-Hydroxymethylcytosine (5hmC) was first identified in T-even bacteriophages during early 1950s using paper chromatography and ultraviolet absorbance spectra [4]. This nucleotide is normally incorporated during DNA synthesis and then further glycosylated by phage encoded glucosyltransferases as a mechanism for protection of the phage DNA from bacterial restriction enzymes during infection [5, 6]. Later, during the 1970s, 5hmC was detected in genomic DNA purified from brain tissue of rats, mice, and frogs and, to a lesser extent, from liver tissue of rats [7]. The same group also observed an increase in 5hmC levels in the adult compared to newborn rat brain, as well as a decrease of 5hmC levels in brains from rats with low protein diets [8]. Unfortunately, these experiments could not be reproduced and this DNA modification was overlooked for several decades [9].

In 2009, 5hmC was rediscovered in mammalian DNA and shown to be present in substantial amounts (~10 to 20% of 5mC) in murine embryonic stem cells (ESCs) [10], Purkinje neurons, and granule cells [11]. These recent studies utilized more advanced analytical techniques, such as 2D thin layer chromatography (TLC) or high-pressure liquid chromatography (HPLC) coupled with mass spectrometry (MS), to confirm the existence of this DNA modification in mammalian DNA. In addition, 5hmC was recently identified in mammalian mitochondrial DNA [12]. As a result of these discoveries, there is a huge amount of interest in developing technologies for genome-wide mapping and site-specific quantification of 5hmC in an effort to decipher its possible role in development and disease.

3.2 TET Enzymes and Their Catalytic Activity

There are three known mammalian 5mC dioxygenases, which catalyze the conversion of 5mC to 5hmC [10]. These proteins belong to the family of ten eleven translocation (TET) enzymes, whose name is based on a common chromosomal translocation in some cancers (described in detail later in this chapter). TET1 was originally named leukemia-associated protein with a CXXC domain (LCX) when it was initially cloned in 2002 [13]. This gene was rediscovered in 2003 along with the two other members of the family and they were renamed ten eleven translocation, or TET, genes [14]. All three TET proteins share a similar catalytic domain structure to 2-oxoglutarate (2-OG) oxygenases. These types of enzymes can oxidize DNA and RNA that is methylated on either the nitrogen (N) or carbon (C) of the base by conversion of 2-OG and oxygen to carbon dioxide and succinate [15]. The TETs were identified based on their similarity to the JBP1 and JBP2 enzymes in trypanosome, which were originally named for their ability to bind to the unique nucleotide β-D-glucosylhydroxymethyl-uracil (base J) and then later were reported to hydroxylate thymine, the first step in the conversion of base J [16]. Proteins with similar

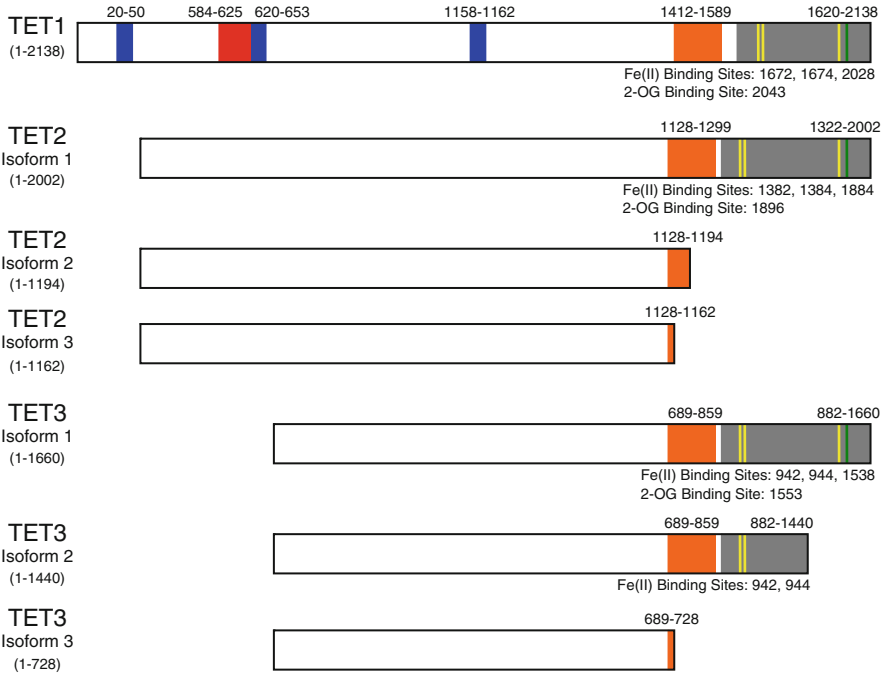

Fig. 3.1 Diagram of TET enzyme isoforms. TET1 is 2138 aa long with multiple putative Nuclear Localization Sequence (NLS), a CXXC motif, and cysteine-rich region N-terminal to the DSBH making up the core catalytic domain. There are three isoforms of TET2, the longest being 2002 aa long. TET2 does not contain any putative NLS or CXXC motif, but does have a core catalytic domain very similar to TET1. TET3 also has three isoforms, of 1660 aa or less in length. Similar to TET2, TET3 does not appear to have any other domains other than the core catalytic domain. Numbers in brackets represent length of proteins in aa or location of domains. *Blue bars* NLS; *red bar* CXXC motif; *orange* bar Cysteine-rich region; *Gray bar* DSBH; *yellow bars* Fe(II) binding sites; *green bar* 2-OG binding site

homology to the TET proteins are found in several lower eukaryote groups, including *Drosophila* [17].

The human TET1 gene is found at chromosomal location 10q21 and is approximately 134 kilobases (kb) long [18]. The resulting transcript contains 12 exons and is approximately 9.6 kb. The TET1 protein consists of ~2,136 amino acids encoding a 236 kilodalton (kDa) enzyme. TET1 is a multidomain protein containing several putative nuclear localization sequences, a binuclear Zn^{+2}-chelating CXXC domain, and a cysteine-rich region preceding the catalytic domain (Fig. 3.1). CXXC domains are frequently found in chromatin binding proteins, including DNA (cytosine-5) methyltransferase-1 (DNMT1), 5-methylcytosine binding proteins (MBDs), and mixed lineage leukemia (MLL) protein [19]. The CXXC domain of TET1 binds to CG-rich sequences of both methylated and unmethylated DNA, with some preference for unmethylated CpGs in cell free assays [19, 20].

The human TET2 gene is found on chromosome 4q24 and contains 11 exons, which can result in three known isoforms produced through alternative splicing. The longest form of TET2 is ~2,002 amino acids and similar to TET1 with approximately 70% homology in their C-terminal regions, including their catalytic domains [18]. There are two shorter isoforms of TET2 (1,164 and 1,194 amino acids long) that both lack catalytic domains due to truncation or introduction of stop codons (Fig. 3.1). The TET3 gene resides on chromosome 2p13. It is approximately 62 kb in length, with a transcript containing nine exons. Similar to TET2, the TET3 protein sequence shares approximately 70% sequence homology to TET1 in the regions surrounding the catalytic domain (Fig. 3.1). Three putative isoforms of TET3 have been identified using complementary DNA screening [18]. These include the full-length protein, as well as two shorter variants that are missing either a small portion, or most of the catalytic domain (Fig. 3.1).

TET2 and TET3 differ from TET1 in that they do not appear to contain any putative nuclear localization sequences or regions similar to a CXXC domain [18]. Interestingly, one study reported that the CXXC4 gene, at 4q22-24, is a very close neighbor to TET2 and may be the result of a chromosomal inversion of the TET2 CXXC domain followed by a translocation [17]. It has been proposed that interaction of CXXC4 and TET2 may be required for appropriate TET2 targeting and activity [17].

The catalytic domains of all 2-oxoglutarate (2-OG) oxygenases contain a double-stranded β-helix (DSBH) [10, 15]. The DSBH domain, in addition to the cysteine-rich region, of TET1 has been found to be both necessary and sufficient for catalytic activity [10]. Furthermore, the DSBH domain contains three Fe(II) binding sites and a 2-OG binding site (details in Fig. 3.1) [18]. Amino acid mutation studies have confirmed the requirement of these domains for TET catalytic activity [21].

The increased homology within the cysteine-rich region and the DSBH domain of TET1, TET2, and TET3 suggests that they have similar catalytic activity. Each protein of this family also contains unique regions indicating that they may have distinct binding affinities to chromatin and/or protein partners, resulting in the establishment of specific 5hmC patterns in various cell types and during different developmental stages. All three forms of the Tet enzymes are known to be catalytically active in cells [22] and tissue-specific expression of TET transcripts has also been reported [23, 24], supporting the above hypothesis.

Triple knockout (TKO, knockout of Dnmt1, Dnmt3a, and Dnmt3b) ESCs display decreased 5hmC levels although they have normal Tet expression. This confirms that the 5mC catalyzed by Dnmts is in fact the substrate for the Tet enzymes [22, 25]. In addition to the oxidation of 5mC to 5hmC, the TET enzymes have recently been reported to have the ability to further oxidize 5hmC to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) [26, 27]. Quantification of the three oxygenated forms of 5mC reveals unequal distribution with much more 5hmC than 5fC or 5caC in genomic DNA [27]. The function of these less frequent enzymatic products of TET enzymes is not well understood, but current knowledge

suggests they may be involved in the DNA demethylation process described later in this chapter [28].

3.3 Technologies and Advancements in 5hmC Detection

Identifying and quantifying 5hmC globally and at specific loci has been, and continues to be, quite a challenge. For example, the most accepted technique for 5mC detection and measurement, bisulfite sequencing, does not differentiate between 5mC and 5hmC or unmodified C and 5caC [28, 29]. Additionally, restriction enzymes have been used for years to specifically digest methylated or unmethylated DNA and recent data shows that many of these enzymes have different specificities or sensitivities for oxidized forms of 5mC or glucosylated 5hmC (5ghmC) [30–33]. Indeed, many of the 5mC-sensitive enzymes that have previously been used to measure DNA methylation are also sensitive to 5hmC [34]. Complicating matters further, 5mC-specific antibodies appear to have no cross reactivity with 5hmC, thus in the past oxidation of 5mC may have been mistaken for demethylation. Since the discovery of 5hmC in mammalian DNA there has been a flurry of new techniques reported to measure this elusive base, either globally or at a specific locus.

There are several techniques that have been shown to evaluate global 5hmC levels. Some are more qualitative than quantitative and each has its own range of sensitivity and accuracy. Initially, the existence of 5hmC (followed by 5fC and 5caC) in mammalian DNA was discovered using restriction enzyme-based TLC [7, 10, 11, 27, 28]. Dot blot of genomic DNA and immunofluorescence in mammalian cells using 5hmC-specific antibodies has also been used extensively to examine global 5hmC levels [22, 25, 35]. These antibodies appear to be sensitive but seem to require several proximal 5hmC sites for measurable binding to occur [36]. More recently, an antibody was developed targeting cytosine 5-methylenesulfonate (CMS), a product of sodium bisulfite treatment of hydroxymethylated DNA that can apparently detect as few as one 5hmC site on DNA [21]. Although these techniques are not truly quantitative, they offer more sensitivity as the input DNA could be as low as several nanograms. Currently, the most sensitive techniques for measuring global 5hmC, 5fC, and 5caC utilize HPLC coupled with mass spectrometry [27, 28, 37]. However, these techniques require unique expertise and complex analytical machinery. A simple, yet very accurate and sensitive, technique for measuring global 5hmC uses the T4 phage enzyme, β-glucosyltransferase (β-GT), and radioactive UDP-[^{3}H]-glucose [38, 39].

The 5hmC and CMS-specific antibodies mentioned above have also been utilized for hydroxymethylcytosine-DNA immunoprecipitation (hMe-DIP) followed by next generation sequencing, DNA array, or PCR [25, 36, 40, 41]. A second technique, (glucosylation, periodate oxidation, biotinylation, or GLIB) uses a glucosylation reaction to attach UDP-6-N3-glucose to 5hmC, which marks these sites with a reactive azide group. The azide group is further reacted with biotin using click

chemistry for subsequent pulldown with a streptavidin matrix [42, 43]. Although data derived from these techniques can be extremely useful in mapping the regions of 5hmC, it still does not offer single base resolution. Single-molecule real-time (SMRT) sequencing is a novel sequencing technique that can discriminate between unmodified cytosine, 5mC, and 5hmC due to variations in polymerase kinetics during the sequencing reaction [44]. It is also possible to differentiate 5mC from 5hmC with nanopore amperometry, as each modification causes unique breaks in current as synthetic DNA molecules are fed through nanopores [45]. Current research is focused towards optimizing the last two methods for genomic DNA samples and for high-throughput analysis, but these technologies are not yet commonly used.

Many restriction enzymes that can differentiate between 5mC and unmodified cytosine, as well as families of enzymes that target 5hmC or 5ghmC are being studied for unique properties that make them useful for measuring 5hmC [30, 33, 46]. For example, MspI and GlaI can fully digest 5mC or 5hmC in their respective target sequences, but after conversion of 5hmC to 5ghmC, digestion by both of these enzymes is blocked [32, 47]. Taq$^{\alpha}$I is a restriction enzyme that is not fully blocked by 5ghmC, but is blocked by biotin-N3-5gmC [31]. Therefore, tagging a 5hmC residue with glucose or a modified glucose may be a valuable tool for epigenetic studies. In contrast to restriction enzymes that are blocked by 5hmC or 5ghmC, but not by unmodified cytosine, another class known as PvuRts1I family show digestion preference for 5hmC or 5ghmC as compared to 5mC and cytosine [30, 33]. Using this class of enzymes for digestion followed by PCR amplification of a region of interest can reveal the level of 5hmC at a specific site. Alternatively, one could use the digested fragments for next generation sequencing for genome-wide mapping of 5hmC.

Novel and more accurate techniques for measuring 5hmC will be available in the near future as the epigenetics field progresses with reference to this modification. We must always consider how to normalize traditional techniques and any new ones that are developed to evaluate various DNA modifications when drawing conclusions about how epigenomic modification patterns relate to biological phenomenon.

3.4 Tet1 Binding and 5hmC in Embryonic Stem Cells

It is important to understand the normal function of TET enzymes and 5hmC in order to comprehend how and why they may be disrupted in disease. The study of mouse ESCs may allow us to gain some insight into these phenomena. Mouse ESCs are derived from the inner cell mass (ICM) of blastocysts and can be cultured in an undifferentiated state with use of leukemia inhibitory factor (LIF) [48]. ESCs can be differentiated into embryoid bodies (EB) with the removal of LIF or into other more specific lineages by addition or removal of cytokines and specific growth factors. As mentioned earlier, ESCs tend to have high levels of 5hmC as compared to other cell

types [10, 27]. It is thought that the TET enzymes and 5hmC may play a significant biological role in ESCs because epigenetic modifications and factors are important for both maintaining an undifferentiated state and for differentiation. *Tet1* and *Tet2* are expressed in ESCs and induced pluripotent stem (iPS) cells, while *Tet3* expression is quite low, suggesting that Tet1 and Tet2 are especially important for maintaining a pluripotent status [22, 49]. Furthermore, expression of *Tet1* and *Tet2* is repressed during differentiation and it appears that Oct4 [49], one of a few transcription factors that are required for ESC pluripotency and dedifferentiation of somatic cells, is involved in regulating Tet1 and Tet2 expression [50].

A number of reports describe Tet1 binding and/or 5hmC status throughout the genome of mouse ESCs and the relationship of these patterns to gene expression [25, 41, 42, 47, 51, 52]. Several techniques were utilized in these studies, including ChIP-seq, GLIB-Seq, hMeDIP-Seq, restriction enzyme-dependent genome-wide sequencing, and hMeDIP-Chip (with 5hmC and CMS-specific antibodies), as well as RNA-Seq and microarray analyses [25, 41, 42, 51, 52]. Even though there are some disagreements between these studies, their overall conclusions are similar. In general, Tet1 binds to CG-rich regions of the genome, which seems to be due, at least in part, to its CXXC domain. Tet1 binds to both active and inactive genes, with more binding in the gene bodies of active genes and increased binding in the promoters and transcriptional start sites (TSS) of inactive genes. Tet1 targeted genes are involved in many cellular pathways, including development, differentiation, and neural processes [22, 25, 49, 52]. Tet1 also appears to be enriched in regions containing the active H3K4me3 mark, as well as the bivalent H3K4me3 and H3K27me3 marks, and to a lesser extent at polycomb repressed genes exhibiting only H3K27me3 mark.

5hmC patterns in the genome are very similar to Tet1 binding. Both 5mC and its oxidative product 5hmC are commonly found in the gene bodies of active genes and in the promoters of inactive genes. Surprisingly, there are a number of Tet1 binding sites that do not appear to contain 5hmC. This suggests that Tet1 may have additional non-catalytic activities or that 5hmC is quickly removed specifically at these loci as part of a DNA demethylation/repair pathway. Several studies indicate that gene body 5hmC is more prevalent in exons than introns [25, 42, 51, 52]; however, results from another group indicated more enrichment in introns [41]. These ambiguities could be due to differences in the techniques utilized and will likely be sorted out in the future with base resolution mapping of the respective mammalian hydroxymethylome. Interestingly, 5hmC is enriched in and around the TSS, which is in contrast to a general reduction in 5mC at these locations [51]. Intergenic regions and repetitive elements appear to have less 5hmC than coding regions. Thus, 5hmC and 5mC coexist in some genomic regions, while also displaying unique patterns of genomic localization. Genome-wide 5hmC patterns have also been reported for human ESCs and they closely match with the description of mouse ESCs [36]. The patterning observed in both mouse and human ESCs suggests that 5hmC may have a more specific role in regulating transcription, while 5mC has additional roles in maintaining genomic integrity and transposon stability.

Upon knockdown of Tet1 expression or gene knockout, there are clear increases in both locus-specific and global 5mC with concomitant decreases in

5hmC globally and at Tet1 target sites [22, 47, 53]. In addition, loss or reduction of Tet1 consistently resulted in both increased and decreased gene expression with gene activation being associated with promoter hypo-hydroxymethylation [41, 51–53]. Tet1 enrichment occurs at almost two-thirds of all genes in mouse ESCs and thus overlaps with a number of chromatin modifying and transcriptional regulatory proteins, such as Suz12, Ezh2, Sin3a, Mbd3, and LIF activated Stat3 [41, 47, 51, 54]. Concomitantly, the binding of these proteins to the chromatin is reduced by Tet1 knockdown [41, 47, 51, 54]. It is not clear whether it is direct interaction with Tet1, possibly via other bridge proteins, or 5hmC that provides a platform for their recruitment to specific regions of the chromatin, except in the cases of Sin3a and Mbd3. These two proteins have been shown to either bind directly to Tet1 or in a complex with Tet1 by co-immunoprecipitation experiments. Mbd3 also appears to bind to 5hmC-modified DNA, which is thought to result in its recruitment to inactive genes [51, 54].

There is an overall enrichment of 5hmC at regulatory protein binding sites, such as gene promoters, enhancers, and insulators further supporting the hypothesis of 5hmC-specific binding proteins [25, 36]. In addition to transcriptional regulation by putative 5hmC binding proteins, active promoters bound by Tet1 may be maintained in an unmethylated state through constant oxidation of 5mC, allowing transcription factors and RNA polymerase to bind. Based on these observations, Tet enzymes can regulate the levels of both 5hmC and 5mC at specific gene sequences in order to direct the binding of transcriptional regulator proteins, resulting in both positive and/or negative effects on its expression.

3.5 Role of Tets and 5hmC in Early Mammalian Development and Embryonic Stem Cells

The mammalian paternal zygotic genome is thought to be actively demethylated upon fertilization of the egg and this demethylated state persists over the next several cell divisions, during which time the maternal genome undergoes passive demethylation [55]. At the blastocyst stage of development, both the maternal and paternal DNAs are remethylated by the de novo methyltransferases. The observation that the paternal genome is demethylated is based primarily on studies utilizing anti-5mC antibody staining and bisulfite sequencing of a small number of loci [56–59]. However, recent data suggests that the lack of staining of the paternal genome by the 5mC antibody is actually due to conversion of 5mC to 5hmC [35, 60]. High levels of 5hmC in the paternal genome persist for several genome replications suggesting that demethylation is not as extensive as was previously thought and may take place only at specific loci [35]. Technological advances that allow for the paternal and maternal DNA to be fully sequenced for epigenetic modifications will help in the future to resolve this important observation.

Tet3 is the most likely Tet family member that oxidizes the paternal DNA as it is expressed at high levels in oocytes and zygotes, but not at later developmental stages

[35, 60, 61]. *Tet1* seems to only be expressed at the two- and four-cell stages and in ESCs, and *Tet2* is only expressed at very low levels throughout fertilization and zygote development, except in ESCs where Tet2 expression is higher [35, 60]. Knockdown of *Tet3* by siRNA injection into the oocyte or conditional knockout of Tet3 in primordial germ cells (PGC) of mice significantly reduces oxidation of 5mC in the paternal genome [60, 61]. Furthermore, the Tet3 responsible for this process appears to be of maternal origin as wild-type (WT) females crossed with Tet3 conditionally null males did not exhibit this defect [61]. Finally, primordial germ cell gene 7 (PGC7) may be involved in protecting the maternal genome from demethylation [62] and knockout of this gene results in oxidation of the maternal genome [60]. It is not clear why only the paternal genome methylation specifically undergoes widespread oxidation during zygote development, but this process is likely involved in locus-specific 5mC erasure and epigenetic reprogramming of the chromatin.

There are several contradicting reports on whether knockdown or knockout of Tet genes alters growth and differentiation of ESCs. Two studies report that knockdown of *Tet1*, but not *Tet2* or *Tet3*, in mouse ESCs results in decreased alkaline phosphatase activity (a marker of healthy ESCs) and pluripotency associated genes, as well as an increase in differentiation markers and altered cell growth and morphology [22, 47]. It is suggested that this may be due to a decrease in *Nanog* expression as reintroduction of Nanog can rescue the phenotype. ChIP analysis shows that Tet1 binds to the Nanog promoter and depletion of Tet1 results in methylation and suppression of the *Nanog* gene. Furthermore, use of Dnmt TKO ES cells prevents the methylation and repression of *Nanog* [22].

In contrast, other studies did not report any effects on morphology or *Nanog* expression with *Tet1* knockdown or knockout in undifferentiated cells [49, 51, 53]. However, there was agreement amongst some reports that *Tet1* knockdown upregulates genes involved in trophectoderm and endoderm development and represses genes involved in neuroectoderm development [22, 49, 53]. Loss of Tet1 function in ESCs results in differentiation toward endoderm/mesoderm and trophoblast lineages. Based on this, and because *Tet1* is primarily expressed in the ICM (not the trophectoderm), it is thought that Tet1 participates in preventing the expression of trophectoderm developmental genes and maintaining proper cellular specification in embryos [22, 49]. *Tet2* knockdown did not seem to affect trophectoderm, endoderm, or mesoderm genes but did slightly increase neuroectoderm markers. In addition, knockdown of either *Tet1* or *Tet2* alters expression of unique subsets of genes suggesting that each enzyme has unique target regions in the genome [49]. *Tet3* knockdown in ESCs had minimal transcriptional effects on the differentiation genes that were examined.

Tet1 knockout ESCs are capable of producing live pups and loss of Tet1 has minimal effects on embryogenesis and mouse development, as Tet1 homozygous null mice maintain proper Mendelian ratios, appear healthy, and are fertile [53]. The only initial observations of aberrant development are that both male and female Tet1 null mice are born at lower body weight (although they are similar to WT mice as adults); they have slightly decreased neutrophil numbers, and smaller litter sizes when inter-crossed. These mice do not appear to have any myeloid or other disorders

[53]. Complete knockout of Tet2 has not yet been reported, but a mouse model has been developed that utilizes a Tet2-LacZ fusion to express an inactive Tet2 protein [63]. However, these mice maintain 20–50% of normal Tet2 transcripts, have no obvious reduction in 5hmC, are normal in overall appearance, and display expected Mendelian ratios. In spite of this, and unlike Tet1 null mice, Tet2 hypomorphs do appear to have aberrant hematopoiesis [63]. Although no changes in Tet gene expression have been reported, it is possible that the different members of the Tet family are compensating for the loss or reduction of Tet1 or Tet2 in these mouse models [53, 63].

Tet3 null mice are unique in that they exhibit neonatal lethality [61]. This lethality was overcome by creation of Tet3 conditional knockout mice [61]. As described above, the parental mice only lack Tet3 expression in PGC and thus are essentially normal, with only the females exhibiting reduced fecundity. The zygotes of these mice have decreased 5hmC levels and aberrant reprogramming of the paternal DNA, which is thought to disrupt prenatal development [61].

Tissue-specific expression of Dnmts and patterning of 5mC is known to be involved in gene regulation. It is hypothesized that Tet enzyme activity and 5hmC may be involved in specific biological functions in different tissues and organs as well. Indeed, TET enzymes display altered expression levels depending on the tissue or the stage of development [22–24, 35, 60]. A number of tissue types have been examined for TET expression, including but not limited to brain, lung, liver, heart, and kidney. *TET1* and *TET2* exhibit varied expression levels in different tissues examined [23] and isoforms 2 and 3 of *TET2* are expressed at a lower level than its isoform 1 [24]. Overall TET2 and its isoforms appear to be the most highly expressed amongst the TET enzymes in many tissues [22, 24]. *TET3* also tends to have consistently high expression across various tissues [23]. All TETs are highly expressed in hematopoietic cells, with *TET2* and *TET3* being the highest. Consistently, hypomorphic expression of Tet2 in mice has been shown to alter hematopoietic development [23, 24, 63].

Several studies have measured global 5hmC in DNA from various tissues using the techniques described above [26–28, 64]. Based on these analyses one would conclude that in addition to tissue-specific expression of TET enzymes, many tissues also display varied global 5hmC levels with some tissues having high, medium, or low levels of 5hmC. In general, tissues of the central nervous system have variable but overall high levels of global 5hmC [26, 27, 64]. Conversely, glandular tissues tend to have low 5hmC levels and the majority of key organs, such as heart, lung, and kidney tend to have midlevels of 5hmC in their genome [26–28]. This is in contrast with the stable global 5mC levels that are observed across most tissues [26]. However, it is important to note that in spite of stable global 5mC levels in various tissues there are locus-specific differences that are involved in maintaining proper tissue phenotype and function. These data suggest that high levels of 5hmC are not indicative of low 5mC levels on a genome-wide basis in somatic tissues, but that locus-specific shifts in the amount of unmodified, methylated, and hydroxymethylated cytosines are important for regulating gene expression in a tissue-specific manner. This is also supported by our work showing tissue-specific levels of 5hmC

at various loci in both mouse and human genomic DNA samples [32]. However, more detailed analysis of 5hmC patterning in various tissues and during development is required, which would help us to understand the roles of TET enzymes and 5hmC in differentiation and development.

3.6 Mutation of 5hmC Pathway Genes in Cancer and the Possible Consequences

TET1 is a common translocation partner of MLL histone methyltransferase at t(10;11)(q22;q23), in acute myeloid leukemia (AML) [13, 14]. The MLL-TET1 translocation has also been less commonly identified in acute lymphoblastic leukemia (ALL) [65]. Apart from the t(10;11)(q22;q23) translocation, no other mutations of TET1 have been reported. The MLL-TET1 fusion protein is predicted to have a molecular mass of approximately 204 kDa and is created by the fusion of the N-terminal part of MLL with the C-terminal part of TET1. The resulting protein contains the AT hooks, subnuclear localization domains, and the CXXC region of MLL fused to the core catalytic domain of TET1 [14]. The catalytic activity of the MLL-TET1 fusion protein is unknown, but it may be a gain of novel function of the fusion protein or loss of MLL and/or TET1 normal function that promotes oncogenesis. Regardless of the precise mechanism(s), MLL translocations correlate with a poor prognosis in ALL and AML patients [66–69].

Similar to TET1, it had been known that the 4q24 chromosomal region was commonly disrupted in hematologic malignancies, but the gene targeted within that region was not clear. It is now known that TET2 is the affected gene at 4q24 in many of these hematologic malignancies. TET2 mutations in myeloproliferative neoplasms (MPN) were identified recently [70–72]. Since then, mutations in TET2 have been observed in myelodysplastic syndrome (MDS), polycythemia vera, essential thrombocythemia, myelofibrosis, blastic plasmacytoid dendritic cell neoplasm (BPDCN), lymphomas, and different types of leukemia [23, 63, 70, 73–82]. Interestingly, certain TET2 mutations are found in specific subsets of these diseases [83].

TET2 mutations range from nonsense and missense mutations to frameshifts and deletions. Essentially all of these mutations are thought to result in loss of function of the TET2 enzyme and are generally somatic in nature. Several common mutations observed in MPN patients were tested for their effects on TET2 activity, including W1291R, E1318G, P1367S, I1873T, and G1913D [21]. All of these mutations are located in the cysteine-rich region or catalytic domain of human TET2. Overexpression of the mutant mouse counterpart of the W1291R (W1211R), P1367S (P1287S), and G1913D (C1834D) mutants in HEK293T cells results in reduced 5hmC as compared to overexpression of the WT Tet2 [21]. In addition, mutations of TET2 often occur on either one or both alleles suggesting that TET2 may either be haploinsufficient or gain an oncogenic function [70, 83]. These results indicate that TET2 functions as a tumor suppressor gene, especially in

hematopoietic cells. However, TET2 mutations may not be enough to cause transformation as it is commonly mutated along with genes in other important pathways, such as JAK and p53 [76, 84, 85].

Tet2 appears to have a direct role in myelopoiesis as *Tet2* knockdown alters differentiation of bone marrow stem cells when grown in the presence of specific cytokines [86]. Furthermore, conditional knockout or reduced expression of Tet2 in mice results in amplification of hematopoietic stem and progenitor cells with skewed numbers of differentiated myeloid and lymphoid lineages [63]. Several studies have attempted to evaluate the effect of TET2 mutations on patient prognosis, albeit in a limited number of samples. Mutations in TET2 correlate with reduced survival time in AML patients [77] and lower survival rate in patients with chronic myelomonocytic leukemia (CMML) [80]. Conversely, TET2 mutations in MDS patients appear to increase survival rate, as well as decrease progression to AML [79].

To date, there is only one report of a genetic aberration associated with TET3. A patient with refractory anemia with ringed sideroblasts (RARS), a specific form of MDS, and idiopathic myelofibrosis carried a deletion of 2p23 where the TET3 gene resides [87]. It is still unknown whether TET3 has a role in myeloproliferative diseases in a similar manner to TET1 and TET2. However, as TET3 is a catalytically active enzyme and has different tissue-specific expression patterns than TET1 and TET2, it remains a possibility that TET3 is involved in the development or progression of these and other diseases or disorders. Genetic studies will be required to test the functional role of TET enzymes in the development and progression of various diseases, including cancer.

As described above, the TET enzymes require cofactors for catalysis, one of which is 2-OG. Two enzymes that are involved in producing 2-OG are the cytosolic isocitrate dehydrogenase 1 (IDH1) and its mitochondrial homolog IDH2 [88]. Interestingly, IDH1 and IDH2 are commonly mutated in several diseases, including gliomas, astrocytomas, leukemias, and MPN [88], where 5hmC and TET expression are abundant. Furthermore, these mutations are not only mutually exclusive with each other but also with TET2 mutations in AML [88].

Mutations of IDH1 and IDH2 can result in a gain-of-function phenotype whereby 2-OG is further reduced by the mutant enzyme to 2-hydroxyglutarate (2-HG) [89, 90]. AML cells harboring mutations in IDH1, IDH2, or TET2 tend to have a hypermethylated phenotype (increased global and locus-specific methylation) and importantly a significant overlap of the genes that are hypermethylated [88]. Overexpression of mutant IDH enzyme results in a global increase in methylation and co-overexpression with TET2 does not result in increased 5hmC levels [88]. The above observation was confirmed in another study that showed inhibition of murine Tet1 and Tet2 in vitro by 2-HG and in vivo by mutant IDH1 [91]. In addition, glioma, astrocytoma, glioblastoma tissue samples harboring IDH1 mutations display decreased 5hmC staining and increased 5mC staining in immunohistological assays, as well as decreased 5hmC with LC-MS analysis [64, 91]. These studies suggest that alterations in 5hmC, either through directly disrupting the TET enzymes or changing availability of cofactors, may be involved in the development and progression of cancer and related diseases.

It is hypothesized that 5hmC is an intermediate in the process of demethylation (described fully in Sect. 3.7) and as a result disruption of the TET protein functions by translocation or mutation may result in a hypermethylated phenotype. Indeed, widespread locus-specific hypermethylation in AML patients with TET2 mutations has been reported [88]. Conversely, another study found that TET2 mutations in leukemia patients are associated with reduced 5hmC levels as expected, but also with global DNA hypomethylation [21]. Another recent report indicated that brain lesions, especially astrocytomas and glioblastomas, have significantly decreased global 5hmC with increasing tumor grade, although these samples did not display clear changes in 5mC levels [64]. Furthermore, several, tumor types appear to have decreased 5hmC when compared to matched normal tissue [39, 92, 93]. The mechanism of global hypo-hydroxymethylation in tumors and the relationship to mutations in TETs is not clear and may be dependent on tumor type and stage.

Hypomethylating agents were originally tested and approved for clinical use in MDS and leukemia patients [94]. The fact that these diseases have especially high rates of mutation in the TET proteins raises the question as to the correlation of TET mutations with treatment efficacy. One study on a very limited number of patients (two) did not confirm that TET2 mutations would improve the efficacy of DNMT inhibitors for the treatment of MDS [95]. In addition, a slightly larger study with AML patients reported that those with mutant TET2 had improved initial response, but did not yield better survival as compared to patients carrying the WT allele [96]. These results emphasize the necessity for studies to be completed using large cohorts of patients identify factors that categorize patients with myeloid disorders, harboring TET mutations, as likely or unlikely to benefit from treatment with demethylating agents. Finally, although TET mutations are clearly predominant in MPN it is still possible that they occur in any number of other diseases and this will likely be a focus of future research.

3.7 Demethylation Pathways of 5hmC and Possible Roles in Cancer Methylation

Reports of methylation cycling in the promoters of specific genes, active demethylation during certain stages of development, and global hypomethylation in tumors have left epigeneticists searching for a DNA demethylase [3]. Several possible demethylation mechanisms have been proposed in the past, including direct enzymatic removal of the methyl group by MBD2 [97], removal of the entire methylated base by a DNA glycosylase in a similar manner to the process of demethylation in plants [98], and deamination followed by base excision repair (BER), including deamination by DNMT3 enzymes in the presence of minimal *S*-adenosyl-L-methionine (AdoMet) [3, 99]. The stability of the carbon–carbon bond of the methyl group and the fifth carbon of the cytosine ring makes it unlikely that demethylation is due to direct removal of the methyl group from cytosine [3]. However, oxidation of methyl groups is a feasible mechanism for removal, especially as

histone demethylases function through oxidation to return histone proteins to an unmodified amino acid state [15].

Before 5hmC was found in mouse ESCs and brain DNA, several groups studied the effects of oxidation of 5mC on methyl binding proteins (MBD) and DNMT1 activity. For example, the MBD MeCP2 was shown to have decreased binding to 5hmC as compared to 5mC [100]. Altered binding of MeCP2 may have serious effects on transcriptional regulation, but would not lead to demethylation. However, DNMT1 was shown to have reduced catalytic activity when the DNA substrate was hemi-hydroxymethylated as opposed to the preferential hemi-methylated substrate [101]. This could have major effects on DNA methylation maintenance during replication, resulting in passive demethylation that is dependent on cell cycling. It is still unknown whether DNMT3a or DNMT3b expressed during S-phase is capable of methylating hemi-hydroxymethylated DNA.

Mammalian 5hmC glycosylases have been described as early as 1988 suggesting that this may be a possible mechanism for removal of this modified nucleobase [102]. Overexpression of TET genes causes increased 5hmC and then subsequent demethylation (based on digestion with methyl-sensitive restriction enzymes) of either endogenous or exogenous methylated DNA that requires a functional BER pathway [20, 103]. Additionally, overexpression of several of the Apobec family of cytidine deaminases causes further demethylation [103]. In fact, viral overexpression of Tet1 in the adult mouse dentate gyrus in the brain leads to substantial increases in global 5hmC, whereas viral overexpression of activation-induced deaminase (AID) in the same tissue causes a decrease in global 5hmC by more than 50%. Overexpression of either Tet1 or AID in adult mouse dentate granule cells results in demethylation and expression of neuronal genes known to display activity-induced DNA demethylation, but no demethylation occurs at non-neuronal promoters [103, 104]. Based on these findings, the following hypothesis has been proposed as one possible mechanism for 5hmC-stimulated demethylation: 5mC is first oxidized by TET enzymes to 5hmC, which is then deaminated by AID/APOBEC cytidine deaminases resulting in 5hmU, then 5hmU is targeted and removed by BER pathways (Fig. 3.2) [103].

Another possible mechanism of demethylation through 5hmC mimics the process of thymine conversion to uracil that is part of the thymidine salvage pathway in which successive oxidation of the 3-methyl group of thymine is completed to produce uracil by decarboxylation [3]. Previously, 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) (the further oxidized forms of 5hmC) could not be easily measured, but as more sensitive techniques were utilized it was clear that these forms of cytosine do exist in mammalian DNA (Fig. 3.2) [26–28]. Mouse ESC, mouse cortex DNA, and DNA from several other somatic tissues contain substantial amounts of each of these modifications, with 5caC being the lowest modified residue [27]. Interestingly, some tissue DNAs contained higher amounts of 5fC than 5hmC, such as liver and spleen [27]. The differences in the global amounts of each modified cytosine could be due to varied rate of conversion from one form to the next, as well as efficiency of removal for 5caC by thymine-DNA glycosylase (TDG) resulting in replacement with unmodified cytosine by

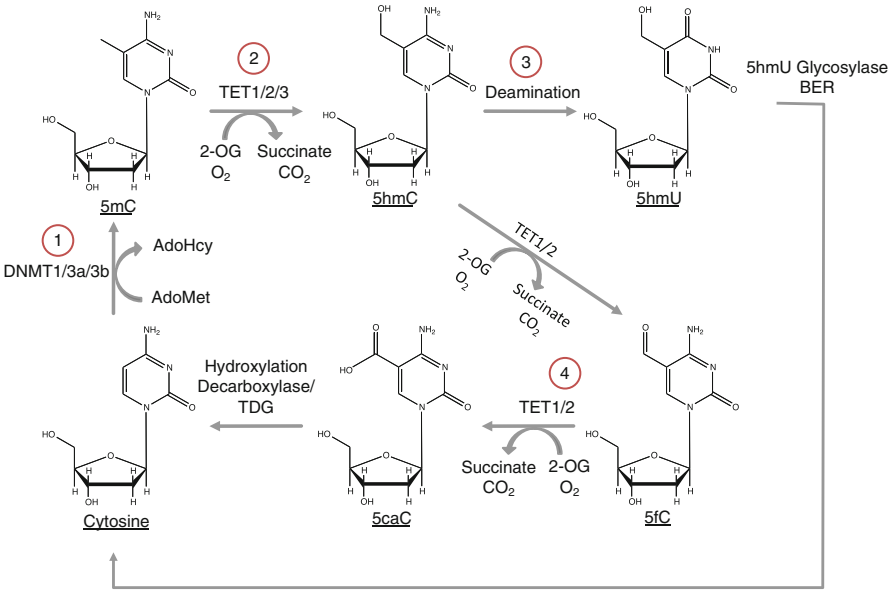

Fig. 3.2 5-Hydroxymethylcytosine and proposed demethylation pathways. (1) Cytosine in an unmodified state can be methylated by any of the three active DNMTs to 5-methylcytosine (5mC) to create the substrate for the TET enzymes. (2) 5mC can be oxidized by any of the three TET family enzymes to 5-hydroxymethylcytosine (5hmC). (3) 5hmC may then be deaminated by unknown enzymes to 5-hydroxymethyluracil (5hmU), which could then be removed by base excision repair pathway enzymes (BER). (4) 5hmC could also be further oxidized by the TET enzymes to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC), at which point the base can be removed by thymine-DNA glycosylase (TDG) or the carboxyl group can be removed by decarboxylases to produce unmethylated cytosine

DNA repair enzymes [28, 105]. Tet1 and Tet2 were both reported to oxidize 5hmC further to 5fC and 5caC both in vitro and in overexpression studies in cultured cells [27, 28].

The knowledge that 5hmC and its derivatives that are converted by the TET enzymes can result in demethylation provide some possible mechanisms for how aberrant methylation could occur in cancers. Loss-of-function mutations in TET2 correlate with hypermethylation and myeloid malignancies that commonly have TET mutations tend to be sensitive to hypomethylating agents [88, 94]. However, one study did correlate TET2 mutations with global hypomethylation in patients with myeloid malignancies [21]. For cancers that display hypomethylation, there are several potential explanations; one possibility is that hypomethylation by 5hmC is an earlier event during cancer progression than loss-of-function mutations that have been reported for TETs, or TET proteins (or other proteins involved in 5hmC-induced demethylation pathways) may be overexpressed or have gain-of-function mutations that are currently unknown. Clearly much research still needs to be done in this particular area to understand demethylation pathways of 5hmC and what enzymes are involved both in normal and disease states.

3.8 Future Perspectives

It was not long ago that the study of DNA methylation was uncharted territory, but now we have a basic understanding of how, when, and where DNA methylation occurs, as well as its role in many biological processes. The identification of 5hmC, and its oxidative products 5fC and 5caC, has complicated our understanding of this process, so now we have to tease out what past data (that may or may not include 5hmC, 5fC, and 5caC) means, and how to acquire more accurate data in the future. This has been and will continue to be a difficult process, but even in the short time since the identification of 5hmC, epigenetics research has moved forward by leaps and bounds, perhaps due to the past experiences with 5mC. Scientists have already developed several techniques to measure global and locus-specific 5hmC across the genome. It is known that there is tissue-specific expression of TETs and 5hmC levels, both globally and at specific loci, and that 5hmC may be involved in DNA demethylation pathways. Even so, there is certainly more research needed to determine the involvement of the TET enzymes and 5hmC in gene regulation, development, and disease.

3.9 Addendum

Two new methods have been reported that allow for single base resolution sequencing of 5hmC [106, 107]. Both techniques depend on the concept that 5fC and 5caC, unlike 5mC or 5hmC, are converted to uracil during sodium bisulfite treatment of the DNA. The first method utilizes potassium perruthenate (KRuO4) to chemically oxidize 5hmC to 5fC followed by rigorous bisulfite treatment and then sequencing of primarily CpG islands in mouse embryonic stem (ES) cell DNA [106]. The second method utilizes a three step process whereby the 5hmC sites are first glucosylated by beta-glucosyltransferase, which is followed by enzymatic oxidation of 5mC to 5caC by recombinant mouse Tet1 catalytic domain, and finally sodium bisulfite conversion and sequencing of human and mouse ES cell DNA. The glucosylated hydroxymethylcytosine residues are resistant to enzymatic oxidation and displayed as C in subsequent PCR based sequencing [107]. In both cases sequencing of both an oxidation pretreated DNA library and a control library must be completed to accurately map both 5mC and 5hmC sites across the genome. Considering that next generation sequencing analysis of bisulfite converted DNA is quite complicated, the data analysis for these methods could be especially difficult. However, these techniques should be useful for identification of 5mC and 5hmC at specific loci using a candidate gene approach in a similar manner to original bisulfite sequencing.

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References

1. Bird AP (1986) CpG-rich islands and the function of DNA methylation. Nature 321:209–213
2. Poulsen HE (2005) Oxidative DNA modifications. Exp Toxicol Pathol 57(Suppl 1):161–169
3. Wu SC, Zhang Y (2010) Active DNA demethylation: many roads lead to Rome. Nat Rev Mol Cell Biol 11:607–620
4. Wyatt GR, Cohen SS (1952) A new pyrimidine base from bacteriophage nucleic acids. Nature 170:1072–1073
5. Josse J, Kornberg A (1962) Glucosylation of deoxyribonucleic acid III alpha- and beta-glucosyl transferases from T4-infected Escherichia coli. J Biol Chem 237:1968–1976
6. Kornberg SR, Zimmerman SB, Kornberg A (1961) Glucosylation of deoxyribonucleic acid by enzymes from bacteriophage-infected Escherichia coli. J Biol Chem 236:1487–1493
7. Penn NW, Suwalski R, O'Riley C, Bojanowski K, Yura R (1972) The presence of 5-hydroxymethylcytosine in animal deoxyribonucleic acid. Biochem J 126:781–790
8. Penn NW (1976) Modification of brain deoxyribonucleic acid base content with maturation in normal and malnourished rats. Biochem J 155:709–712
9. Kothari RM, Shankar V (1976) 5-Methylcytosine content in the vertebrate deoxyribonucleic acids: species specificity. J Mol Evol 7:325–329
10. Tahiliani M, Koh KP, Shen Y, Pastor WA, Bandukwala H, Brudno Y, Agarwal S, Iyer LM, Liu DR, Aravind L, Rao A (2009) Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. Science 324:930–935
11. Kriaucionis S, Heintz N (2009) The nuclear DNA base 5-hydroxymethylcytosine is present in Purkinje neurons and the brain. Science 324:929–930
12. Shock LS, Thakkar PV, Peterson EJ, Moran RG, Taylor SM (2011) DNA methyltransferase 1, cytosine methylation, and cytosine hydroxymethylation in mammalian mitochondria. Proc Natl Acad Sci USA 108:3630–3635
13. Ono R, Taki T, Taketani T, Taniwaki M, Kobayashi H, Hayashi Y (2002) LCX, leukemia-associated protein with a CXXC domain, is fused to MLL in acute myeloid leukemia with trilineage dysplasia having t(10;11)(q22;q23). Cancer Res 62:4075–4080
14. Lorsbach RB, Moore J, Mathew S, Raimondi SC, Mukatira ST, Downing JR (2003) TET1, a member of a novel protein family, is fused to MLL in acute myeloid leukemia containing the t(10;11)(q22;q23). Leukemia 17:637–641
15. Loenarz C, Schofield CJ (2009) Oxygenase catalyzed 5-methylcytosine hydroxylation. Chem Biol 16:580–583
16. Yu Z, Genest PA, ter Riet B, Sweeney K, DiPaolo C, Kieft R, Christodoulou E, Perrakis A, Simmons JM, Hausinger RP, van Luenen HG, Rigden DJ, Sabatini R, Borst P (2007) The protein that binds to DNA base J in trypanosomatids has features of a thymidine hydroxylase. Nucleic Acids Res 35:2107–2115
17. Iyer LM, Tahiliani M, Rao A, Aravind L (2009) Prediction of novel families of enzymes involved in oxidative and other complex modifications of bases in nucleic acids. Cell Cycle 8:1698–1710
18. Mohr F, Dohner K, Buske C, Rawat VP (2011) TET genes: new players in DNA demethylation and important determinants for stemness. Exp Hematol 39:272–281
19. Frauer C, Rottach A, Meilinger D, Bultmann S, Fellinger K, Hasenoder S, Wang M, Qin W, Soding J, Spada F, Leonhardt H (2011) Different binding properties and function of CXXC zinc finger domains in Dnmt1 and Tet1. PLoS One 6:e16627
20. Zhang H, Zhang X, Clark E, Mulcahey M, Huang S, Shi YG (2010) TET1 is a DNA-binding protein that modulates DNA methylation and gene transcription via hydroxylation of 5-methylcytosine. Cell Res 20:1390–1393
21. Ko M, Huang Y, Jankowska AM, Pape UJ, Tahiliani M, Bandukwala HS, An J, Lamperti ED, Koh KP, Ganetzky R, Liu XS, Aravind L, Agarwal S, Maciejewski JP, Rao A (2010) Impaired hydroxylation of 5-methylcytosine in myeloid cancers with mutant TET2. Nature 468:839–843

22. Ito S, D'Alessio AC, Taranova OV, Hong K, Sowers LC, Zhang Y (2010) Role of Tet proteins in 5mC to 5hmC conversion, ES-cell self-renewal and inner cell mass specification. Nature 466:1129–1133
23. Langemeijer SM, Aslanyan MG, Jansen JH (2009) TET proteins in malignant hematopoiesis. Cell Cycle 8:4044–4048
24. Langemeijer SM, Kuiper RP, Berends M, Knops R, Aslanyan MG, Massop M, Stevens-Linders E, van Hoogen P, van Kessel AG, Raymakers RA, Kamping EJ, Verhoef GE, Verburgh E, Hagemeijer A, Vandenberghe P, de Witte T, van der Reijden BA, Jansen JH (2009) Acquired mutations in TET2 are common in myelodysplastic syndromes. Nat Genet 41:838–842
25. Ficz G, Branco MR, Seisenberger S, Santos F, Krueger F, Hore TA, Marques CJ, Andrews S, Reik W (2011) Dynamic regulation of 5-hydroxymethylcytosine in mouse ES cells and during differentiation. Nature 473:398–402
26. Globisch D, Munzel M, Muller M, Michalakis S, Wagner M, Koch S, Bruckl T, Biel M, Carell T (2010) Tissue distribution of 5-hydroxymethylcytosine and search for active demethylation intermediates. PLoS One 5:e15367
27. Ito S, Shen L, Dai Q, Wu SC, Collins LB, Swenberg JA, He C, Zhang Y (2011) Tet proteins can convert 5-methylcytosine to 5-formylcytosine and 5-carboxylcytosine. Science 333:1300–1303
28. He YF, Li BZ, Li Z, Liu P, Wang Y, Tang Q, Ding J, Jia Y, Chen Z, Li L, Sun Y, Li X, Dai Q, Song CX, Zhang K, He C, Xu GL (2011) Tet-mediated formation of 5-carboxylcytosine and its excision by TDG in mammalian DNA. Science 333:1303–1307
29. Huang Y, Pastor WA, Shen Y, Tahiliani M, Liu DR, Rao A (2010) The behaviour of 5-hydroxymethylcytosine in bisulfite sequencing. PLoS One 5:e8888
30. Wang H, Guan S, Quimby A, Cohen-Karni D, Pradhan S, Wilson G, Roberts RJ, Zhu Z, Zheng Y (2011) Comparative characterization of the PvuRts1I family of restriction enzymes and their application in mapping genomic 5-hydroxymethylcytosine. Nucleic Acids Res 39:9294–9305
31. Song CX, Yu M, Dai Q, He C (2011) Detection of 5-hydroxymethylcytosine in a combined glycosylation restriction analysis (CGRA) using restriction enzyme Taq(alpha)I. Bioorg Med Chem Lett 21:5075–5077
32. Kinney SM, Chin HG, Vaisvila R, Bitinaite J, Zheng Y, Esteve PO, Feng S, Stroud H, Jacobsen SE, Pradhan S (2011) Tissue-specific distribution and dynamic changes of 5-hydroxymethylcytosine in mammalian genomes. J Biol Chem 286:24685–24693
33. Szwagierczak A, Brachmann A, Schmidt CS, Bultmann S, Leonhardt H, Spada F (2011) Characterization of PvuRts1I endonuclease as a tool to investigate genomic 5-hydroxymethylcytosine. Nucleic Acids Res 39:5149–5156
34. Roberts RJ, Vincze T, Posfai J, Macelis D (2010) REBASE—a database for DNA restriction and modification: enzymes, genes and genomes. Nucleic Acids Res 38:D234–D236
35. Iqbal K, Jin SG, Pfeifer GP, Szabo PE (2011) Reprogramming of the paternal genome upon fertilization involves genome-wide oxidation of 5-methylcytosine. Proc Natl Acad Sci USA 08:3642–3647
36. Stroud H, Feng S, Morey Kinney S, Pradhan S, Jacobsen SE (2011) 5-Hydroxymethylcytosine is associated with enhancers and gene bodies in human embryonic stem cells. Genome Biol 12:R54
37. Le T, Kim KP, Fan G, Faull KF (2011) A sensitive mass spectrometry method for simultaneous quantification of DNA methylation and hydroxymethylation levels in biological samples. Anal Biochem 412:203–209
38. Szwagierczak A, Bultmann S, Schmidt CS, Spada F, Leonhardt H (2010) Sensitive enzymatic quantification of 5-hydroxymethylcytosine in genomic DNA. Nucleic Acids Res 38:e181
39. Terragni J, Bitinaite J, Zheng Y, Pradhan S (2012) Biochemical characterization of recombinant Beta-glucosyltransferase and analysis of global 5-hydroxymethylcytosine in unique genomes. Biochemistry 51:1009–1019
40. Wu H, D'Alessio AC, Ito S, Wang Z, Cui K, Zhao K, Sun YE, Zhang Y (2011) Genome-wide analysis of 5-hydroxymethylcytosine distribution reveals its dual function in transcriptional regulation in mouse embryonic stem cells. Genes Dev 25:679–684

41. Wu H, D'Alessio AC, Ito S, Xia K, Wang Z, Cui K, Zhao K, Sun YE, Zhang Y (2011) Dual functions of Tet1 in transcriptional regulation in mouse embryonic stem cells. Nature 473:389–393
42. Pastor WA, Pape UJ, Huang Y, Henderson HR, Lister R, Ko M, McLoughlin EM, Brudno Y, Mahapatra S, Kapranov P, Tahiliani M, Daley GQ, Liu XS, Ecker JR, Milos PM, Agarwal S, Rao A (2011) Genome-wide mapping of 5-hydroxymethylcytosine in embryonic stem cells. Nature 473:394–397
43. Song CX, Szulwach KE, Fu Y, Dai Q, Yi C, Li X, Li Y, Chen CH, Zhang W, Jian X, Wang J, Zhang L, Looney TJ, Zhang B, Godley LA, Hicks LM, Lahn BT, Jin P, He C (2011) Selective chemical labeling reveals the genome-wide distribution of 5-hydroxymethylcytosine. Nat Biotechnol 29:68–72
44. Flusberg BA, Webster DR, Lee JH, Travers KJ, Olivares EC, Clark TA, Korlach J, Turner SW (2010) Direct detection of DNA methylation during single-molecule, real-time sequencing. Nat Methods 7:461–465
45. Wanunu M, Cohen-Karni D, Johnson RR, Fields L, Benner J, Peterman N, Zheng Y, Klein ML, Drndic M (2010) Discrimination of methylcytosine from hydroxymethylcytosine in DNA molecules. J Am Chem Soc 133:486–492
46. Bair CL, Black LW (2007) A type IV modification dependent restriction nuclease that targets glucosylated hydroxymethyl cytosine modified DNAs. J Mol Biol 366:768–778
47. Freudenberg JM, Ghosh S, Lackford BL, Yellaboina S, Zheng X, Li R, Cuddapah S, Wade PA, Hu G, Jothi R (2011) Acute depletion of Tet1-dependent 5-hydroxymethylcytosine levels impairs LIF/Stat3 signaling and results in loss of embryonic stem cell identity. Nucleic Acids Res 40(8):3364–3377
48. Zhou GB, Meng QG, Li N (2010) In vitro derivation of germ cells from embryonic stem cells in mammals. Mol Reprod Dev 77:586–594
49. Koh KP, Yabuuchi A, Rao S, Huang Y, Cunniff K, Nardone J, Laiho A, Tahiliani M, Sommer CA, Mostoslavsky G, Lahesmaa R, Orkin SH, Rodig SJ, Daley GQ, Rao A (2011) Tet1 and Tet2 regulate 5-hydroxymethylcytosine production and cell lineage specification in mouse embryonic stem cells. Cell Stem Cell 8:200–213
50. Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126:663–676
51. Williams K, Christensen J, Pedersen MT, Johansen JV, Cloos PA, Rappsilber J, Helin K (2011) TET1 and hydroxymethylcytosine in transcription and DNA methylation fidelity. Nature 473:343–348
52. Xu Y, Wu F, Tan L, Kong L, Xiong L, Deng J, Barbera AJ, Zheng L, Zhang H, Huang S, Min J, Nicholson T, Chen T, Xu G, Shi Y, Zhang K, Shi YG (2011) Genome-wide regulation of 5hmC, 5mC, and gene expression by Tet1 hydroxylase in mouse embryonic stem cells. Mol Cell 42:451–464
53. Dawlaty MM, Ganz K, Powell BE, Hu YC, Markoulaki S, Cheng AW, Gao Q, Kim J, Choi SW, Page DC, Jaenisch R (2011) Tet1 is dispensable for maintaining pluripotency and its loss is compatible with embryonic and postnatal development. Cell Stem Cell 9:166–175
54. Yildirim O, Li R, Hung JH, Chen PB, Dong X, Ee LS, Weng Z, Rando OJ, Fazzio TG (2011) Mbd3/NURD Complex Regulates Expression of 5-Hydroxymethylcytosine Marked Genes in Embryonic Stem Cells. Cell 147:1498–1510
55. Reik W, Dean W, Walter J (2001) Epigenetic reprogramming in mammalian development. Science 293:1089–1093
56. Mayer W, Niveleau A, Walter J, Fundele R, Haaf T (2000) Demethylation of the zygotic paternal genome. Nature 403:501–502
57. Oswald J, Engemann S, Lane N, Mayer W, Olek A, Fundele R, Dean W, Reik W, Walter J (2000) Active demethylation of the paternal genome in the mouse zygote. Curr Biol 10:475–478
58. Santos F, Hendrich B, Reik W, Dean W (2002) Dynamic reprogramming of DNA methylation in the early mouse embryo. Dev Biol 241:172–182
59. Hajkova P, Jeffries SJ, Lee C, Miller N, Jackson SP, Surani MA (2010) Genome-wide reprogramming in the mouse germ line entails the base excision repair pathway. Science 329:78–82

60. Wossidlo M, Nakamura T, Lepikhov K, Marques CJ, Zakhartchenko V, Boiani M, Arand J, Nakano T, Reik W, Walter J (2011) 5-Hydroxymethylcytosine in the mammalian zygote is linked with epigenetic reprogramming. Nat Commun 2:241
61. Gu TP, Guo F, Yang H, Wu HP, Xu GF, Liu W, Xie ZG, Shi L, He X, Jin SG, Iqbal K, Shi YG, Deng Z, Szabo PE, Pfeifer GP, Li J, Xu GL (2011) The role of Tet3 DNA dioxygenase in epigenetic reprogramming by oocytes. Nature 477:606–610
62. Nakamura T, Arai Y, Umehara H, Masuhara M, Kimura T, Taniguchi H, Sekimoto T, Ikawa M, Yoneda Y, Okabe M, Tanaka S, Shiota K, Nakano T (2007) PGC7/Stella protects against DNA demethylation in early embryogenesis. Nat Cell Biol 9:64–71
63. Quivoron C, Couronne L, Della Valle V, Lopez CK, Plo I, Wagner-Ballon O, Do Cruzeiro M, Delhommeau F, Arnulf B, Stern MH, Godley L, Opolon P, Tilly H, Solary E, Duffourd Y, Dessen P, Merle-Beral H, Nguyen-Khac F, Fontenay M, Vainchenker W, Bastard C, Mercher T, Bernard OA (2011) TET2 inactivation results in pleiotropic hematopoietic abnormalities in mouse and is a recurrent event during human lymphomagenesis. Cancer Cell 20:25–38
64. Kraus TF, Globisch D, Wagner M, Eigenbrod S, Widmann D, Munzel M, Muller M, Pfaffeneder T, Hackner B, Feiden W, Schuller U, Carell T, Kretzschmar HA (2012) Low values of 5-hydroxymethylcytosine (5hmC), the "sixth base", are associated with anaplasia in human brain tumours. International journal of cancer. J Int Cancer Jan 10. doi: 10.1002/ijc.27429. [Epub ahead of print]
65. Burmeister T, Meyer C, Schwartz S, Hofmann J, Molkentin M, Kowarz E, Schneider B, Raff T, Reinhardt R, Gokbuget N, Hoelzer D, Thiel E, Marschalek R (2009) The MLL recombinome of adult CD10-negative B-cell precursor acute lymphoblastic leukemia: results from the GMALL study group. Blood 113:4011–4015
66. Pui CH, Chessells JM, Camitta B, Baruchel A, Biondi A, Boyett JM, Carroll A, Eden OB, Evans WE, Gadner H, Harbott J, Harms DO, Harrison CJ, Harrison PL, Heerema N, Janka-Schaub G, Kamps W, Masera G, Pullen J, Raimondi SC, Richards S, Riehm H, Sallan S, Sather H, Shuster J, Silverman LB, Valsecchi MG, Vilmer E, Zhou Y, Gaynon PS, Schrappe M (2003) Clinical heterogeneity in childhood acute lymphoblastic leukemia with 11q23 rearrangements. Leukemia 17:700–706
67. Pui CH, Gaynon PS, Boyett JM, Chessells JM, Baruchel A, Kamps W, Silverman LB, Biondi A, Harms DO, Vilmer E, Schrappe M, Camitta B (2002) Outcome of treatment in childhood acute lymphoblastic leukaemia with rearrangements of the 11q23 chromosomal region. Lancet 359:1909–1915
68. Liedtke M, Cleary ML (2009) Therapeutic targeting of MLL. Blood 113:6061–6068
69. Chou WC, Chou SC, Liu CY, Chen CY, Hou HA, Kuo YY, Lee MC, Ko BS, Tang JL, Yao M, Tsay W, Wu SJ, Huang SY, Hsu SC, Chen YC, Chang YC, Kuo KT, Lee FY, Liu MC, Liu CW, Tseng MH, Huang CF, Tien HF (2011) TET2 mutation is an unfavorable prognostic factor in acute myeloid leukemia patients with intermediate-risk cytogenetics. Blood 118:3803–3810
70. Delhommeau F, Dupont S, Della Valle V, James C, Trannoy S, Masse A, Kosmider O, Le Couedic JP, Robert F, Alberdi A, Lecluse Y, Plo I, Dreyfus FJ, Marzac C, Casadevall N, Lacombe C, Romana SP, Dessen P, Soulier J, Viguie F, Fontenay M, Vainchenker W, Bernard OA (2009) Mutation in TET2 in myeloid cancers. N Engl J Med 360:2289–2301
71. Tefferi A, Levine RL, Lim KH, Abdel-Wahab O, Lasho TL, Patel J, Finke CM, Mullally A, Li CY, Pardanani A, Gilliland DG (2009) Frequent TET2 mutations in systemic mastocytosis: clinical, KITD816V and FIP1L1-PDGFRA correlates. Leukemia 23:900–904
72. Tefferi A, Lim KH, Levine R (2009) Mutation in TET2 in myeloid cancers. N Engl J Med 361:1117
73. Tefferi A, Pardanani A, Lim KH, Abdel-Wahab O, Lasho TL, Patel J, Gangat N, Finke CM, Schwager S, Mullally A, Li CY, Hanson CA, Mesa R, Bernard O, Delhommeau F, Vainchenker W, Gilliland DG, Levine RL (2009) TET2 mutations and their clinical correlates in polycythemia vera, essential thrombocythemia and myelofibrosis. Leukemia 23:905–911
74. Tefferi A, Lim KH, Abdel-Wahab O, Lasho TL, Patel J, Patnaik MM, Hanson CA, Pardanani A, Gilliland DG, Levine RL (2009) Detection of mutant TET2 in myeloid malignancies other than myeloproliferative neoplasms: CMML, MDS, MDS/MPN and AML. Leukemia 23: 1343–1345

75. Makishima H, Jankowska AM, McDevitt MA, O'Keefe C, Dujardin S, Cazzolli H, Przychodzen B, Prince C, Nicoll J, Siddaiah H, Shaik M, Szpurka H, Hsi E, Advani A, Paquette R, Maciejewski JP (2011) CBL, CBLB, TET2, ASXL1, and IDH1/2 mutations and additional chromosomal aberrations constitute molecular events in chronic myelogenous leukemia. Blood 117:e198–e206
76. Jardin F, Ruminy P, Parmentier F, Troussard X, Vaida I, Stamatoullas A, Lepretre S, Penther D, Duval AB, Picquenot JM, Courville P, Capiod JC, Tilly H, Bastard C, Marolleau JP (2011) TET2 and TP53 mutations are frequently observed in blastic plasmacytoid dendritic cell neoplasm. Br J Haematol 153:413–416
77. Abdel-Wahab O, Mullally A, Hedvat C, Garcia-Manero G, Patel J, Wadleigh M, Malinge S, Yao J, Kilpivaara O, Bhat R, Huberman K, Thomas S, Dolgalev I, Heguy A, Paietta E, Le Beau MM, Beran M, Tallman MS, Ebert BL, Kantarjian HM, Stone RM, Gilliland DG, Crispino JD, Levine RL (2009) Genetic characterization of TET1, TET2, and TET3 alterations in myeloid malignancies. Blood 114:144–147
78. Jankowska AM, Szpurka H, Tiu RV, Makishima H, Afable M, Huh J, O'Keefe CL, Ganetzky R, McDevitt MA, Maciejewski JP (2009) Loss of heterozygosity 4q24 and TET2 mutations associated with myelodysplastic/myeloproliferative neoplasms. Blood 113:6403–6410
79. Kosmider O, Gelsi-Boyer V, Cheok M, Grabar S, Della-Valle V, Picard F, Viguie F, Quesnel B, Beyne-Rauzy O, Solary E, Vey N, Hunault-Berger M, Fenaux P, Mansat-De Mas V, Delabesse E, Guardiola P, Lacombe C, Vainchenker W, Preudhomme C, Dreyfus F, Bernard OA, Birnbaum D, Fontenay M (2009) TET2 mutation is an independent favorable prognostic factor in myelodysplastic syndromes (MDSs). Blood 114:3285–3291
80. Kosmider O, Gelsi-Boyer V, Ciudad M, Racoeur C, Jooste V, Vey N, Quesnel B, Fenaux P, Bastie JN, Beyne-Rauzy O, Stamatoulas A, Dreyfus F, Ifrah N, de Botton S, Vainchenker W, Bernard OA, Birnbaum D, Fontenay M, Solary E (2009) TET2 gene mutation is a frequent and adverse event in chronic myelomonocytic leukemia. Haematologica 94:1676–1681
81. Kohlmann A, Grossmann V, Klein HU, Schindela S, Weiss T, Kazak B, Dicker F, Schnittger S, Dugas M, Kern W, Haferlach C, Haferlach T (2010) Next-generation sequencing technology reveals a characteristic pattern of molecular mutations in 72.8% of chronic myelomonocytic leukemia by detecting frequent alterations in TET2, CBL, RAS, and RUNX1. J Clin Oncol 28:3858–3865
82. Nibourel O, Kosmider O, Cheok M, Boissel N, Renneville A, Philippe N, Dombret H, Dreyfus F, Quesnel B, Geffroy S, Quentin S, Roche-Lestienne C, Cayuela JM, Roumier C, Fenaux P, Vainchenker W, Bernard OA, Soulier J, Fontenay M, Preudhomme C (2010) Incidence and prognostic value of TET2 alterations in de novo acute myeloid leukemia achieving complete remission. Blood 116:1132–1135
83. Hellstrom-Lindberg E (2010) Significance of JAK2 and TET2 mutations in myelodysplastic syndromes. Blood Rev 24:83–90
84. Swierczek SI, Yoon D, Bellanne-Chantelot C, Kim SJ, Saint-Martin C, Delhommeau F, Najman A, Prchal JT (2011) Extent of hematopoietic involvement by TET2 mutations in JAK2V(1)F polycythemia vera. Haematologica 96:775–778
85. Rocquain J, Carbuccia N, Trouplin V, Raynaud S, Murati A, Nezri M, Tadrist Z, Olschwang S, Vey N, Birnbaum D, Gelsi-Boyer V, Mozziconacci MJ (2010) Combined mutations of ASXL1, CBL, FLT3, IDH1, IDH2, JAK2, KRAS, NPM1, NRAS, RUNX1, TET2 and WT1 genes in myelodysplastic syndromes and acute myeloid leukemias. BMC Cancer 10:401
86. Ko M, Bandukwala HS, An J, Lamperti ED, Thompson EC, Hastie R, Tsangaratou A, Rajewsky K, Koralov SB, Rao A (2011) Ten-Eleven-Translocation 2 (TET2) negatively regulates homeostasis and differentiation of hematopoietic stem cells in mice. Proc Natl Acad Sci USA 108:14566–14571
87. Gelsi-Boyer V, Trouplin V, Adelaide J, Bonansea J, Cervera N, Carbuccia N, Lagarde A, Prebet T, Nezri M, Sainty D, Olschwang S, Xerri L, Chaffanet M, Mozziconacci MJ, Vey N, Birnbaum D (2009) Mutations of polycomb-associated gene ASXL1 in myelodysplastic syndromes and chronic myelomonocytic leukaemia. Br J Haematol 145:788–800
88. Figueroa ME, Abdel-Wahab O, Lu C, Ward PS, Patel J, Shih A, Li Y, Bhagwat N, Vasanthakumar A, Fernandez HF, Tallman MS, Sun Z, Wolniak K, Peeters JK, Liu W, Choe

SE, Fantin VR, Paietta E, Lowenberg B, Licht JD, Godley LA, Delwel R, Valk PJ, Thompson CB, Levine RL, Melnick A (2010) Leukemic IDH1 and IDH2 mutations result in a hypermethylation phenotype, disrupt TET2 function, and impair hematopoietic differentiation. Cancer Cell 18:553–567
89. Dang L, White DW, Gross S, Bennett BD, Bittinger MA, Driggers EM, Fantin VR, Jang HG, Jin S, Keenan MC, Marks KM, Prins RM, Ward PS, Yen KE, Liau LM, Rabinowitz JD, Cantley LC, Thompson CB, Vander Heiden MG, Su SM (2009) Cancer-associated IDH1 mutations produce 2-hydroxyglutarate. Nature 462:739–744
90. Ward PS, Patel J, Wise DR, Abdel-Wahab O, Bennett BD, Coller HA, Cross JR, Fantin VR, Hedvat CV, Perl AE, Rabinowitz JD, Carroll M, Su SM, Sharp KA, Levine RL, Thompson CB (2010) The common feature of leukemia-associated IDH1 and IDH2 mutations is a neomorphic enzyme activity converting alpha-ketoglutarate to 2-hydroxyglutarate. Cancer Cell 17:225–234
91. Xu W, Yang H, Liu Y, Yang Y, Wang P, Kim SH, Ito S, Yang C, Xiao MT, Liu LX, Jiang WQ, Liu J, Zhang JY, Wang B, Frye S, Zhang Y, Xu YH, Lei QY, Guan KL, Zhao SM, Xiong Y (2011) Oncometabolite 2-hydroxyglutarate is a competitive inhibitor of alpha-ketoglutarate-dependent dioxygenases. Cancer Cell 19:17–30
92. Haffner MC, Chaux A, Meeker AK, Esopi DM, Gerber J, Pellakuru LG, Toubaji A, Argani P, Iacobuzio-Donahue C, Nelson WG, Netto GJ, De Marzo AM, Yegnasubramanian S (2011) Global 5-hydroxymethylcytosine content is significantly reduced in tissue stem/progenitor cell compartments and in human cancers. Oncotarget 2:627–637
93. Li W, Liu M (2011) Distribution of 5-hydroxymethylcytosine in different human tissues. J Nucleic Acids 2011:870726
94. Kantarjian H, O'Brien S, Cortes J, Wierda W, Faderl S, Garcia-Manero G, Issa JP, Estey E, Keating M, Freireich EJ (2008) Therapeutic advances in leukemia and myelodysplastic syndrome over the past 40 years. Cancer 113:1933–1952
95. Pollyea DA, Raval A, Kusler B, Gotlib JR, Alizadeh AA, Mitchell BS (2010) Impact of TET2 mutations on mRNA expression and clinical outcomes in MDS patients treated with DNA methyltransferase inhibitors. Hematol Oncol 29:157–160
96. Itzykson R, Kosmider O, Cluzeau T, Mansat-De Mas V, Dreyfus F, Beyne-Rauzy O, Quesnel B, Vey N, Gelsi-Boyer V, Raynaud S, Preudhomme C, Ades L, Fenaux P, Fontenay M (2011) Impact of TET2 mutations on response rate to azacitidine in myelodysplastic syndromes and low blast count acute myeloid leukemias. Leukemia 25:1147–1152
97. Detich N, Theberge J, Szyf M (2002) Promoter-specific activation and demethylation by MBD2/demethylase. J Biol Chem 277:35791–35794
98. Feng S, Jacobsen SE, Reik W (2010) Epigenetic reprogramming in plant and animal development. Science 330:622–627
99. Metivier R, Gallais R, Tiffoche C, Le Peron C, Jurkowska RZ, Carmouche RP, Ibberson D, Barath P, Demay F, Reid G, Benes V, Jeltsch A, Gannon F, Salbert G (2008) Cyclical DNA methylation of a transcriptionally active promoter. Nature 452:45–50
100. Valinluck V, Tsai HH, Rogstad DK, Burdzy A, Bird A, Sowers LC (2004) Oxidative damage to methyl-CpG sequences inhibits the binding of the methyl-CpG binding domain (MBD) of methyl-CpG binding protein 2 (MeCP2). Nucleic Acids Res 32:4100–4108
101. Valinluck V, Sowers LC (2007) Endogenous cytosine damage products alter the site selectivity of human DNA maintenance methyltransferase DNMT1. Cancer Res 67:946–950
102. Cannon SV, Cummings A, Teebor GW (1988) 5-Hydroxymethylcytosine DNA glycosylase activity in mammalian tissue. Biochem Biophys Res Commun 151:1173–1179
103. Guo JU, Su Y, Zhong C, Ming GL, Song H (2011) Hydroxylation of 5-methylcytosine by TET1 promotes active DNA demethylation in the adult brain. Cell 145:423–434
104. Ma DK, Jang MH, Guo JU, Kitabatake Y, Chang ML, Pow-Anpongkul N, Flavell RA, Lu B, Ming GL, Song H (2009) Neuronal activity-induced Gadd45b promotes epigenetic DNA demethylation and adult neurogenesis. Science 323:1074–1077

105. Maiti A, Drohat AC (2011) Thymine DNA glycosylase can rapidly excise 5-formylcytosine and 5-carboxylcytosine: Potential implications for active demethylation of CpG sites. J Biol Chem 286:35334–35338
106. Booth MJ, Branco MR, Ficz G, Oxley D, Krueger F, Reik W, Balasubramanian S. (2012) Quantitative sequencing of 5-methylcytosine and 5-hydroxymethylcytosine at single-base resolution. Science 336:934–937
107. Yu M, Hon GC, Szulwach KE, Song CX, Zhang L, Kim A, Li X, Dai Q, Shen Y, Park B, Min JH, Jin P, Ren B, He C (2012) Base-resolution analysis of 5-hydroxymethylcytosine in the mammalian genome. Cell 149:1368–1380

Chapter 4
Altered Histone Modifications in Cancer

Moray J. Campbell and Bryan M. Turner

Abstract In human health and disease the choreographed actions of a wide armory of transcription factors govern the regulated expression of coding and nonprotein coding genes. These actions are central to human health and are evidently aberrant in cancer. Central components of regulated gene expression are a variety of epigenetic mechanisms that include histone modifications. The post-translational modifications of histones are widespread and diverse, and appear to be spatial-temporally regulated in a highly intricate manner. The true functional consequences of these patterns of regulation are still emerging. Correlative evidence supports the idea that these patterns are distorted in malignancy on both a genome-wide and a discrete gene loci level. These patterns of distortion also often reflect the altered expression of the enzymes that control these histone states. Similarly gene expression patterns also appear to reflect a correlation with altered histone modifications at both the candidate loci and genome-

Chromatin-modifying enzymes: The nomenclature for enzymes involved in protein methylation, demethylation, and acetylation has recently been rationalized (Allis CD et al (2007) New nomenclature for chromatin-modifying enzymes. Cell 131:633–636). In this review, we use the new nomenclature for lysine methyltransferases (KMT), lysine demethylases (KDM), and lysine acetyltransferases (KAT). Histone deacetylases (HDACs) have retained their original nomenclature. To maintain a link between the new nomenclature and the literature, we use both the new designation and the original published designation(s), e.g., KDM5A/JARID1A/RBP2.

Histone modifications: We use the Brno nomenclature for histone modifications (Turner BM (2005) Reading signals on the nucleosome with a new nomenclature for modified histones. Nat Struct Mol Biol 12:110–112). For example, histone H3 tri-methylated at lysine 4 is shown as H3K4me3.

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wide level. Clarity is emerging in resolving these relationships between histone modification status and gene expression patterns. For example, altered transcription factor interactions with the key co-activator and co-repressors, which in turn marshal many of the histone-modifying enzymes, may distort regulation of histone modifications at specific gene loci. In turn these aberrant transcriptional processes can trigger other altered epigenetic events such as DNA methylation and underline the aberrant and specific gene expression patterns in cancer. Considered in this manner, altered expression and recruitment of histone-modifying enzymes may underline the distortion to transcriptional responsiveness observed in malignancy. Insight from understanding these processes addresses the challenge of targeted epigenetic therapies in cancer.

Abbreviations

AR	Androgen receptor
ChIP	Chromatin immunoprecipitation
CoA	Co-activator complex
E_2	Estradiol
ERα	Estrogen receptor alpha
ES	Embryonic stem cell
HDAC	Histone deacetylase
JMJD	Jumonji domain containing protein
JARID	Jumonji AT-rich interactive domain
KAT	Lysine acetyltransferase
KDM	Lysine demethylase
KMT	Lysine methyltransferase
LSD1	Lysine-specific demethylase 1
NCOR	Nuclear co-repressor
NR	Nuclear receptor
PSA	Prostate-specific antigen
SET	Su(var), enhancer of zeste and trithorax
TF	Transcription factor
TSA	Trichostatin A
TSS	Transcription start site

4.1 Altered Histone Modifications in Cancer

4.1.1 The Nucleosome and Its Modified Forms

Of the various protein–DNA interactions that are central to genome function, those between the histones and DNA are among the most intimate. A histone–DNA complex, the nucleosome, is the basic unit of chromatin structure in nearly

all eukaryotes, It comprises 146 bp of DNA wrapped in 1¾ superhelical turns around a core of eight histones, two each of H2A, H2B, H3, and H4. The structure of the nucleosome core particle has been defined in great detail by X-ray crystallography [3].

Despite its extreme conservation through evolution and its consistent crystal structure, the nucleosome in vivo is subject to a variety of enzyme-driven modifications that, potentially at least, alter its structure. Chromatin-modifying enzymes directly manipulate nucleosome structure or change nucleosome position along the DNA fiber [4]. DNA translocating enzymes such as polymerases, which pull and twist the DNA fiber as part of their normal activities, distort nucleosomes in their paths. Chromatin must deform reversibly in order to accommodate torsional and tensional stress generated by these enzymes ([5] and references therein). Nucleosome remodeling can dissociate the histone core, providing opportunities to enzymatically modify internal histone regions (see below), or to incorporate histone variants. All core histones, apart from H4, have nonallelic variant forms that differ in amino acid sequence and are associated with specific cellular and genomic functions [6].

4.1.1.1 Post-translational Modification of Histones

The most widespread and complex source of nucleosome variability is the enzyme catalyzed, post-translational modification of selected histone amino acids. All four core histones are subject to such changes, which include acetylation of lysines, methylation of lysines and arginines, phosphorylation of serines and threonines, and attachment of the small peptides ubiquitin and SUMO [7]. Advances in mass spectrometry and proteomics [8] have led to the identification of previously unsuspected chemical changes, including *O*-glycosylation of serines and threonines [9], formylation and crotonylation of lysines, and hydroxylation of serines [10]. They have also revealed that modifications occur both along the N-terminal tail domains, unstructured regions that are exposed on the nucleosome surface, and on residues in the globular internal regions that mediate histone–histone and histone–DNA interactions [11]. Histone modifications are put in place and removed by families (often large) of modifying and de-modifying enzymes and are consistently dynamic. The level of any particular modification reflects a steady-state balance between the actions of these two sets of enzymes.

The internal histone regions mediate the interactions that give the nucleosome its characteristic structure and their modification can, potentially, exert a direct structural effect. Yeast mutants with internal substitutions (some mimicking modifications) commonly cause functional changes, particularly altered gene silencing and increased sensitivity to DNA damaging agents [12, 13]. Acetylation of H3K56, on the lateral face of the nucleosome, is incorporated into chromatin at sites of DNA damage and repair [14, 15] and at replication forks [16]. These are all situations in which the nucleosome is partially dissociated, and during which internal residues will be accessible to modifying enzymes. Structural changes brought about by

H3K56 acetylation increase nucleosome mobility, thereby increasing DNA accessibility and, in the appropriate context, facilitating transcription [13, 17, 18].

Each core histone has an unstructured N-terminal tail domain that protrudes outside the nucleosomal DNA. These regions are not necessary for in vitro nucleosome assembly and crystallization [19] but contain many amino acids that are susceptible to post-translational modification [7]. How do these tail regions contribute to chromatin structure and function? Studies on the in vitro thermal mobility of nucleosomes [20] and earlier genetic and biochemical studies in yeast [21, 22] show that tails play a role in nucleosome mobility and higher order chromatin structure, but these roles are only revealed by removal of all, or most, of the tail, raising the question of how post-translational modifications could directly influence their function. Hyperacetylation of the tails of H2B, H3, and H4, each of which have 4–5 acetylatable lysines, will cause a significant loss of net positive charge and might influence higher order chromatin structures, even though the nucleosome itself is unaffected. An attempt to distinguish between the effects of lysine-specific and global acetylation of the H4 tail domain in yeast gave mixed results. For H4 lysines 5, 8, and 12, the level of acetylation (i.e., the number of lysines acetylated) seemed to be a more important determinant of transcription than the individual lysine involved, but H4 lysine 16 exerted independent effects [23]. Of course, methylation of lysines and arginines causes no change in net charge.

4.1.1.2 Chemical Signals on the Nucleosome Surface

An alternative explanation for the functional effects of histone tail modifications is that they act *indirectly* by generating, on the nucleosome surface, a variety of chemical signals that provide binding sites for nonhistone proteins. These binding proteins, in turn, regulate chromatin structure and function. This hypothesis was proposed 20 years ago [24, 25] and has since been extensively validated, not least by the identification of families of proteins carrying binding domains that recognize specific histone modifications [26, 27]. Bromodomains bind specifically to acetylated lysines, while chromodomains and several others bind to methylated lysines at selected positions on specific histones. Binding domains sometimes distinguish between lysines carrying one, two, or three methyl groups [26, 27].

A good example of how binding domains work is provided by the heterochromatin protein HP1, which is essential for heterochromatin formation in Drosophila and mammals. HP1 binds specifically, via its chromodomain, to H3 methylated at lysine 9 (H3K9me). H3K9me is located on heterochromatin in vivo and heterochromatin cannot form if the required methyl transferase is knocked out in mice [28]. Further, detailed studies of binding of HP1 to nucleosome arrays carrying methylated H3K9 provide likely mechanisms for both chromatin condensation and for the ability of heterochromatin to spread in vivo [29]. Other histone modifications have been associated with specific chromatin states. H4K36ac seems to be involved in the elongation phase of ongoing transcription [30], H4K20me3 is a marker for centric (constitutive) heterochromatin [31], and H3K27me3 is associated with long-term

gene silencing [32]. However, it is important to avoid oversimplifying a complex situation. Binding to any given modified residue will inevitably be influenced by modification at adjacent residues and functional outcomes are usually determined by the combinatorial action of different modifications. For example, phosphorylation of H3 serine 10 can displace HP1 bound to H3 methylated at lysine 9 [33]. Epigenomics approaches are beginning to reveal combinations of modifications that are consistently associated with functionally defined genomic regions, particularly promoters and enhancers [34–36].

The nucleosome can be seen as a gatekeeper that controls the access of transcription factors and other DNA binding proteins to DNA. Access is regulated by a variety of processes that change nucleosome structure, either directly (chromatin-remodeling enzymes, modification of internal amino acids) or indirectly (histone tail modifications). The enzyme families that carry out these processes are all susceptible to disruption, either through genetic mutation or environmental agents, triggering alterations in genome function that can sometimes precipitate changes in cell behavior and disease. Unraveling these complex chromatin-modifying enzyme systems will bring enormous benefits in the form of improved understanding of the etiology of diseases such as cancer and opening up new routes to therapy.

4.1.2 Histone Modification Status Is Regulated by Antagonistic Enzymes

Each histone modification is governed by antagonistic groups of enzymes that are able either to add or remove the modification in question. For example, histone acetyltransferases (KATs) catalyze the transfer of an acetyl group from acetyl-CoA to the ε-amino group of targeted lysine residues, and in this manner can neutralize the positive charge of the lysines. As a result the electrostatic interactions between histone and DNA are reduced. It is often suggested that this electrostatic effect can result in an open chromatin conformation that is more conducive to transcription [37, 38]. However, the role of the histone tails in maintaining higher order chromatin structure is not clear and while charge-mediated changes may be important in some contexts, they cannot provide a complete explanation for the functional affects of histone modifications. The actions of KATs are countered by HDACs. Broadly, acetylation is associated with gene activation and deacetylation with gene repression. However, for other modifications there is often not such a strict relationship between modification and function. For example, histone methyltransferases (KMTs) can either promote or inhibit transcription depending on the specific residue that is targeted and its genomic location relative to a gene's transcription start site (TSS). The functional identification of enzymes involved in setting and removing histone modifications has revealed an increasingly numerous battery of proteins and complexes. Many of these enzymes are either cofactors or binding partners for transcription factors (TF). Alternatively transcription regulatory factors can contain intrinsic histone-modifying capacity.

It is also apparent that at least some histone modifications can be regulated on a larger chromosomal scale or even globally, whereas other modifications have a much more restricted pattern. For example, H3 methylated at K79 (H3K79me3) is widely distributed across euchromatic regions in yeast and protects against the spreading of telomeric heterochromatin [39, 40] while H3K27me3, a mark put in place by the polycomb repressive complex, is spread across groups of genes (e.g., the HOX clusters) to bring about their coordinated silencing [41, 42]. Alternatively, marks such as H3K4me3 are closely associated with local genomic features, particularly promoters, enhancers, and TSSs [43, 44].

The KAT superfamily includes at least 20 different and diverse proteins including CLOCK and NCOA1. Several subfamilies exist including the P300/CBP family, e.g., p300; GCN5 family, e.g., KAT2A; the MYST family, e.g., MYST1; SRC/p160 nuclear receptor co-activator family, e.g., NCOA1. Eighteen HDAC are known in humans that are classified into four classes based on homology that include the HDAC1-11 and 7 SIRT members. Twenty-eight different KMT are known to act on histones, at least in vitro [1]. KMT are abundant and diverse reflecting the importance of the methylated state of key residues for the control of evolutionarily conserved transcriptional programs, for example, associated with development. There are at least 30 KMTs, including key families such as EZ, SUV39, and SET. At least 20 demethylases (KDM) are divided into two major groups that include the LSD family members, e.g., KDM1A/LSD1 and the Jumonji family, e.g., JHDM3 and JARID proteins containing ARID domains.

Two points are particularly important in considering the extent of redistribution and altered patterns of histone modifications in cancer. The first is that the steady-state level of each modification represents a dynamic balance between the effects of the modifying and de-modifying enzymes, with turnover likely to vary from one part of the genome to another, between cell types, and is intimately associated with cell cycle status, cell–cell interactions, and cell lineage commitment. Secondly, many, if not all, of the enzymes are either dependent upon, or influenced by, metabolites and components present in the intra- or extracellular environment. At the simplest level, many of these enzymes depend on cofactors such as acetyl CoA, NAD, and *S*-adenosyl methionine for their activity, and in turn these levels will depend on the metabolic and redox state of the cell. More subtle effects can be derived from metabolism. For example, naturally occurring inhibitors, such as short chain fatty acids (inhibitors of Class I HDACs) and nicotinamide (an inhibitor of the NAD-dependent deacetylase SIRT1) can be derived intrinsically within a cell or tissue and may naturally influence epigenetic status, for example, in the cell lining the lumen of the gut [45–47]. The effects of metabolic changes on gene expression are a strongly re-emergent area in cancer biology [48–50] and the generation of linked transcriptomic and metabolomics data is revealing the key functional associations in malignancy [51–53]. Thus the nucleosome, through the array of histone modifications it carries and the enzymes that put them in place, is a finely tuned sensor of the metabolic state of the cell and the composition of its environment. In this manner, nucleosome structure provides a platform through which external environmental and internal variables can influence genomic function.

4.2 Disruption of Histone Modifications in Cancer

Given that dynamic histone modifications are required for the precise control of DNA structure, during DNA repair and transcription, it is not surprising that there is significant evidence for the disruption of these events in malignancy. Understanding the differential recruitment and activity of proteins that govern histone modifications is key to understanding the roles that altered histone modifications can play in cancer initiation and development. Currently, a key focus in cancer biology is dissecting the mechanisms that alter the local and global recruitment and activity of histone-modifying complexes. It is anticipated that the insight generated will address the central challenge of separating which epigenetic processes directly drive cancer initiation and progression, from those that are merely a consequence of altered genomic structure such as mutation, copy number variation, and cytogenetic rearrangement. Insight into the contribution of altered histone functions to cancer progression can be gleamed by considering global and gene-loci specific alterations to histone modifications.

4.2.1 Global Distortions to Histone Modifications

A number of histone modifications are intimately associated with higher order chromatin structures and chromatin packaging and therefore changing the distribution of these global marks can have profound impact on the structure of chromatin in the nucleus. In turn such altered structures may be either more prone to aberrant DNA repair or promote genomic instability [54]. In prostate cancer, for example, quantifying global levels of five selected histone modifications in tissue sections by immunocytochemistry allowed discrimination between groups of patients with distinct risks of tumor recurrence [55, 56]. Quantitative analysis of just two modifications (H3K18ac and H3K4me2) was shown to provide useful prognostic information. The mechanisms underpinning these intriguing observations remain unknown.

The Polycomb complex (PcC) is a highly conserved inducer of repressive chromatin and sustains the H3K27me3 mark. This repression was shown to extend to multiple target genes associated with differentiation, often during development. Consequently, an emergent area in malignancy is the focus on aberrant PcC function to repress differentiation programs inappropriately. Increased H3K27me3 has been shown to have prognostic value in prostate and other cancers. These findings, however, reported the prognostic value to arise from the opposite patterns. Thus, increased levels of H3K27me3 are correlated with poor prognosis in esophageal cancer [57, 58], whereas in prostate cancer low levels have the poorer prognosis [59, 60].

The enzymes that control H3K27 methylation status are members of the enhancer of zeste homolog (EZH) that is the catalytic subunit of the polycomb repressive complex 2 [61]. These proteins are overexpressed in many cancers and in certain cases appear to correlate with poor prognosis or more aggressive disease.

However, although there are some correlations with increased H3K27me3 status, these are not universal in terms of the level of the mark. This may instead reflect the dynamics of turnover of the mark, and therefore the H3K27me3 status needs to be correlated with the enzymes that both add and subtract his mark.

Other modifications do appear to be altered in their global distribution in malignancy. For example, loss of H4K16 acetylation and H4K20me3 appears to be diminished globally in cancer cells, and indeed were some of the first histone marks to be characterized as being altered in malignancy [62, 63]. The consequence of these alterations probably reflect the role that certain modifications have in cross-talking with the mechanism of DNA methylation and indeed reduced levels of these marks were associated with DNA hypomethylation. Down-regulation of MYST1/MOF, one of the KAT that targets H4K16, may in part explain these altered patterns [64]. Reenforcing the concept of antagonistic enzymes, H4K16 is deacetylated by SIRT1 which is also up-regulated in several cancers and may have prognostic significance of its own [65]. Furthermore, the MYST family of KATs is associated with global changes in histone marks associated with chromatin packaging, DNA repair, and the control of developmental transcriptional programs (reviewed in [66]).

The control of lysine methylation states, however, is frequently more complex than acetylation states, and there are multiple enzymes controlling this modification. A major contributor to this complexity is the fact that the lysine epsilon amino group can accommodate one, two, or three methyl groups. All three methylation states are found in vivo and are often associated with distinct functional outcomes. Lysine methylation often proceeds in two steps, with mono and di-methylation governed by one class of enzyme and subsequent tri-methylation being regulated by a subsequent enzyme. For example, SET7 is able to catalyze the generation of H3K20me2, which then forms a substrate for the SUV class enzymes that generates the fully methylated state H3K20me3. Reflecting this, there is some evidence that levels of SUV family members are reduced in cancer in association with gene silencing [67, 68].

Further examples of a global alteration of histone status linked with cancer progression are those modifications that drive nucleosome movement. One of the key modifications in this regard is the internal lysine H3K56 that is targeted for acetylation by the KATs, CBP/p300 and GCN5, and has recently been shown to facilitate nucleosome disassembly and transcriptional activation. Inhibitor studies and expression profiling both suggest that the altered levels of H3K56ac distort the DNA damage response and maybe a trigger for genomic instability. Parallel studies have also revealed that H3K56ac is also involved in modulation of chromatin structure during DNA replication and repair; consequently, disruption to this process can also lead to genomic instability [18, 69–71]. Perhaps reflecting the importance of the regulation of this mark, multiple HDACs have been implicated in its control and include the NAD-dependent SIRTs.

Global changes in histone modifications have also been linked to stem cell differentiation. Undifferentiated embryonic stem (ES) cells show global enrichment in histone modifications associated with transcriptional activity and depletion in modifications associated with silent chromatin [72, 73]. By several criteria, ES cell

nuclear DNA is packaged in an unusual form of chromatin that appears to be more "open" than that in differentiated cells and is transcriptionally hyperactive [74]. How elevated histone modification levels are generated, and whether they are a cause or a consequence of open, hyperactive chromatin, remains to be determined.

Knocking down, individually, the histone demethylases KDM2A/JMJD1A and KDM4C/JMJD2C in mouse ES cells, globally increased the level of histone modifications usually associated with silent chromatin, namely, H3K9me2. In addition to their global effects, KDM2A/JMJD1A and KDM4C/JMJD2C were also shown to target, and regulate, specific genes, including *Tcl1*, a potential regulator of self-renewal, and *Nanog*, a key determinant of pluripotency [75]. Thus, key chromatin-modifying enzymes can exert both global and gene-specific effects that in turn influence differentiation. Intriguingly, both demethylase genes were themselves positively regulated by the key transcription factor Oct4, showing how a transcription factor might trigger a feed-forward signal to bring about a genome-wide change in the epigenetic landscape through regulation of genes encoding histone-modifying enzymes. In adult stem cell compartments, regulation of specific histone demethylating enzymes has also emerged as critical in activating differentiation programs, for example, the control of neural stem cell differentiation by the retinoic acid receptor, a member of the nuclear receptor (NR) superfamily [76]. A similar relationship between a transcription factor, global histone modifications, and adult stem cell differentiation is seen in studies of epidermal stem cells [77]. Quiescent stem cells are induced to leave their niche in the interfollicular epidermis and hair follicle bulge by activation of MYC, a process accompanied by globally increased H4 acetylation and di-methylation of H3K9 and H4K20. Together these studies illustrate how key transcription factors combine with environmental factors to influence and regulate the stem cell niche and control differentiation outputs.

Finally, the enzymes that govern histone methylation are also distorted in cancer with both loss and gain of function. Expression patterns of histone-modifying enzymes are even able to discriminate between tumor samples and their normal counterparts and cluster the tumor samples according to cell type [78]. This indicates that changes in the expression of histone-modifying enzymes have important and tumor-specific roles in cancer development. Thus, overexpression of G9a, an H3K9 KMT, occurs in lung and breast cancers and associates with aggressiveness [79]. Similarly enzymes that de-acetylate H3K9, and allow it to be methylated, are also overexpressed in cancers, including breast cancer. These enzymes may also be playing separate roles, and therefore expression is selected in malignancy on a different basis, for example, in gene regulation and DNA repair. It is possible that increases in HDAC levels are a homeostatic response in which the cell attempts to compensate for the aberrant increase in KAT activity (or vice versa). What is important from a functional point of view is not the absolute levels of KATs or HDACs, but the new steady-state levels of the (histone) modifications they regulate.

More precise specificity is dependent on the combination of both the enzyme *and* target gene(s). For example, mutation of KDM6A/UTX results in the inability to relieve H3K37me3 repression [80, 81]. Gain of function also occurs, for example, increased targeting of methyltransferases KMT1A/SUV39H1 to *CDKN1A* leads to

sustained H3K9me2 and transcriptional silencing that in turn can be targeted with the enzyme inhibitor, chaetocin [82, 83]. Similarly, the KMTs/MLLs are overexpressed in prostate cancer [84–86] and sustain levels of H3K27me3 at key targets such as DAP2IB, an RAS regulatory molecule, thereby leading to metastasis [87]. These observations illustrate deregulation of the enzymes that control histone lysine methylation is common but most likely highly targeted. This contextual nature is typified by KDM1A/LSD1 [88], which can target the demethylation of either H3K9me2 or H3K4me3 and thereby drive both gene activation [89, 90] and repression [91]. In this manner, KDM1A/LSD1 may mediate parallel repression and activation of target genes and play a key role in the malignant evolution of AR signaling in prostate cancer.

4.2.2 Altered Histone Modification Patterns at Discrete Gene Loci

Histone modifications therefore appear to operate at a level of restricted action, at discrete loci, exemplified by lysine methylation. Functional outcomes depend not only on which lysine on which histone is methylated, but also on whether the lysine carries one, two, or three methyl groups and its genomic position on a given loci with respect to the TSS. The different degrees of methylation are put in place, and removed, by a diverse group of enzymes. In particular, KDMs seem to have a particularly close association with key transcription factors that in turn are also implicated in malignancy such as MYC and members of the NR superfamily. Ligand binding or cofactor associations are able to influence the activity or even the specificity of these enzymes and thereby regulate functional outcomes (usually a change in gene expression) [92].

The modification of H3K9Ac and H3K9me2 serves to illustrate key concepts concerning histone status and specific gene expression. These marks are mutually exclusive and reciprocal, being associated with gene activation and repression, respectively. Loss of H3K9me2 is often associated with elevated gene expression. Recent studies have underscored the targeted changes in lysine methylation status and specifically illustrated that the KDM that targets H3K9me2 and the KMT that targets H3K4me at the gene TSS (to activate gene expression) are within the same complex associated with the ERα and therefore facilitate this two-step gene activation process [93]. Naturally, given that gene expression in cancer is uniformly neither up or down-regulated, the global expression of these marks is also not uniformly altered. Rather patterns are nuanced and suggest specific loci are deregulated.

Another example of this specificity emerges from considering KDM1A/LSD1 that can demethylate H3 mono- and di-methylated at either K4 or K9, and, remarkably, this specificity can be regulated in vitro by the protein cofactors, CoREST or BHC80, with which it is associated [94, 95]. Thus, KDM1A/LSD1 acts as an H3K4 demethylase (i.e., can remove a potentially activating modification) on NRSF targets and an H3K9 demethylase (i.e., can remove a potentially repressive modification)

on AR and ERα target promoters. Catalytic activity/specificity can also be regulated by adjacent histone modifications. H3K9 acetylation inhibits H3K4 demethylation (on the same tail) in vitro [96, 97]. Local patterns of modification are set by the combined actions of methylating and demethylating enzymes and the methylases too are influenced by other histone modifications. Further details of the gene-specific interactions have also emerged. JMJD2C demethylates H3K9me3, while KDM1A/LSD1 demethylates H3K9me2/me1 at promoters such as *PSA* and *KLK2* to remove H3K9 methylation associated with transcriptional silencing.

Therefore, the specific complex that KDM1A/LSD1 interacts with profoundly alters the transcriptional outputs, for example, of the AR, since demethylation of H3K9 has a gene activating effect, while demethylation of H3K4 has a gene silencing effect. The balance of these actions is in part controlled by the regulation of phosphorylation of H3 at threonine 6 (H3T6) by protein kinase C beta I. This prevents KDM1A/LSD1 from targeting H3K4me2 during AR-dependent gene activation and prevents it from limiting transcriptional activation. Also reflecting shared functions PKCbeta(I) co-localizes with AR and KDM1A/LSD1 on target gene promoters and phosphorylates H3T6 after androgen-induced gene expression. Therefore, it appears that androgen-dependent phosphorylation leads to the new chromatin mark H3T6ph, which in turn prevents removal of active methyl marks from H3K4 and forms a positive feed-forward loop of gene regulation [91]. More recently, KDM1A/LSD1 has been shown to drive AR-stimulated gene transrepression of the AR itself and thereby form a negative feedback loop of gene regulation [98]. Thus, the complex within which this one regulatory enzyme associates, its targeting to different genes, and the position of the response element, relative to the TSS, can all combine to determine how different H3K methylation states are governed.

4.2.3 Interplay Between Altered Transcriptional Signals and Epigenetic States

In normal cells a highly choreographed balance of histone modifications occurs during the dynamic regulation of coding and noncoding genes. These patterns are generated by the highly integrated actions of transcriptional networks [99] and are evident in many aspects of biology. For example, in development; in homeostasis to control the circadian rhythm [100], tissue self-renewal, and the response to hypoxia [83, 101]; in immune function to regulate inflammation [102]. Many of these processes are disrupted in malignancy and generally in cancer cells there is a loss of dynamic transcriptional patterns and signaling complexity is reduced [103]. Consequently, an area where altered histone modifications appear to associate with the cancer phenotype is in distortion of transcriptional control of key cellular processes.

Epigenetic events play a central role for transcriptional complexes and the various components in these multimeric complexes sequentially initiate, sustain, and

finally terminate transcription [104]. In this manner, transcription can work as a type of biological ratchet, with histone modifications being associated with the various states by generating chromatin states that are either receptive or resistant to transcription (reviewed in [27]). For example, different histone modifications can control the rate and magnitude of transcription (reviewed in [105]). These events are intertwined with low-level CpG methylation [106–108]. Thus, the histone modifications and other epigenetic events including DNA methylation processes combine during transcription to generate highly flexible chromatin states that are either transcriptionally receptive and resistant [101]. That is, the specific transcriptional potential of a gene is flexibly controlled by the combination of epigenetic events. These events are varied in space across the gene loci, and in time through the course of the transcriptional cycle. Current challenges in the field of cancer epigenetics, therefore, are to reveal how altered histone modifications directly drive distorted transcriptional programs, and what patterns exist on a genome-wide scale to distort networks of transcription. This will help to define how these altered histone states are genuine drivers in cancer progression.

Precisely how transcriptional programs evolve during malignancy is emerging. Genome-wide approaches are now allowing workers to ascribe broader views of the biology of transcription factor families, now that all members are known, and questions can be addressed in more detailed biological contexts. These findings suggest that the actions of the many key transcription factors are distilled through interactions with multiple cellular processes thereby generating an extremely flexible and integrated signaling module. In malignancy, however, these transcriptional choices and phenotypic outputs become restricted, for example, as seen with the emergence of a novel AR-transcriptome in androgen deprivation therapy-resistant prostate cancer [109].

Importantly, these epigenetic regulatory mechanisms operate in response to signals from the cellular microenvironment of the tumor, including signals from associated stromal (noncancerous) cells [110, 111]. The “niche” in which cells find themselves is an important determinant of their epigenetic properties [112] and raises the possibility that histone marks can be modified by environmental conditions that alter metabolic and redox status, leading to a heritable alteration in cell phenotype, an “epigenetic mutation.” Such lesions are not restricted to single nucleotides, but rather can be targeted to larger regions and therefore comparable to genetic deletions and amplifications. They can act alongside conventional genetic and cytogenetic alterations, either inherited or de novo, to cause the bi-allelic silencing of tumor suppressor genes that can be the first step in development of a cancer [113]. These concepts are illustrated by considering key transcription factor families implicated strongly in cancer initiation and progression.

4.2.3.1 The MYC/MAX/MAD Family

The MYC/MAX/MAD family forms heterodimeric complexes with MAX as the central partner to activate the expression of a diverse range of genes. Deregulated

and elevated expression of c-MYC has been documented in a wide range of human malignancies, associated frequently with aggressive and poorly differentiated tumors [114]. MYC has the potential to target a large proportion (11%) of all genes in the human genome [115], but the set of genes to which it actually binds in any particular cell is regulated by a variety of factors, including interacting proteins. For example, the MAD family of transcritpional repressors is , like MYC, able to bind MAX proteins and antagonize the activity of MYC by competing for MAX binding at E-box sequences in target gene promoters, actively repressing transcription of MYC target genes [116].

The specificity and affinity of MYC binding is influenced by the configuration of the chromatin packaging at potential binding sites, and particularly by patterns of histone modification [117]. MYC was found to bind E-boxes in regions enriched for several histone modifications generally associated with euchromatin, such as acetylated H3 (specifically H3K9ac, H3K14ac, and H3K18ac), but showed the strongest association with H3K4me3. Reciprocally, MYC was inversely correlated with the repressive polycomb group mark H3K27me3. On some promoters, MYC associated with both H3K4me3 and H3K27me3, a bivalent state that is common in ES cells but seems rarer in lineage committed cells [118]. Overall, it seems more likely that H3K4me3 recruits MYC rather than H3K27me3 excluding MYC binding. [117].

MYC function can be controlled interactions with JARID1A/RBP2 and JARID1B/PLU-1 [119, 120]. These enzymes are both specific for H3 methylated at lysine 4 (H3K4me1,2,3) and may help to regulate this modification at MYC binding sites. There is emerging evidence that this process is disrupted by increased association with histone demethylase NO66/MAPJD to alter the potential interactions with genes involved in proliferation of lung cancer cells [121]. A gene encoding a related protein, MINA53 (myc-induced nuclear antigen) is a MYC target that is overexpressed in lung cancer, for example [122, 123]. Together these findings suggest that the co-association of MYC with different histone-modifying enzymes, for example, through the consequence of altered enzyme expression, distorts and restricts the MYC transcriptome in malignancy.

In the light of these developments, MYC function has been reassessed to reveal the regulation of unexpected gene targets, some of which inhibit proliferation and induce programmed cell death [124], contrary to the accepted view of MYC as an oncogene promoting growth and survival. These findings suggest that the malignant function of MYC represents selection for a subset of its potential actions.

4.2.3.2 The NR Superfamily

The NR superfamily also illustrates the key concepts of distorted and selected transcription in cancer due to altered regulation of histone modifications. NRs are the largest superfamily of transcription factors in humans and generally form active heterodimers to control networks that regulate homeostasis, energy metabolism, and xenobiotic handling. These receptors are intimately associated with the control of self-renewal in a number of epithelial systems, notably the prostate and mammary

glands. For example, studies in the prostate have established that the androgen receptor (AR) cooperates with WNT and mTOR pathways [125, 126] to induce proliferation. Equally other receptors, such VDR, PPARs, and RARs, exert mitotic restraint, at least in part by antagonizing WNT signaling and activation of cell cycle arrest through regulation of gene targets such as *CDKN1A* (encodes $p21^{(waf1/cip1)}$) and *IGFBP3* [127–134].

Cancer is typified by the actions of individual receptors becoming selective and the NR network collectively displaying a loss of transcriptional plasticity. The AR transcriptional program evolves towards increased targeting of proliferative gene promoters and decreased targeting of pro-differentiation genes [135, 136]. Similarly, within breast cancer the transcriptional actions of the ERα appear to become increasingly selective for gene targets associated with proliferation and survival and away from targets associated with differentiation [137–139]. Equally in a range of solid tumors and myeloid leukemia, NRs that normally exert mitotic restraint, such as the VDR, RARs, and PPARs, become skewed, with selective silencing of antiproliferative target genes [129, 140–144]. Combined, oncogenic transcriptional rigidity reflects the simultaneous distorted regulation of target loci such that proliferative and survival signals are enhanced and antimitotic inputs are either limited or lost. This filtering of transcriptional choices during cancer progression has significant therapeutic implications. For example, the oncogenic actions of the TMPRSS2/ETS fusion, a common event in prostate cancer [145], are critical precisely because the *TMPRSS2* promoter is sustained in an AR-responsive state.

More recently, genome-wide ChIP approaches have revealed considerable variability in the networks of interactions capable of bringing about varied transcriptional responses [146–148]. For example, in prostate cancer, as the disease progresses, there are altered levels of H3K4me1 and 2 on gene enhancer regions in the so-called AR-independent state, where cells have evolved resistance to antiandrogen therapies. In this new state, the targeted increase of H3K4Me1 and 2 at different enhancer regions allows the cells to initiate a different AR transcriptional program [109].

4.2.3.3 Hypoxia-Inducible Factor-1 Alpha

The hypoxia response of hypoxia-inducible factor-1 alpha (HIF-1A) also illustrates how transcriptional actions are selectively distorted by epigenetic processes in cancer cells. Within a normal cell, the levels of oxygen are monitored sensitively by a transcriptional circuit that governs the function of HIF-1A. In normoxic conditions, HIF levels are kept low level by destruction by an E3 ubiquitin ligase containing the VHL tumor suppressor protein, where oxygen serves as a co-substrate. Also oxygen impedes the interactions of HIF1α with the KATs CBP/p300 thus limiting the capacity to initiate activating histone marks. In hypoxia, HIF-1α becomes stabilized and active, and promotes a stable interaction with CBP/p300 and therefore facilitates transcription [83]. Genome-wide analyses of HIF binding sites identified a number of KDMs as downstream targets, notably

JMJD1A and JMJD2B, thus providing the capacity to affect the epigenetic status of the cell. In part, this may contribute to maintenance of transcriptional activity under stress. It may also support the observed aberrant and selective HIF1α transcriptional responses [149].

Taken together these findings support the concept that the actions of major transcription factor families are selective at several levels to govern the expression of sub-transcriptomes that are phenotypically related. The flexibility of transcriptional actions includes the exact choice of target sequence, the timing, amplitude, and magnitude of transcription and integration with other transcriptional programs and signal transduction events. In malignancy, the dexterity of targeting and regulation is blunted and instead transcription factors become addicted to specific sub-transcriptomes, for example, those associated with blockade of programmed cell death and progression through the cell cycle.

4.2.4 Loss- and Gain-of-Function of Transcriptional Co-activators and Co-repressors

One means by which transcriptional actions are distorted is through the altered expression of associated cofactors that either have an intrinsic or associated capacity to regulate histone modifications. The diversity of co-activator and co-repressors is extreme and they have been the subject of numerous reviews [150–154]. Several examples are strongly illustrative of underlying mechanisms of transcriptional regulation. In essence, the altered expression and function of these key proteins alters the equilibrium of key histone modifications and thereby allowing the gene regulatory actions of a given transcription factor to become more or less pronounced.

Co-activators and co-repressors each display both loss and gain of function, and can result in similar phenotypes. Thus, the loss of a co-activator can lead to suppressed ability of a transcription factor to transactivate a given target. Similarly, the gain of function of co-repressors can limit transactivation ability and enhance transrepression. The opposite patterns will in turn enhance the transactivation function.

For example, NCOA3/SRC3 is situated within a common area of chromosomal amplification in breast cancer on chromosome 20q. Initially, cDNAs were isolated from this region that contained a putative target gene that was termed AIB1 (for "amplified in breast cancer-1"). Subsequently, this gene was found to be a member of the SRC co-activator family and was amplified and overexpressed in breast and ovarian cancer cell lines, as well as in breast cancer biopsies [155]. NCOA3/SRC3/AIB1 interacts with ERs in a ligand-dependent fashion and enhances the regulation of target genes. Specifically the protein has intrinsic KAT activity and also acts to recruit other CBP/p300 in an allosteric manner [156]. Therefore, increased expression increases the ability of the ERα to transactivate a given gene target. Subsequently, this protein was identified NCOA3 and shown to be a potent histone acetyl transferase able to enhance the function of multiple NRs [157–159].

Compared to their co-activator cousins, the co-repressors are somewhat under-explored. Again, these key proteins, originally identified for their repressive interactions with NR illustrate how deregulated functions can alter chromatin and thereby attenuate gene regulation. NCOR1 and NCOR2/SMRT were cloned in 1995 using NR as bait [160, 161], and both proteins exist in large multimeric complexes (~2.0 MDa) [162] with histone deactylases and other histone-modifying enzymes (reviewed in [153]). These complexes are recruited to many different transcription factors to repress gene activity during the transcriptional cycle. These transcription factors include: NR, MAD/MXI, MYOD, ETO, CBF, FOXP, AP-1, and NF-κB factors. The importance of targeted *basal repression* by co-repressors is evident in the lethality of the *Ncor1*$^{-/-}$ and *Ncor2/Smrt*$^{-/-}$ mice. These models reveal enhanced function of transcription factors, notably Pparγ in adipocytes [163] and FoxP in cardiomyocytes [164]. Dynamic mechanisms have also emerged whereby NCOR1 and NCOR2/SMRT complexes can be recruited to activate transcription factors leading to transrepression [165, 166]. Finally, an emerging theme is the pattern of active de-repression where loss of co-repressor association, following activated transcription factor, leads to up-regulation of target genes independently of the sustained presence of the transcription factor [167].

Well-established oncogenic roles for NCOR1 and NCOR2/SMRT have been elucidated in acute promyelocytic leukemia (PML) that results from a fusion between the NR, RARα, and either the PML or promyelocytic leukemia zinc finger (PLZF) genes [142]. Both chimeric proteins sustain NCOR1 interactions and consequently RARα-mediated cell differentiation is blocked, in part, as a result of maintaining a condensed chromatin structure around the promoters of RARα target genes that govern normal hematopoietic differentiation [168, 169]. In the PML-RAR fusion, this can be overcome by pharmacological dosing with retinoic acid. The PLZF-RAR fusion is resistant to retinoic acid alone and treatment with a combination of retinoic acid and HDAC inhibitors has shown promising results. Similarly, in acute myeloid leukemia (AML), the AML1/ETO fusion protein promotes leukemogenesis by recruiting NCOR1 and again impeding transcriptional regulation [170]. The importance of NCOR1 binding in the treatment of these disease states exemplifies the relevance of the co-repressors in firstly driving critical oncogenic events, but secondly providing a rational targeted strategy towards HDACs.

Expression profiling in solid tumors has revealed altered NCOR1 and NCOR2/SMRT expression and localization, for example, in breast, bladder, and prostate cancers [129, 141, 143, 171–173]. However, to date, uncertainty remains over their precise role in solid tumors, especially in the case of breast and prostate cancers where the etiology of disease is intimately driven by the actions of steroid hormone NRs. Indeed, the ability of the ligand-free NR to bind NCOR1 and NCOR2/SMRT is important to therapeutic exploitation with receptor antagonists such as Tamoxifen in the case of breast cancer. Therefore, ambiguity exists over the extent and timing of NCOR1 and NCOR2/SMRT expression changes, as they relate to initiation and progression of disease. Secondly, it remains unclear how changes in NCOR1 and NCOR2/SMRT expression relate to different NRs and other transcription factors that exert either pro- or antimitotic and survival effects. Resolving these ambiguities

has significant therapeutic implications in terms of targeting co-repressors as either epigenetic mono-therapies using HDAC inhibitors or in combinations with transcription factor targeting.

In prostate cancer cells, elevated levels of NCOR2/SMRT have been detected and suppress VDR responsiveness [129]. Similarly, PPAR actions are disrupted and can be targeted selectively by using HDAC inhibitor co-treatments [174, 175]. More specifically, elevated NCOR1, and to a lesser extent NCOR2/SMRT correlated with, and functionally drove, the selective insensitivity of PPARα/γ receptors towards dietary derived and therapeutic ligands [175] most clearly in androgen-independent disease. Similar roles for NCOR1 and NCOR2/SMRT appear in the development of breast cancer and Tamoxifen resistance [171]. Elevated levels of NCOR1 occur in ERα negative disease and in turn attenuate antimitotic actions of VDR. Again, this molecular lesion can be targeted in ERα negative breast cancer cell lines with co-treatments of VDR ligand (e.g., $1\alpha,25(OH)_2D_3$) plus HDAC inhibitors resulting in selective re-expression of VDR target genes, notably *VDUP1* and *GADD45A* [143]. Together, the studies in breast and prostate cancer suggest that NR show specificity in their interactions with co-repressors. NCOR1 appears to be involved in the regulation of receptors such as the VDR and PPARs and NCOR2/SMRT with steroid hormone receptors, reflecting the emergent specificities of NR interactions in the murine knockout models.

4.3 Consequences of Altered Histone Modification States

4.3.1 Higher Order Chromatin Interactions Associated with Transcription

Another theme that has emerged concerning epigenetic regulation of transcription is higher order chromosomal interactions. It seems that large-scale chromatin rearrangement, through looping, is frequent and widespread. Loops can be inter- or intra-chromosomal and are guided by transcription factors, key pioneer factors, and chromatin-modifying enzymes [176, 177]. Improved microscopy techniques have recently shown nascent RNA on the surface of protein dense transcription factories ("gene hubs") that seem to correspond to structures previously termed "nuclear speckles" [178].

A clear example of these interactions has been illustrated in the transcriptional responses of B-cells where translocation of genes occurs from separate chromosomes and nuclear regions to common sites referred to as transcription factories. These sites contain significant levels of RNA Pol II, and other proteins, including factors required for elongation, chromatin remodeling, capping, splicing, and nonsense-mediated decay. Recruitment of genes to transcription factories is highly selective, with certain genes and chromosome regions co-localizing far more frequently than expected by chance. Intriguingly, sites of chromosome translocation

associated with various cancers often co-localize. For example, *Myc* and *Igh* tend to co-localize and their fusion, in human lymphoid cells, is a common cause of Burkitt's Lymphoma. These rapid movements are associated with movements of the nuclear architecture and involve ATP-dependent mechanisms that involve a chromosome locus usually located at the nuclear periphery being rapidly translocated to the interior in a direction perpendicular to the nuclear membrane [179].

Again, the NR superfamily illustrates these aspects of the deregulation of epigenetic states. NRs appear to interact with more dominant more widely binding pioneer factors. For example, ERα interacts with pioneer factors and KDMs. This interaction is involved with micro-chromatin reorganization at response elements, and also with higher order chromatin reorganization. Active ATP-dependent transport mechanisms have recently been shown to be an essential intermediate step in gene activation by ERα and act to move discrete chromosomal regions together into interchromatin hubs. These granules are subsequently joined to the surface of nuclear structures rich in splicing and transcriptional machinery that may reflect the previously termed "nuclear speckles" [180].

This suggests a role for KDM1A/LSD1 in directing docking of the ERα-gene hub complex with the nuclear speckles, but the exact function of KDM1/LSD1 in this process remains unclear. If this role is catalytic rather than purely structural, it is possible that the substrate involved is a nonhistone protein. It will also be of interest to determine whether KDM1/LSD1 or related enzymes play a role in directing *MYC* and *IGH* alleles to transcription factories. The recent development of improved microscopy techniques which has shown nascent RNA appearing on the surface of protein dense transcription factories should aid in clarifying this situation [178], as well as further work investigating the relationship between nuclear speckles and transcription factories.

4.3.2 Directing DNA Methyltransferase Specificity and Stable Gene Silencing

There is compelling evidence that histone and DNA methylation processes disrupt transcriptional actions, both alone and together. For example, one consequence of NCOR1 and NCOR2/SMRT association at target genes is the loss of H3K9ac and accumulation of H3K9me2, allowing the potential for hypermethylation at adjacent CpG regions. Further links exist between NCOR1 and DNA methylation through its interaction with KAISO [181]. Correlative studies reveal that a number of key AR and VDR target genes are silenced by increased CpG methylation [182, 183]. At high density regions of CpG methylation, spanning hundreds of base pairs, the entire region acquires H3K9 and -K27 methylation, loses H3K4 methylation, and recruits heterochromatin binding protein 1 (HP1) [101]. The recruitment of HP1 through interaction with MBD1 leads to recruitment of both an H3K9 methylase (KMT1A/SUV39H1) [184] and DNA methyltransferases (DNMTs) [185]; enzymes that add repressive methylation marks to histones and CpG.

DNMT3L and UHFR1 also provide potential links between DNA methylation and absence of H3K4 methylation and presence of H3K9 methylation, respectively (reviewed in [186]).

Thus, these processes become self-reinforcing. It is not precisely clear, however, in mammalian cells whether either the H3K9 methylation or the high density of CpG methylation is required first to set up this heterochromatic structure. However in *Neurospora crassa*, loss of HP1 (which requires H3K9 methylation for binding to chromatin) leads to loss of DNA methylation [187]. This situation describes stable heterochromatic silencing of genomic regions and is in contrast to the dynamic changes at a locus with active epigenetic regulation of transcription in response to NR activation. However, even in such actively regulated regions, dynamic changes in DNA methylation appear to occur. For example, these have been measured in response to NR actions [106–108].

This differential regulation of histone methylation has profound implications for transcriptional control. DNA methylation and H3K4 methylation are mutually exclusive, while H3K9 methylation is strongly associated with DNA methylation, for example, through the formation of heterochromatin by HP1 binding and histone deacetylation. In the absence of DNA methylation, these inter-relationships are highly dynamic, with target gene promoters often poised to be subsequently pushed towards a fully active, or a more stably repressed state. For example, CpG island promoter regions of non-expressed genes do in fact show low-level RNA POLII association and modest transcriptional initiation. It seems that the presence of H3K4me3 methylation holds these promoters in a chromatin structure that is accessible to the transcriptional machinery, poised to recruit specific transcription factors to drive high level, efficient transcription. In turn this prevents H3K9me2 and DNA methylation. Aberrant DNA methylation of these CpG islands in cancer cells reduces this plasticity and coincides with loss of H3K4 methylation, gain of H3K9 methylation along with other heterochromatin marks, and stable transcriptional silencing [101].

The distributions of these histone modifications and DNA methylation patterns in cell line models are being organized by research consortia, for example, ENCODE [188]. Again, these genome-wide datasets also appear to support the idea that these histone marks are strongly associated with features of genomic architecture, such as gene regions, TSS, and enhancer regions where regulatory transcription factors can bind.

The links between sustained repressive histone modifications in the enhancer or promoter regions of a gene locus and altered DNA methylating events are targets for exploitation. Importantly, these epigenetic lesions are individually highly targetable with clinically available small molecular weight inhibitors targeted to specific histone deacetylation events and more recently this has been extended to include histone methylation events [189], coupled with agents that target CpG methylation (reviewed in [190]). Thus, comprehensive understanding of the key co-repressors in malignancy, delineating the key transcription factors interactions and the critical targets that are thereby dysregulated, may have considerable prognostic utility, specifically through the capacity to stratify patients for specific tailored epigenetic therapies.

References

1. Allis CD et al (2007) New nomenclature for chromatin-modifying enzymes. Cell 131: 633–636
2. Turner BM (2005) Reading signals on the nucleosome with a new nomenclature for modified histones. Nat Struct Mol Biol 12:110–112
3. Luger K, Mader AW, Richmond RK, Sargent DF, Richmond TJ (1997) Crystal structure of the nucleosome core particle at 2.8 A resolution. Nature 389:251–260
4. Flaus A, Owen-Hughes T (2011) Mechanisms for ATP-dependent chromatin remodelling: the means to the end. FEBS J 278:3579–3595
5. Recouvreux P et al (2011) Linker histones incorporation maintains chromatin fiber plasticity. Biophys J 100:2726–2735
6. Talbert PB, Henikoff S (2010) Histone variants—ancient wrap artists of the epigenome. Nat Rev Mol Cell Biol 11:264–275
7. Kouzarides T (2007) Chromatin modifications and their function. Cell 128:693–705
8. Ngara R, Ndimba R, Borch-Jensen J, Jensen ON, Ndimba B (2012) Identification and profiling of salinity stress-responsive proteins in Sorghum bicolor seedlings. J Proteomics 75: 4139–4150
9. Zhang S, Roche K, Nasheuer HP, Lowndes NF (2011) Modification of histones by sugar beta-N-acetylglucosamine (GlcNAc) occurs on multiple residues, including histone H3 serine 10, and is cell cycle-regulated. J Biol Chem 286:37483–37495
10. Tan M et al (2011) Identification of 67 histone marks and histone lysine crotonylation as a new type of histone modification. Cell 146:1016–1028
11. Cosgrove MS (2007) Histone proteomics and the epigenetic regulation of nucleosome mobility. Expert Rev Proteomics 4:465–478
12. Hyland EM et al (2005) Insights into the role of histone H3 and histone H4 core modifiable residues in Saccharomyces cerevisiae. Mol Cell Biol 25:10060–10070
13. Xu F, Zhang K, Grunstein M (2005) Acetylation in histone H3 globular domain regulates gene expression in yeast. Cell 121:375–385
14. Masumoto H, Hawke D, Kobayashi R, Verreault A (2005) A role for cell-cycle-regulated histone H3 lysine 56 acetylation in the DNA damage response. Nature 436:294–298
15. Vempati RK (2012) DNA damage in the presence of chemical genotoxic agents induce acetylation of H3K56 and H4K16 but not H3K9 in mammalian cells. Mol Biol Rep 39:303–308
16. Clemente-Ruiz M, Gonzalez-Prieto R, Prado F (2011) Histone H3K56 acetylation, CAF1, and Rtt106 coordinate nucleosome assembly and stability of advancing replication forks. PLoS Genet 7:e1002376
17. Watanabe S et al (2010) Structural characterization of H3K56Q nucleosomes and nucleosomal arrays. Biochim Biophys Acta 1799:480–486
18. Xie W et al (2009) Histone h3 lysine 56 acetylation is linked to the core transcriptional network in human embryonic stem cells. Mol Cell 33:417–427
19. Luger K, Richmond TJ (1998) The histone tails of the nucleosome. Curr Opin Genet Dev 8:140–146
20. Ferreira H, Somers J, Webster R, Flaus A, Owen-Hughes T (2007) Histone tails and the H3 alphaN helix regulate nucleosome mobility and stability. Mol Cell Biol 27:4037–4048
21. Lenfant F, Mann RK, Thomsen B, Ling X, Grunstein M (1996) All four core histone N-termini contain sequences required for the repression of basal transcription in yeast. EMBO J 15:3974–3985
22. Ling X, Harkness TA, Schultz MC, Fisher-Adams G, Grunstein M (1996) Yeast histone H3 and H4 amino termini are important for nucleosome assembly in vivo and in vitro: redundant and position-independent functions in assembly but not in gene regulation. Genes Dev 10:686–699
23. Dion MF, Altschuler SJ, Wu LF, Rando OJ (2005) Genomic characterization reveals a simple histone H4 acetylation code. Proc Natl Acad Sci USA 102:5501–5506

24. Turner BM (1993) Decoding the nucleosome. Cell 75:5–8
25. Turner BM, Birley AJ, Lavender J (1992) Histone H4 isoforms acetylated at specific lysine residues define individual chromosomes and chromatin domains in Drosophila polytene nuclei. Cell 69:375–384
26. Kutateladze TG (2011) SnapShot: histone readers. Cell 146:842–842.e1
27. Taverna SD, Li H, Ruthenburg AJ, Allis CD, Patel DJ (2007) How chromatin-binding modules interpret histone modifications: lessons from professional pocket pickers. Nat Struct Mol Biol 14:1025–1040
28. Liu Z et al (2010) Jmjd1a demethylase-regulated histone modification is essential for cAMP-response element modulator-regulated gene expression and spermatogenesis. J Biol Chem 285:2758–2770
29. Canzio D et al (2011) Chromodomain-mediated oligomerization of HP1 suggests a nucleosome-bridging mechanism for heterochromatin assembly. Mol Cell 41:67–81
30. Kolasinska-Zwierz P et al (2009) Differential chromatin marking of introns and expressed exons by H3K36me3. Nat Genet 41:376–381
31. Kourmouli N et al (2004) Heterochromatin and tri-methylated lysine 20 of histone H4 in animals. J Cell Sci 117:2491–2501
32. Ringrose L, Paro R (2007) Polycomb/trithorax response elements and epigenetic memory of cell identity. Development 134:223–232
33. Mateescu B, England P, Halgand F, Yaniv M, Muchardt C (2004) Tethering of HP1 proteins to chromatin is relieved by phosphoacetylation of histone H3. EMBO Rep 5:490–496
34. Ernst J et al (2011) Mapping and analysis of chromatin state dynamics in nine human cell types. Nature 473:43–49
35. Kharchenko PV et al (2011) Comprehensive analysis of the chromatin landscape in Drosophila melanogaster. Nature 471:480–485
36. Rada-Iglesias A et al (2011) A unique chromatin signature uncovers early developmental enhancers in humans. Nature 470:279–283
37. Korolev N, Lyubartsev AP, Nordenskiold L (2006) Computer modeling demonstrates that electrostatic attraction of nucleosomal DNA is mediated by histone tails. Biophys J 90: 4305–4316
38. Perico A, La Penna G, Arcesi L (2006) Electrostatic interactions with histone tails may bend linker DNA in chromatin. Biopolymers 81:20–28
39. Takahashi YH et al (2011) Dot1 and histone H3K79 methylation in natural telomeric and HM silencing. Mol Cell 42:118–126
40. Jones B et al (2008) The histone H3K79 methyltransferase Dot1L is essential for mammalian development and heterochromatin structure. PLoS Genet 4:e1000190
41. Lee MG et al (2007) Demethylation of H3K27 regulates polycomb recruitment and H2A ubiquitination. Science 318:447–450
42. Agger K et al (2007) UTX and JMJD3 are histone H3K27 demethylases involved in HOX gene regulation and development. Nature 449:731–734
43. Pekowska A et al (2011) H3K4 tri-methylation provides an epigenetic signature of active enhancers. EMBO J 30:4198–4210
44. Robertson AG et al (2008) Genome-wide relationship between histone H3 lysine 4 mono- and tri-methylation and transcription factor binding. Genome Res 18:1906–1917
45. Zeissig S et al (2007) Butyrate induces intestinal sodium absorption via Sp3-mediated transcriptional up-regulation of epithelial sodium channels. Gastroenterology 132:236–248
46. Augenlicht LH et al (2002) Short chain fatty acids and colon cancer. J Nutr 132: 3804S–3808S
47. Tanaka Y, Bush KK, Klauck TM, Higgins PJ (1989) Enhancement of butyrate-induced differentiation of HT-29 human colon carcinoma cells by 1,25-dihydroxyvitamin D3. Biochem Pharmacol 38:3859–3865
48. Cuezva JM et al (2002) The bioenergetic signature of cancer: a marker of tumor progression. Cancer Res 62:6674–6681

49. Racker E, Spector M (1981) Warburg effect revisited: merger of biochemistry and molecular biology. Science 213:303–307
50. Hsu PP, Sabatini DM (2008) Cancer cell metabolism: Warburg and beyond. Cell 134:703–707
51. Cavill R et al (2011) Consensus-phenotype integration of transcriptomic and metabolomic data implies a role for metabolism in the chemosensitivity of tumour cells. PLoS Comput Biol 7:e1001113
52. Su G, Burant CF, Beecher CW, Athey BD, Meng F (2011) Integrated metabolome and transcriptome analysis of the NCI60 dataset. BMC Bioinformatics 12(Suppl 1):S36
53. Sharon D, Chen R, Snyder M (2010) Systems biology approaches to disease marker discovery. Dis Markers 28:209–224
54. Misteli T, Soutoglou E (2009) The emerging role of nuclear architecture in DNA repair and genome maintenance. Nat Rev Mol Cell Biol 10:243–254
55. Seligson DB et al (2005) Global histone modification patterns predict risk of prostate cancer recurrence. Nature 435:1262–1266
56. Kurdistani SK (2007) Histone modifications as markers of cancer prognosis: a cellular view. Br J Cancer 97:1–5
57. He LR et al (2009) Prognostic impact of H3K27me3 expression on locoregional progression after chemoradiotherapy in esophageal squamous cell carcinoma. BMC Cancer 9:461
58. Tzao C et al (2009) Prognostic significance of global histone modifications in resected squamous cell carcinoma of the esophagus. Mod Pathol 22:252–260
59. Yu J et al (2007) A polycomb repression signature in metastatic prostate cancer predicts cancer outcome. Cancer Res 67:10657–10663
60. Wei Y et al (2008) Loss of trimethylation at lysine 27 of histone H3 is a predictor of poor outcome in breast, ovarian, and pancreatic cancers. Mol Carcinog 47:701–706
61. Hansen KH et al (2008) A model for transmission of the H3K27me3 epigenetic mark. Nat Cell Biol 10:1291–1300
62. Fraga MF et al (2005) Loss of acetylation at Lys16 and trimethylation at Lys20 of histone H4 is a common hallmark of human cancer. Nat Genet 37:391–400
63. Tryndyak VP, Kovalchuk O, Pogribny IP (2006) Loss of DNA methylation and histone H4 lysine 20 trimethylation in human breast cancer cells is associated with aberrant expression of DNA methyltransferase 1, Suv4-20h2 histone methyltransferase and methyl-binding proteins. Cancer Biol Ther 5:65–70
64. Pfister S et al (2008) The histone acetyltransferase hMOF is frequently downregulated in primary breast carcinoma and medulloblastoma and constitutes a biomarker for clinical outcome in medulloblastoma. Int J Cancer 122:1207–1213
65. Bosch-Presegue L, Vaquero A (2011) The dual role of sirtuins in cancer. Genes Cancer 2:648–662
66. Rius M, Lyko F (2011) Epigenetic cancer therapy: rationales, targets and drugs. Oncogene Dec 19. doi:10.1038/onc.2011.601
67. Pogribny IP et al (2006) Histone H3 lysine 9 and H4 lysine 20 trimethylation and the expression of Suv4-20h2 and Suv-39h1 histone methyltransferases in hepatocarcinogenesis induced by methyl deficiency in rats. Carcinogenesis 27:1180–1186
68. Lakshmikuttyamma A, Scott SA, DeCoteau JF, Geyer CR (2010) Reexpression of epigenetically silenced AML tumor suppressor genes by SUV39H1 inhibition. Oncogene 29:576–588
69. Das C, Lucia MS, Hansen KC, Tyler JK (2009) CBP/p300-mediated acetylation of histone H3 on lysine 56. Nature 459:113–117
70. Williams SK, Truong D, Tyler JK (2008) Acetylation in the globular core of histone H3 on lysine-56 promotes chromatin disassembly during transcriptional activation. Proc Natl Acad Sci USA 105:9000–9005
71. Adkins MW, Williams SK, Linger J, Tyler JK (2007) Chromatin disassembly from the PHO5 promoter is essential for the recruitment of the general transcription machinery and coactivators. Mol Cell Biol 27:6372–6382

72. Meshorer E, Misteli T (2006) Chromatin in pluripotent embryonic stem cells and differentiation. Nat Rev Mol Cell Biol 7:540–546
73. Spivakov M, Fisher AG (2007) Epigenetic signatures of stem-cell identity. Nat Rev Genet 8:263–271
74. Meshorer E, Gruenbaum Y (2008) Gone with the Wnt/Notch: stem cells in laminopathies, progeria, and aging. J Cell Biol 181:9–13
75. Loh YH, Zhang W, Chen X, George J, Ng HH (2007) Jmjd1a and Jmjd2c histone H3 Lys 9 demethylases regulate self-renewal in embryonic stem cells. Genes Dev 21:2545–2557
76. Jepsen K et al (2007) SMRT-mediated repression of an H3K27 demethylase in progression from neural stem cell to neuron. Nature 450:415–419
77. Frye M, Fisher AG, Watt FM (2007) Epidermal stem cells are defined by global histone modifications that are altered by Myc-induced differentiation. PLoS One 2:e763
78. Ozdag H et al (2006) Differential expression of selected histone modifier genes in human solid cancers. BMC Genomics 7:90
79. Watanabe H et al (2008) Deregulation of histone lysine methyltransferases contributes to oncogenic transformation of human bronchoepithelial cells. Cancer Cell Int 8:15
80. Wang JK et al (2010) The histone demethylase UTX enables RB-dependent cell fate control. Genes Dev 24:327–332
81. van Haaften G et al (2009) Somatic mutations of the histone H3K27 demethylase gene UTX in human cancer. Nat Genet 41:521–523
82. Cherrier T et al (2009) p21(WAF1) gene promoter is epigenetically silenced by CTIP2 and SUV39H1. Oncogene 28:3380–3389
83. Pollard PJ et al (2008) Regulation of Jumonji-domain-containing histone demethylases by hypoxia-inducible factor (HIF)-1alpha. Biochem J 416:387–394
84. Scharer CD et al (2009) Genome-wide promoter analysis of the SOX4 transcriptional network in prostate cancer cells. Cancer Res 69:709–717
85. Varambally S et al (2002) The polycomb group protein EZH2 is involved in progression of prostate cancer. Nature 419:624–629
86. Kondo Y et al (2008) Gene silencing in cancer by histone H3 lysine 27 trimethylation independent of promoter DNA methylation. Nat Genet 40:741–750
87. Min J et al (2010) An oncogene-tumor suppressor cascade drives metastatic prostate cancer by coordinately activating Ras and nuclear factor-kappaB. Nat Med 16:286–294
88. Shi Y et al (2004) Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. Cell 119:941–953
89. Wissmann M et al (2007) Cooperative demethylation by JMJD2C and LSD1 promotes androgen receptor-dependent gene expression. Nat Cell Biol 9:347–353
90. Metzger E et al (2005) LSD1 demethylates repressive histone marks to promote androgen-receptor-dependent transcription. Nature 437:436–439
91. Metzger E et al (2010) Phosphorylation of histone H3T6 by PKCbeta(I) controls demethylation at histone H3K4. Nature 464:792–796
92. Shi Y (2007) Histone lysine demethylases: emerging roles in development, physiology and disease. Nat Rev Genet 8:829–833
93. Shi L et al (2011) Histone demethylase JMJD2B coordinates H3K4/H3K9 methylation and promotes hormonally responsive breast carcinogenesis. Proc Natl Acad Sci USA 108:7541–7546
94. Lee MG, Wynder C, Cooch N, Shiekhattar R (2005) An essential role for CoREST in nucleosomal histone 3 lysine 4 demethylation. Nature 437:432–435
95. Shi YJ et al (2005) Regulation of LSD1 histone demethylase activity by its associated factors. Mol Cell 19:857–864
96. Forneris F, Binda C, Vanoni MA, Battaglioli E, Mattevi A (2005) Human histone demethylase LSD1 reads the histone code. J Biol Chem 280:41360–41365
97. Forneris F et al (2006) A highly specific mechanism of histone H3-K4 recognition by histone demethylase LSD1. J Biol Chem 281:35289–35295

98. Cai C et al (2011) Androgen receptor gene expression in prostate cancer is directly suppressed by the androgen receptor through recruitment of lysine-specific demethylase 1. Cancer Cell 20:457–471
99. Yosef N, Regev A (2011) Impulse control: temporal dynamics in gene transcription. Cell 144:886–896
100. Alenghat T et al (2008) Nuclear receptor corepressor and histone deacetylase 3 govern circadian metabolic physiology. Nature 456(7224):997–1000
101. Mohn F, Schubeler D (2009) Genetics and epigenetics: stability and plasticity during cellular differentiation. Trends Genet 25:129–136
102. De Santa F et al (2007) The histone H3 lysine-27 demethylase Jmjd3 links inflammation to inhibition of polycomb-mediated gene silencing. Cell 130:1083–1094
103. Cui Q et al (2007) A map of human cancer signaling. Mol Syst Biol 3:152
104. Dobrzynski M, Bruggeman FJ (2009) Elongation dynamics shape bursty transcription and translation. Proc Natl Acad Sci USA 106(8):2583–2588
105. Goldberg AD, Allis CD, Bernstein E (2007) Epigenetics: a landscape takes shape. Cell 128:635–638
106. Le May N et al (2010) NER factors are recruited to active promoters and facilitate chromatin modification for transcription in the absence of exogenous genotoxic attack. Mol Cell 38:54–66
107. Metivier R et al (2008) Cyclical DNA methylation of a transcriptionally active promoter. Nature 452:45–50
108. Kangaspeska S et al (2008) Transient cyclical methylation of promoter DNA. Nature 452:112–115
109. Wang Q et al (2009) Androgen receptor regulates a distinct transcription program in androgen-independent prostate cancer. Cell 138:245–256
110. Hu M et al (2005) Distinct epigenetic changes in the stromal cells of breast cancers. Nat Genet 37:899–905
111. Orimo A, Weinberg RA (2006) Stromal fibroblasts in cancer: a novel tumor-promoting cell type. Cell Cycle 5:1597–1601
112. Li L, Neaves WB (2006) Normal stem cells and cancer stem cells: the niche matters. Cancer Res 66:4553–4557
113. Feinberg AP, Ohlsson R, Henikoff S (2006) The epigenetic progenitor origin of human cancer. Nat Rev Genet 7:21–33
114. Vita M, Henriksson M (2006) The Myc oncoprotein as a therapeutic target for human cancer. Semin Cancer Biol 16:318–330
115. Fernandez PC et al (2003) Genomic targets of the human c-Myc protein. Genes Dev 17:1115–1129
116. Adhikary S, Eilers M (2005) Transcriptional regulation and transformation by Myc proteins. Nat Rev Mol Cell Biol 6:635–645
117. Guccione E et al (2006) Myc-binding-site recognition in the human genome is determined by chromatin context. Nat Cell Biol 8:764–770
118. Bernstein BE et al (2006) A bivalent chromatin structure marks key developmental genes in embryonic stem cells. Cell 125:315–326
119. Secombe J, Li L, Carlos L, Eisenman RN (2007) The Trithorax group protein Lid is a trimethyl histone H3K4 demethylase required for dMyc-induced cell growth. Genes Dev 21:537–551
120. Secombe J, Eisenman RN (2007) The function and regulation of the JARID1 family of histone H3 lysine 4 demethylases: the Myc connection. Cell Cycle 6:1324–1328
121. Suzuki C et al (2007) Identification of Myc-associated protein with JmjC domain as a novel therapeutic target oncogene for lung cancer. Mol Cancer Ther 6:542–551
122. Ogasawara S et al (2010) Accelerated expression of a Myc target gene Mina53 in aggressive hepatocellular carcinoma. Hepatol Res 40:330–336
123. Komiya K et al (2010) Mina53, a novel c-Myc target gene, is frequently expressed in lung cancers and exerts oncogenic property in NIH/3T3 cells. J Cancer Res Clin Oncol 136:465–473

124. Watt FM, Frye M, Benitah SA (2008) MYC in mammalian epidermis: how can an oncogene stimulate differentiation? Nat Rev Cancer 8:234–242
125. Li H, Kim JH, Koh SS, Stallcup MR (2004) Synergistic effects of coactivators GRIP1 and beta-catenin on gene activation: cross-talk between androgen receptor and Wnt signaling pathways. J Biol Chem 279:4212–4220
126. Yang X et al (2006) Complex regulation of human androgen receptor expression by Wnt signaling in prostate cancer cells. Oncogene 25(24):3436–3444
127. Campbell MJ, Elstner E, Holden S, Uskokovic M, Koeffler HP (1997) Inhibition of proliferation of prostate cancer cells by a 19-nor-hexafluoride vitamin D3 analogue involves the induction of p21waf1, p27kip1 and E-cadherin. J Mol Endocrinol 19:15–27
128. Degenhardt T, Matilainen M, Herzig KH, Dunlop TW, Carlberg C (2006) The insulin-like growth factor binding protein 1 gene is a primary target of peroxisome proliferator-activated receptors. J Biol Chem 281(51):39607–39619
129. Khanim FL et al (2004) Altered SMRT levels disrupt vitamin D(3) receptor signalling in prostate cancer cells. Oncogene 23:6712–6725
130. Kubota T et al (1998) 19-nor-26,27-bishomo-vitamin D3 analogs: a unique class of potent inhibitors of proliferation of prostate, breast, and hematopoietic cancer cells. Cancer Res 58:3370–3375
131. Liu M, Lee MH, Cohen M, Bommakanti M, Freedman LP (1996) Transcriptional activation of the Cdk inhibitor p21 by vitamin D3 leads to the induced differentiation of the myelomonocytic cell line U937. Genes Dev 10:142–153
132. Palmer HG et al (2004) The transcription factor SNAIL represses vitamin D receptor expression and responsiveness in human colon cancer. Nat Med 10:917–919
133. Saramaki A, Banwell CM, Campbell MJ, Carlberg C (2006) Regulation of the human p21(waf1/cip1) gene promoter via multiple binding sites for p53 and the vitamin D3 receptor. Nucleic Acids Res 34:543–554
134. Thorne J, Campbell MJ (2008) The vitamin D receptor in cancer. Proc Nutr Soc 67:115–127
135. Hendriksen PJ et al (2006) Evolution of the androgen receptor pathway during progression of prostate cancer. Cancer Res 66:5012–5020
136. Taneja SS et al (2004) ART-27, an androgen receptor coactivator regulated in prostate development and cancer. J Biol Chem 279:13944–13952
137. Ross-Innes CS et al (2012) Differential oestrogen receptor binding is associated with clinical outcome in breast cancer. Nature 481(7381):389–393
138. Ceschin DG et al (2011) Methylation specifies distinct estrogen-induced binding site repertoires of CBP to chromatin. Genes Dev 25:1132–1146
139. Welboren WJ et al (2009) ChIP-Seq of ERalpha and RNA polymerase II defines genes differentially responding to ligands. EMBO J 28:1418–1428
140. Rashid SF et al (2001) Synergistic growth inhibition of prostate cancer cells by 1 alpha,25 Dihydroxyvitamin D(3) and its 19-nor-hexafluoride analogs in combination with either sodium butyrate or trichostatin A. Oncogene 20:1860–1872
141. Abedin SA et al (2009) Elevated NCOR1 disrupts a network of dietary-sensing nuclear receptors in bladder cancer cells. Carcinogenesis 30(3):449–456
142. Lin RJ et al (1998) Role of the histone deacetylase complex in acute promyelocytic leukaemia. Nature 391:811–814
143. Banwell CM et al (2006) Altered nuclear receptor corepressor expression attenuates vitamin D receptor signaling in breast cancer cells. Clin Cancer Res 12:2004–2013
144. Ting HJ, Bao BY, Reeder JE, Messing EM, Lee YF (2007) Increased expression of corepressors in aggressive androgen-independent prostate cancer cells results in loss of 1alpha,25-dihydroxyvitamin D3 responsiveness. Mol Cancer Res 5:967–980
145. Tomlins SA et al (2005) Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. Science 310:644–648
146. Anderson SP et al (2004) Overlapping transcriptional programs regulated by the nuclear receptors peroxisome proliferator-activated receptor {alpha}, retinoid X receptor and liver X receptor in mouse liver. Mol Pharmacol 66(6):1440–1452

147. Bookout AL et al (2006) Anatomical profiling of nuclear receptor expression reveals a hierarchical transcriptional network. Cell 126:789–799
148. Handschin C, Meyer UA (2005) Regulatory network of lipid-sensing nuclear receptors: roles for CAR, PXR, LXR, and FXR. Arch Biochem Biophys 433:387–396
149. Xia X et al (2009) Integrative analysis of HIF binding and transactivation reveals its role in maintaining histone methylation homeostasis. Proc Natl Acad Sci USA 106:4260–4265
150. Malik S, Roeder RG (2010) The metazoan Mediator co-activator complex as an integrative hub for transcriptional regulation. Nat Rev Genet 11:761–772
151. Xu J, Wu RC, O'Malley BW (2009) Normal and cancer-related functions of the p160 steroid receptor co-activator (SRC) family. Nat Rev Cancer 9:615–630
152. Taatjes DJ, Marr MT, Tjian R (2004) Regulatory diversity among metazoan co-activator complexes. Nat Rev Mol Cell Biol 5:403–410
153. Perissi V, Jepsen K, Glass CK, Rosenfeld MG (2010) Deconstructing repression: evolving models of co-repressor action. Nat Rev Genet 11:109–123
154. Battaglia S, Maguire O, Campbell MJ (2010) Transcription factor co-repressors in cancer biology: roles and targeting. Int J Cancer 126:2511–2519
155. Anzick SL et al (1997) AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer. Science 277:965–968
156. Demarest SJ et al (2002) Mutual synergistic folding in recruitment of CBP/p300 by p160 nuclear receptor coactivators. Nature 415:549–553
157. Zhao C et al (2003) Elevated expression levels of NCOA3, TOP1, and TFAP2C in breast tumors as predictors of poor prognosis. Cancer 98:18–23
158. Doi M, Hirayama J, Sassone-Corsi P (2006) Circadian regulator CLOCK is a histone acetyltransferase. Cell 125:497–508
159. Esteyries S et al (2008) NCOA3, a new fusion partner for MOZ/MYST3 in M5 acute myeloid leukemia. Leukemia 22:663–665
160. Horlein AJ et al (1995) Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor. Nature 377:397–404
161. Chen JD, Evans RM (1995) A transcriptional co-repressor that interacts with nuclear hormone receptors. Nature 377:454–457
162. Li J et al (2000) Both corepressor proteins SMRT and N-CoR exist in large protein complexes containing HDAC3. EMBO J 19:4342–4350
163. Yu C et al (2005) The nuclear receptor corepressors NCoR and SMRT decrease PPARgamma transcriptional activity and repress 3T3-L1 adipogenesis. J. Biol, Chem
164. Jepsen K, Gleiberman AS, Shi C, Simon DI, Rosenfeld MG (2008) Cooperative regulation in development by SMRT and FOXP1. Genes Dev 22:740–745
165. Tiefenbach J et al (2006) SUMOylation of the corepressor N-CoR modulates its capacity to repress transcription. Mol Biol Cell 17:1643–1651
166. Surjit M et al (2011) Widespread negative response elements mediate direct repression by agonist-liganded glucocorticoid receptor. Cell 145:224–241
167. Heikkinen S et al (2011) Nuclear hormone 1{alpha},25-dihydroxyvitamin D3 elicits a genome-wide shift in the locations of VDR chromatin occupancy. Nucleic Acids Res 39(21):9181–9193
168. Muller-Tidow C et al (2010) Profiling of histone H3 lysine 9 trimethylation levels predicts transcription factor activity and survival in acute myeloid leukemia. Blood 116:3564–3571
169. Hoemme C et al (2008) Chromatin modifications induced by PML-RARalpha repress critical targets in leukemogenesis as analyzed by ChIP-Chip. Blood 111:2887–2895
170. Minucci S et al (2000) Oligomerization of RAR and AML1 transcription factors as a novel mechanism of oncogenic activation. Mol Cell 5:811–820
171. Girault I et al (2003) Expression analysis of estrogen receptor alpha coregulators in breast carcinoma: evidence that NCOR1 expression is predictive of the response to tamoxifen. Clin Cancer Res 9:1259–1266
172. Zhang Z et al (2005) NCOR1 mRNA is an independent prognostic factor for breast cancer. Cancer Lett 237(1):123–129

173. Kim JY, Son YL, Lee YC (2009) Involvement of SMRT corepressor in transcriptional repression by the vitamin D receptor. Mol Endocrinol 23:251–264
174. Chang TH, Szabo E (2002) Enhanced growth inhibition by combination differentiation therapy with ligands of peroxisome proliferator-activated receptor-gamma and inhibitors of histone deacetylase in adenocarcinoma of the lung. Clin Cancer Res 8:1206–1212
175. Battaglia S et al (2010) Elevated NCOR1 disrupts PPAR signaling in prostate cancer and forms a targetable epigenetic lesion. Carcinogenesis 31(9):1650–1660
176. Bau D et al (2011) The three-dimensional folding of the alpha-globin gene domain reveals formation of chromatin globules. Nat Struct Mol Biol 18:107–114
177. Li Q, Barkess G, Qian H (2006) Chromatin looping and the probability of transcription. Trends Genet 22:197–202
178. Eskiw CH, Rapp A, Carter DR, Cook PR (2008) RNA polymerase II activity is located on the surface of protein-rich transcription factories. J Cell Sci 121:1999–2007
179. Mitchell JA, Fraser P (2008) Transcription factories are nuclear subcompartments that remain in the absence of transcription. Genes Dev 22:20–25
180. Hu Q et al (2008) Enhancing nuclear receptor-induced transcription requires nuclear motor and LSD1-dependent gene networking in interchromatin granules. Proc Natl Acad Sci USA 105:19199–19204
181. Yoon HG, Chan DW, Reynolds AB, Qin J, Wong J (2003) N-CoR mediates DNA methylation-dependent repression through a methyl CpG binding protein Kaiso. Mol Cell 12:723–734
182. Yegnasubramanian S et al (2004) Hypermethylation of CpG islands in primary and metastatic human prostate cancer. Cancer Res 64:1975–1986
183. Asatiani E et al (2005) Deletion, methylation, and expression of the NKX3.1 suppressor gene in primary human prostate cancer. Cancer Res 65:1164–1173
184. Fujita N et al (2003) Methyl-CpG binding domain 1 (MBD1) interacts with the Suv39h1-HP1 heterochromatic complex for DNA methylation-based transcriptional repression. J Biol Chem 278:24132–24138
185. Esteve PO et al (2006) Direct interaction between DNMT1 and G9a coordinates DNA and histone methylation during replication. Genes Dev 20:3089–3103
186. Cheng X, Blumenthal RM (2010) Coordinated chromatin control: structural and functional linkage of DNA and histone methylation. Biochemistry 49:2999–3008
187. Freitag M, Hickey PC, Khlafallah TK, Read ND, Selker EU (2004) HP1 is essential for DNA methylation in Neurospora. Mol Cell 13:427–434
188. Birney E et al (2007) Identification and analysis of functional elements in 1% of the human genome by the ENCODE pilot project. Nature 447:799–816
189. Schulte JH et al (2009) Lysine-specific demethylase 1 is strongly expressed in poorly differentiated neuroblastoma: implications for therapy. Cancer Res 69:2065–2071
190. Graham JS, Kaye SB, Brown R (2009) The promises and pitfalls of epigenetic therapies in solid tumours. Eur J Cancer 45:1129–1136

Chapter 5
Nucleosome Occupancy and Gene Regulation During Tumorigenesis

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Abstract Nucleosomes are the basic structural units of eukaryotic chromatin. In recent years, it has become evident that nucleosomes and their position, in concert with other epigenetic mechanisms (such as DNA methylation, histone modifications, changes in histone variants, as well as small noncoding regulatory RNAs) play essential roles in the control of gene expression. Here, we discuss the mechanisms and factors that regulate nucleosome position and gene expression in normal and cancer cells.

5.1 Introduction

Nucleosomes are the basic units of eukaryotic chromatin, each one containing ~146 bp of DNA wrapped around an octamer of histone core proteins (H3, H4, H2A, and H2B), which in turn are separated by linker DNA of variable length [1]. At least five epigenetic mechanisms have been shown to act in concert to regulate gene expression by modifying chromatin structure, namely DNA methylation, histone modifications, nucleosome remodeling, and changes in histone variants as well as small noncoding regulatory RNAs [2]. In addition to playing a pivotal role in chromatin structure, nucleosomes display differential occupancy at promoter regions, thereby regulating gene expression by altering DNA accessibility. For instance, a nucleosome-depleted region (NDR) at transcriptional start sites correlates with gene expression, whereas the positioning of a nucleosome over the transcriptional start site results in gene repression [2, 3]. The position of nucleosomes is determined and influenced by a number of factors, including DNA sequence, DNA methylation, histone modifications and histone variants, chromatin remodelers, and

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transcription factor binding [4]. We discuss how these factors act in normal cells and how abnormalities in these factors impact nucleosome occupancy and gene expression in cancer cells.

5.2 Regulation of Nucleosome Position in Normal Cells

5.2.1 DNA Sequence Preferences

The sequences that regulate nucleosome position fall into two categories: motifs that are preferred (included within the nucleosome) and motifs that avoided (excluded from the nucleosome) [5]. Preferred sequences were originally characterized as particular dinucleotides, including CG and GC dinucleotides, occurring with approximately 10 bp periodicity, although nucleosomes may also prefer longer DNA motifs [4, 6]. The sequences that are disfavored by nucleosomes include various 5-mers and long tracts of As (10–20 bp or more), possibly due to their resistance to the structural distortions required for DNA wrapping and nucleosome formation [4, 7]. Such organization helps restrict nucleosome access to those regions to ensure proper gene expression pattern [7]. An example of regions containing both preferred and disfavored sequences with restricted nucleosome positioning are the Alu repeats [6, 8]. More recently, however, the concept of intrinsically DNA-encoded positioning as an organizational determinant of the 5′ end of genes has been challenged. In this regard, studies showed that the majority of the human genome displays great flexibility in nucleosome positioning, although DNA sequence can strongly drive the organization of nucleosomes at specific sites [9]. It has also been shown that these intrinsic signals can be overridden, confirming that additional factors are involved in nucleosome organization [9, 10].

5.2.2 Nucleosomes and DNA Methylation

DNA methylation in mammals occurs at CpG dinucleotides, which are distributed along the genome in clusters (CpG islands) or in regions containing high concentration of repeat sequences, and acts as a relatively stable gene silencing mechanism [2]. The majority of isolated CpGs tend to be methylated in mammals. In contrast, the majority of the CpG islands, which represent 60% of all human promoters, remain largely unmethylated [2, 3]. However, a number of CpG island promoters, such as those of imprinted genes, are methylated resulting in monoallelic gene expression in normal cells [3]. CpG islands can also be found within or in between transcriptional units (orphan CpG islands) [3] and can be associated with novel promoter regions and to be active in a tissue-specific manner [3]. DNA methylation also appears to be important for the regulation of non-CpG island promoters and the

tissue-specific expression of the genes that they control including *MASPIN*, *OCT-4*, *LAMB3*, and *RUNX3* promoter 1 [11–14]. Methylation is also observed in repetitive genomic sequences, which include transposable elements and noncoding DNA, where it helps maintain genomic stability [15, 16]. DNA methylation is established by the activity of three DNA methyltransferases (DNMTs): DNMT1, which preferentially methylates hemimethylated DNA during replication, and DNMT3A and DNMT3B, which are replication-independent, have been shown to establish de novo DNA methylation. Furthermore, DNMT3A and 3B were shown to be recruited to sites methylated by DNMT1 thereby contributing to propagate the methylated state [17, 18].

CpG DNA methylation causes steric interference in the formation of nucleosomes in vitro, suggesting that methylation may play a role in nucleosome occupancy [4]. However, more recent in vivo studies demonstrate that the nucleosome architecture plays a role in the shaping of DNA methylation patterns [19]. This is in agreement with studies from our laboratory showing that nucleosomes are required for stable DNMT3A/3B anchoring [17, 18, 20] and that nucleosome occupancy precedes de novo DNA methylation in vivo [14]. While the direction of the relationship is still under investigation, it is clear that nucleosome position and methylation are interrelated.

5.2.3 Nucleosomes and Histone Modifications

The N-terminus of histones can undergo a variety of modifications in specific residues, including acetylation, methylation, ubiquitination, sumoylation, and phosphorylation [21]. Histone modifications work in a combinatorial fashion to alter chromatin accessibility by disrupting interactions between nucleosomes or by regulating the recruitment of nonhistone proteins [4, 22]. Specific patterns of histone modifications characterize genomic regions. For instance, active promoter regions are enriched in trimethylated H3 at lysine 4 (H3K4me3), whereas inactive promoters are enriched in trimethylated H3 at lysine 27 and trimethylated H3 at lysine 9 (H3K27me3 and H3K9me3), and regulatory enhancers are enriched in monomethylated H3 at lysine 4 (H3K4me1) [21]. Such patterns are dynamic and regulated by enzymes that can add or remove the modifications. These include histone methyltransferases (HMTs) and demethylases (HDMTs), which introduce and remove methyl groups, respectively, and histone acetyltransferases (HATs) and deacetylases (HDACs), which introduce and remove acetyl groups, respectively. Histone acetylation is an important marker of transcriptional activity; for instance, acetylated histone H3 (acH3) can also be found at well-positioned nucleosomes flanking the AR binding site of 20% of AR enhancers, upon hormone stimulation [23]. In addition, acH4K16 can be found at well-positioned nuclesomes flanking unmethylated CpG islands at the promoter regions of some tumor-suppressor genes [24]. In addition, although histone modifications themselves are not likely to have a direct impact in nucleosome

positioning, their ability to recruit chromatin remodeler proteins and other factors may have a substantial impact in nucleosome organization [4].

5.2.4 ATPase-Dependent Chromatin Remodelers and Histone Variants

ATP-dependent chromatin remodelers can be grouped in families based on subunit composition and activity: the SWI/SNF family includes the SWI/SNF, INO80, and SWR1 complexes; the ISWI family comprises the RSF, ACF/CHRAC, WICH, and NURF complexes; and the CHD family which includes NURD complexes [25, 26]. These complexes directly affect nucleosome positioning by actively mobilizing nucleosomes or introducing histone variants.

5.2.4.1 SWItch/Sucrose Non-fermenting

These complexes consist of 9–12 subunits, which include one of two ATPases: Brahma homologue (BRM/SMARCA2) or Brahma-related gene 1 (BRG1/SMARCA4), a set of "core" subunits, including SNF5 and BAF53a/b, and a number of variable subunits [27]. A number of the variable subunits are mutually exclusive; for example, AT-rich interactive proteins (ARID) 1A and ARID1B (BAF250a and BAF250b) [25, 27] do not coexist in the same complex and Polybromo 1 (PBRM1 or BAF180), bromodomain-containing 7 (BRD7), and BAF200 are only present in complexes lacking ARID1 proteins [27]. Complexes containing ARID1 proteins are named BAF whereas complexes containing PBRM1 are known as PBAF [27]. The variety of subunits allows for a combinatorial assemblage that leads to functional diversity as evidenced by the developmental stage-specific composition of SWItch/sucrose non-fermenting (SWI/SNF) complexes [25]. SWI/SNF complexes remodel chromatin by sliding or by ejecting or inserting nucleosomes thereby contributing to either transcriptional activation or repression [27, 28]; interestingly, they are primarily enriched at distal regulatory regions rather than at promoters [25]. SWI/SNF complexes also associate and act in concert with histone modifying complexes, including HDACs, HATs, and protein arginine methyltransferases (PRMT4/CARM1 and 5), to regulate gene expression [27, 29, 30].

5.2.4.2 INO80 and SWR1

These complexes consist of core proteins (the ATPase, helicases, and actin-related proteins) and additional subunits [31]. INO80 complexes contain the INO80 ATPase [31] whereas the SWR1 complexes (SRCAP and TRAAP/Tip60) contain the ATPases SRCAP or p400 and share a number of subunits [31]. The INO80 complex displays helicase activity and catalyzes nucleosome sliding *in cis*, and is involved in

chromosome segregation [32], the DNA, and damage repair response, and facilitates recombination-mediated events [25, 33, 34]. INO80 recruitment to damaged sites has been recently shown to depend on actin-related protein 8 [35]. SRCAP complex directs the incorporation of H2A.Z into nucleosomes by exchange of H2A/H2B dimers for H2A.Z/H2B dimers in a replication-independent manner [36, 37]. SRCAP-mediated deposition of H2A.Z is required for gene reactivation in colon cancer cells treated with the DNA methylase inhibitor Azacitidine [38]. P400-containing complexes play a role in DNA repair by destabilizing nucleosomes and promoting chromatin ubiquitination [39]. It has been suggested that TRAAP/Tip60 (p400) complexes are involved in the deposition of H2A.Z into chromatin in an acetylation-dependent manner. P400-mediated H2A.Z deposition is important for estrogen receptor-mediated gene expression [40] whereas SRCAP appears to be important for the androgen receptor-stimulated expression of Kallikrein 3/prostate specific antigen (KLK3/PSA) and cell proliferation in prostate cancer cells [41].

H2A.Z deposition is associated with several nucleosomes surrounding the transcriptional start site of active and poised promoters, and nucleosomes and H2A.Z are lost preferentially at the −1 nucleosome upon gene activation [42]. In addition, enrichment in H2A.Z, and also the histone H3 variant histone H3.3, has been found at distal regulatory regions such as enhancers [42, 43]. During mitosis, the H2A.Z-containing +1 nucleosome of active genes shift upstream to occupy the transcriptional start site of genes silenced during mitosis, significantly reducing NDRs [44]. Interestingly, H2A.Z has also been shown to play an inhibitory role in cell cycle arrest, providing evidence that H2A.Z localization at regulatory regions may contribute to the positive or negative regulation of gene transcription [42]. Differential H2A.Z acetylation patterns at promoters may contribute to the opposing functions of H2A.Z, as the presence of acetylated H2A.Z has been shown to correlate with gene activation in prostate cancer cells [45] and to be anti-correlated with DNA methylation [46, 47].

5.2.4.3 ISWI Complexes

Similar to SWI/SNF complexes, the combinatorial assembly of subunits allows for a multiplicity of ISWI complexes that display specific functions, including transcriptional repression, DNA replication, and heterochromatin formation. The remodeling spacing factor (RSF), ATP-utilizing chromatin assembly and remodeling factor (ACF), chromatin accessibility (CHRAC), and WICH complexes share the hSNF2H ATPase [25], while the nucleosome remodeling factor (NURF) complexes contain the hSNF2L ATPase. All ISWI complexes display ATPase and nucleosome spacing and remodeling activities and RSF, in particular, promotes regular spacing between nucleosomes and stimulates transcriptional activation [25]. In addition, WICH complexes are important for DNA replication of pericentromeric heterochromatin and the WSTF subunit of this complex binds and stabilizes H2A.X by phosphorylation after DNA damage [25]. NURF complexes have also been shown to play a role in the regulation of chromatin barriers; for example, the

transcription factor USF1 (upstream stimulatory factor 1) recruits NURF and an HMT to the insulator of the beta-globin gene to retain its active configuration [48].

5.2.4.4 NURD Complexes

These complexes are formed by the CHD ATPases CHD3 or CHD4 (or Mi-2a or Mi2b), HDACs, and additional subunits and contain both HDAC and remodeling activity [25]. NURD complexes play a role in transcription, cell differentiation, cell cycle checkpoint control, and metastasis, and are recruited to sites of DNA damage by poly-ADP-ribose polymerase (PARP) [25, 49, 50]. The methyl CpG binding domain 2/3 (MBD2 and 3) subunits of these complexes are thought to be involved in protein–protein interaction and are mutually exclusive, whereas the metastasis associated gene 1 and 2 (MTA1 and 2) subunits bind to specific transcription factors thereby targeting the complex to different genomic loci [50].

5.2.5 Transcription Factor Binding

The position of nucleosomes can be directly affected by transcription factors as they compete for DNA access [4]. Transcription factors often bind at NDRs. For example, OCT-4 is required for establishing and maintaining of an NDR at the distal OCT-4 enhancer and the proximal NANOG promoter regions, which are necessary for gene expression [14]. We have recently reported that a percentage of androgen receptor (AR) enhancers show a NDR in the absence of ligand, and that androgen treatment and subsequent AR recruitment increase the number of enhancers with NDRs without changes in footprint [51]. The pioneering factor GATA-2 is required for the maintenance of the NDR at the AR enhancer of TMPRSS2 in the absence of ligand [51]. The presence of GATA-2 at the enhancer may facilitate AR binding, as proposed by the model of transcription factor cooperativity of Segal and Widom [4]. In contrast, other transcription factors are frequently bound to nucleosome occupied regions; for instance, P53 binding occurs preferentially to regions with high intrinsic nucleosome occupancy [52]. Thus, the relationship between nucleosome occupancy and transcription factor binding is context-specific.

5.3 Aberrant Epigenetic Regulation and Epigenetic Switching in Cancer Cells

Genetic and epigenetic changes play important roles in cancer initiation and progression [53, 54]. During tumorigenesis, the cell epigenome undergoes global changes, including a genome-wide reduction in DNA methylation, an increase in localized DNA methylation at CpG island promoters, and changes in histone

modification profiles [55]; in addition, cancer cells display aberrant expression of chromatin-modifying enzymes [56]. The events leading to these epigenetic abnormalities are still not fully understood. Epigenetic changes are mitotically inherited and may promote tumorigenesis by either silencing tumor suppressor genes [57] or by activating oncogenes [2].

Because of the interaction amongst chromatin remodeling complexes [58] and between these complexes and DNMTs [59, 60], genetic mutations in enzymes or other subunits of chromatin remodeling complexes may lead to profound epigenetic changes, including aberrant nucleosome position, DNA methylation, histone composition, and/or histone modifications [2]. In addition, deregulated expression of proteins involved in the recruitment of remodeling complexes to specific loci may alter nucleosome localization and/or retention at such sites, contributing to the propagation of abnormal epigenetic states [2]. All these changes will in turn lead to aberrant gene expression patterns and genomic instability, which ultimately may predispose or give rise to disease [2]. The mechanisms contributing to the altered epigenetic landscape of cancer cells are discussed below.

5.3.1 Mutations in DNA Methylation Enzymes

CpG island methylation at gene promoters affects gene expression and abnormal patterns of DNA methylation have been implicated in carcinogenesis [53, 54]. Hypomethylation of retrotransposons may lead to their reactivation and genomic translocation or to the activation of alternative transcripts. These DNA methylation changes have also been shown to correlate with changes in nucleosome occupancy [2]. For instance, LINE-1 is hypomethylated and nucleosome depleted in colon cancer [61] and bladder cancer, where it induced the expression of an alternate transcript of the *MET* oncogene [16]. Hypomethylation of centromeric regions and/or of pericentromeric satellite sequences may lead to abnormal chromosome segregation and genomic instability [62]. Perhaps the best example of chromosome instability is a germ line mutation in DNMT3B, which underlies a chromosome instability and immunodeficiency syndrome [63]. In addition, DNA hypomethylation may lead to loss of imprinting (LOI), resulting in biallelic expression of a monoallelic gene [2, 64], which often occurs in a variety of cancer types [64]. Re-expression of normally silenced genes or microRNAs (miRNA) can also occur due to DNA hypomethylation; examples of these events are *R-RAS*, *MASPIN*, and *Cyclin D2* in gastric cancer; *MAGE* in melanoma; *HPV16* (human papillomavirus 16) in cervical cancer; *S100A4* in colon cancer; and the *let-7a-3* miRNA in lung adenocarcinomas [2, 62].

Site-specific hypermethylation and silencing of tumor suppressor genes has also reported in cancer and correlates with changes in nucleosome occupancy [65]. Genes that regulate cell cycle progression, and DNA repair, such as *RB* (retinoblastoma), *MLH1* (endometrial cancer), *p16* (glioma, lymphoma, multiple myeloma), and *p15* (lymphoma and multiple myeloma), *BRCA1* and *BRCA2* (lung and ovarian cancer), *APC* (lung, breast, and colorectal cancer), *PTEN* (brain and thyroid gland

cancers), *XRCC5* (lung and ovarian cancer), and estrogen receptor (prostate cancer) have all been reported to be hypermethylated in cancer [2, 62]. DNA hypermethylation can also indirectly inactivate other genes by silencing transcription factors that control their expression. For example, hypermethylation has been found at the *RUNX3* promoter in esophageal cancer and at the *GATA-4* and *-5* promoters in colorectal and gastric cancers [2, 62]. In addition, inactivation of miRNAs by hypermethylation has been observed in a variety of cancer types including bladder and prostate (mir-127), endometrial (mir-152, mir-129-2), pancreatic (mir-132), oral (mir-137 and miR-193a), gastrointestinal (mir-34b/c), and colorectal (mir-137) cancers, and in ALL (mir-124a), and other hematological malignancies (mir-124-1) [66–75]. A new class of noncoding RNA (mirtrons) has been also shown to be susceptible to epigenetic silencing in urothelial cell carcinoma [73]. DNMT1 mutations have been described in colorectal cancer and DNMT3A mutations and decreased protein levels have been shown to occur in myelodysplastic syndromes (MDS) and AML, and in primary prostate tumors, respectively [76–80], *DNMT1*, *DNMT3A,* and *DNMT3B* appear to be largely overexpressed in a variety of cancer types and may contribute to ectopic hypermethylation [81].

Recent studies have pointed to the existence of both passive and active mechanisms of DNA demethylation [82]. Active demethylation occurs during early embryogenesis and is mediated by the formation of cytosine intermediaries, for instance 5-hydroxy-methyl cytosine or 5-methyl uracil, via the action of enzymes such as ten-eleven-translocation (TET) or activation-induced cytidine deaminase (AID), respectively [82].

TET1 translocations have been reported to occur in AML [83] and TET2 mutations have been frequently found in myelodysplasia and in myeloid malignancies [84–90]. In addition, TET2 promoter hypermethylation was observed in a fraction of gliomas [91].

AID promotes somatic hypermutation and class switch recombination of immunoglobulin (Ig) genes in germinal center (GC) B cells and aberrant AID expression has been implicated in the progression of chronic myeloid leukemia (CML) into fatal blast crisis [92].

Because DNA methylation stabilizes nucleosome occupancy, mutations in DNMTs and in enzymes involved in DNA demethylation are likely to cause large-scale epigenetic alterations in cancer cells; in addition, de novo functions generated by fusion with their translocation partners may also contribute to tumorigenesis [93].

5.3.2 Mutations in Genes Encoding Histone Modifiers

Genome-wide analyses of histone modifications in cancer cells have revealed global changes in various histone marks [2]. These changes may affect the recruitment of transcription factors and chromatin remodeler complexes to specific genomic loci, thereby affecting nucleosome positioning.

5.3.2.1 HATs and HDACs

In cancer cells, there is a global reduction in the active acH4K16 and H3K4me3 marks, and in the repressive H4K20me3 mark [94] as well as a gain in the repressive H3K27me3 mark [95]. Acetylation patterns are disrupted in colon, uterus, lung tumors, and in leukemias as a result of translocations or mutations in the genes that encode some of the HATs and HDACs (for instance, HDAC2) or due to mistargeting of the fusion products [94]. HDAC overexpression has also been observed; for example, the levels of the dedicated H4K16 HDAC SIRT1 were found to be high in hepatocellular carcinoma [96] and colon cancer [97, 98].

5.3.2.2 HMTs and HDMTs

Alterations in HMTs and HDMTs have also been shown to be involved in tumorigenesis. Mixed lineage leukemia (MLL) 1–4, SETD1A, and SETD1B are H3K4 HMTs that exist as multiprotein complexes that contain core subunits and various unique subunits including HATs, tumor suppressor gene products, mRNA-processing factors, and nuclear hormone receptors. MLLs play critical roles during development and in adult tissues; they regulate gene transcription directly by introducing the active H3K4me3 mark, and indirectly via their partnership with other chromatin remodeling complexes and co-regulators [99]. In addition, a potential role for MLL complexes in alternative splicing has been proposed [99]. Mutations in MLL1 and MLL3 genes have been reported in 59% of bladder cancer patients [100]. Chromosomal rearrangements in the *MLL1* gene occur preferentially in hematopoietic cells [101] and result in a multiplicity of fusion proteins with new properties and binding partners that contribute to the development of hematological malignancies [101]. Mutations in *MLL2* [102, 103] and MLL2 decreased expression levels as well as mutations and deletions in *MLL3* have also been reported (Table 5.1) [79, 104–106]. Deletions in MLL5, a member of the MLL family that lacks the HMT and DNA binding domains [107], have been shown in leiomyomata (benign uterine fibroids) [108] and low expression of MLL5 was associated with poorer outcome in acute myeloid leukemia (AML) patients [109]. Genomic alterations in other HMTs have also been reported; for instance, mutations in SETD2, an H3K36 HMT, were found in renal clear cell carcinoma [110].

Members of the polycomb group (Pc-G) of repressor proteins have been shown to be deregulated in cancer. The Pc-G HMT EZH2 (enhancer of zeste homologue 2), a subunit of the polycomb repressor complexes (PRC) 2 and PRC3, is not expressed in adult tissues [111]. However, it is overexpressed in several tumor types (Table 5.1) [112, 113]. EZH2 has been shown to interact with DNMTs in human cell lines, suggesting that it may also play a role in controlling DNA methylation [114]. Overexpression of BMI-1 (B lymphoma Mo-MLV insertion region 1 homolog), a component of PRC1, was also observed in a variety of tumors (Table 5.1) [115–117].

Table 5.1 Summary of epigenetic abnormalities found in cancer

Gene	Function	Alteration	Tumor type	Reference
DNMT1	DNA methylase	Mutation	7% of colorectal	[78]
DNMT3A	DNA methylase	Mutation	MDS; 22% AML	[74–77]
TET1	5'methylcytosine hydroxylase	Chromosome translocation	AML	[81]
TET2	5'methylcytosine hydroxylase	Mutation	MDS, myeloid malignancies	[82–88]
		Silencing	Gliomas	[89]
AID	5'cytidine deaminase	Aberrant expression	CML	[90]
SIRT1	H4K16 HDAC	Overexpression	HCC, colon	[94–96]
MLL1	H3K4 HMT	Mutation	Bladder TCC	[98]
		Chromosome translocation	Hematopoietic	[99]
MLL2	H3K4 HMT	Mutation	Non-Hodgkin lymphoma, B-cell lymphoma	[100, 101]
		Low expression	Prostate (primary)	[77]
MLL3	H3K4 HMT	Mutation	Bladder TCC, glioblastoma, breast, pancreas	[98, 102–104]
		Deletion	Colon	[99]
		Low expression	Prostate (primary)	[77]
MLL5	tumor suppressor gene	Deletion	Uterine fibroids	[106]
		Low expression	AML	[107]
SETD2	H3K36 HMT	Mutation	RCCC	[108]
EZH2	H3K27 HMT	Overexpression	Breast, prostate, bladder, colon, pancreas, liver, gastric, uterine tumors, melanoma, lymphoma, myeloma, and Ewing's sarcoma	[110, 111]
BMI-1	PRC1 subunit	Overexpression	Ovarian, mantle cell lymphomas, and Merkel cell carcinomas	[113–115]
NSD1	H3K36/H4K20 HMT	Chromosome rearrangement	AML	[116]
		Amplification	Lung	[117]
		Silencing	Neuroblastoma	[118]
		Low expression	Prostate (primary)	[77]

G9a	H3K9 HMT	Overexpression	HCC	[111]
LSD1	H3K4/H3K9 HDMT	Mutation	Prostate	[119]
UTX	H3K27 HDMT	Mutation	Bladder, breast, kidney, lung, pancreas, esophagus, colon, uterus, brain, hematological malignancies	[98, 108, 120]
JARID1C	H3K4 HDMT	Mutation	RCCC	[108]
JMJD2C	H3K9/H3K36 HDMT	Overexpression	Esophageal cancer	[121]
JARID1B	H3K4 HDMT	Overexpression	Testicular and breast	[121]
GASC1	H3K9	Amplification	Squamous cell carcinoma	[122]
SNF5	BAF subunit	Mutation	Kidney malignant rhabdoid tumors, atypical rhabdoid/teratoid tumors (extra-renal), epithelioid sarcomas, small cell hepatoblastomas, extraskeletal myxoid chondrosarcomas, and undifferentiated sarcomas	[130–134]
		Loss of expression	Renal medullary carcinoma, metastatic melanoma	[135]
BRG1	ATPase of BAF	Mutations	Lung, rhabdoid, medulloblastoma	[138–141]
BRM	ATPase of BAF	Low expression	Prostate	[142]
		Mutations	Basal cell carcinoma	[143, 144]
ARID1A	Subunit of BAF	Mutations	50% of ovarian clear cell carcinomas, 30% of endometrioid carcinomas, endometrial carcinomas	[150–154]
		Deletions	33% of primary pancreatic adenocarcinomas	[155]
		Genomic rearrangements	Bladder TCC	[98]
		Low expression	56% of ER-/PR-/HER2-breast tumors	[156]
ARID2	PBAF subunit	Mutation	HCC	[159]
BRD7	PBAF subunit	Mutations and deletions	Breast	[161]
		Hypermethylation	Nasopharyngeal	[165]
PBRM1	PBAF subunit	Mutations	41% RCCC, breast	[166, 167]
SRCAP	ATPase of SWR1	Overexpression	Prostate (primary)	[77]
		Low expression	Prostate (metastatic)	[77]

(continued)

Table 5.1 (continued)

Gene	Function	Alteration	Tumor type	Reference
Tip60	Acetylase of SWR1	Low expression (monoallelic expression loss)	Lymphomas, head and neck, breast	[170]
		Low expression	Colon	[171]
P400	ATPase of SWR1	Low expression	Colon	[172]
hSNF2	ATPase of RSF, ACF, CHRAC, WICH and NURF	Overexpression	AML	[175]
BPTF	NURD subunit	Amplification	55% of neuroblastomas, 27% lung tumors	[173, 174]
RBBP7/RbAp46	Helicase	Overexpression	79% of breast tumors	[175]
RBBP4/RbAp48	Helicase	Overexpression	AML	[176]
CHD4	ATPase of NURD	Mutation with loss of expression	55.7% colorectal and 56.4% gastric cancer	[178]
MTA1	NURD subunit	Overexpression	Breast, colorectal, gastric, pancreatic, ovarian, prostate, esophageal, endometrial, NSCLC, HCC, and diffuse B cell lymphoma	[49]
MTA3	NURD subunit	Loss of expression	Advanced breast carcinoma	[49]
CHD5	ATP-dependent helicase	Deletion and mutations	Ovarian, prostate, neuroblastoma, hematopoietic	[178–181]
		Hypermethylation	Gliomas, laryngeal squamous carcinoma, colon, gastric, ovarian, and breast	[178, 182–185]
CHD7	ATP-dependent helicase	Mutations	Gastric and colorectal	[177]

HCC Hepatocellular carcinoma; *NSCLC* non-small cell lung carcinoma, *AML* acute myeloid leukemia; *MDS* myelodysplastic syndromes; *CML* chronic myeloid leukemia; *TCC* transitional cell carcinoma; *RCCC* renal clear cell carcinoma; *ER* estrogen receptor; *PR* progesterone receptor; *HER2* human epidermal growth factor receptor 2; *HMT* histone methyltransferase; *HDMT* histone demethylase; *DNMT* DNA methylase, *PRC1* polycomb repressor complex 1

Other HMTs have been shown to display aberrant expression patterns or chromosome rearrangements. Nuclear receptor binding SET domain protein 1 (NSD1) has been reported to undergo chromosome rearrangements in pediatric AML [118], to be amplified in some lung cancer cases [119] and to be silenced by DNA methylation in neuroblastomas [120]. In addition, the H3K9me3 HMT G9a was found to be upregulated in hepatocellular carcinoma [113].

Lysine-specific histone demethylases, such as LSD1, lysine (K)-specific demethylase 6A (KDM6A/UTX), and Jumonji C-domain containing proteins (JARID1A-D), have been implicated in cancer progression (Table 5.1). For instance, mutations in LSD1 have been reported in prostate cancer [121], whereas KDM6A/UTX was found mutated in many tumors (Table 5.1) [100, 110, 122]. Mutations in KDM5C/JARID1C were observed in renal cell carcinoma lacking VHL [110]. In addition, overexpression of KDM4C/JMJD2C and JARID1B/PLU-1 was found in esophageal cancer and in breast and testicular tumors, respectively, whereas genomic amplification of GASC1 was observed in squamous cell carcinoma [123, 124]. Thus, mutations and aberrant expression of histone modifiers may alter or block the recruitment of chromatin remodelers and transcription factors to specific loci, thereby affecting nucleosome positioning and gene expression patterns.

5.3.3 Mutations in Genes Encoding Subunits of Chromatin Remodeler Complexes

ATPase-dependent chromatin remodeler complexes directly control the position of nucleosomes or alter their stability by introducing histone variants. Thus, aberrant expression of their subunits will cause changes in nucleosome composition, location, and stability.

5.3.3.1 SWItch/Sucrose Non-fermenting

Because of their important role in controlling fundamental processes such as cell proliferation, migration, and differentiation [27], the aberrant expression of SWI/SNF components will have profound effects on cell function. Indeed, mutations in several subunits have been recently identified in tumors of various origins. Since genomic instability is largely absent in tumors harboring defective SWI/SNF complexes, it is likely that perturbations in nucleosome positioning, misslocalization, and excessive formation of complexes with opposing functions contribute to the development of these aggressive cancers [27].

The SWI/SNF subunit SNF5 helps recruit this complex to specific genomic sites and is required for the expression of genes associated with cell proliferation, including *P53* and the cell cycle inhibitor *p16INK4a* [125–127], adipocyte differentiation [128], and inhibition of cell migration [129]. *SNF5* loss, however, does not result in genome instability [130] nor does it inactivate SWI/SNF complexes completely, as

tumorigenesis in the absence of *SNF5* is dependent on BRG1 activity [131]. Thus, it is thought that tumorigenesis arises from aberrant activity of the remaining complexes [131]. *SNF5* mutations have been found in rhabdoid and other tumors (Table 5.1) [132–136]. Loss of the SNF5 protein was also observed in renal medullary carcinomas and in advanced and metastatic melanomas, where it correlated with poor survival rates [137].

Although complexes containing the catalytic subunits BRM or BRG1 display some functional redundancy, they also play distinct roles [27, 28]. *BRG1* mutations have been shown to occur in cancer cell lines of various origins [138, 139] and in primary lung tumors [140, 141], medulloblastoma [142], and rhabdoid tumors [143]. Reduced BRM protein levels occur in prostate tumors [144], and mutations have been found in basal cell carcinoma [145, 146]. In addition, BRM has been shown to be postranslationally regulated in cancer cell lines [28].

BAF250A/ARID1A binds to DNA without sequence specificity [147, 148] and its recruitment represses the expression of cell cycle-related genes in differentiated mouse calvaria cells [149, 150]. In addition, BAF250A/ARID1A is required for normal cell cycle arrest in senescent human fibroblasts [151]. *ARID1A/BAF250a* mutations have been recently described in ovarian clear cell [152–154] and endometrioid carcinomas (Table 5.1) [153]. Frequent mutations in low- and high grade endometrial carcinomas have also been observed [155, 156]. Heterozygous deletions and mutations in *ARID1A/BAF250a* have been reported to exist in 33% of primary pancreatic adenocarcinomas [157]. Genetic aberrations in *ARID1A* were recently reported in transitional cell carcinoma (TCC) of the bladder [100] and low ARID1A expression was found to be significantly associated with larger tumor size and grade and the ER-/PR-/HER2-phenotype in breast cancer cases (Table 5.1) [158]. ARID1A/BAF250a expression was also found to be severely reduced in breast (T47D), renal clear cell (Caki-1 and Caki-2), and cervical (C33A) cancer cell lines [159]. BAF250b/ARID1B containing complexes include components of an E3 ubiquitin ligase that was found to target H2BK20 for monoubiquitination in a nucleosomal context, an upstream event for trimethylation of H3K4 and gene activation [160]. BAF250b/ARID1B and BAF250a/ARID1A have also been shown to play opposing roles in the control of cell cycle genes in osteoblast differentiation in mice [149, 150]; however, no mutations in human *BAF250b/ARID1B* have been described to date. In contrast, inactivating mutations in *ARID2*, which encodes a component of PBAF that facilitates transcriptional activation by nuclear receptors, have been reported in four subtypes of hepatocellular carcinomas (HCC) [161].

BRD7 and BAF180/PBRM1 are regulators of replicative senescence in human cells by controlling P53 transcriptional activity towards a subset of its target genes required for replicative and oncogenic stress senescence induction [162]. BRD7 has also been shown to either activate or repress the expression of a number of genes by protein–protein interaction. BRD7 physically interacts with P53 and the acetylase P300 [162, 163], disheveled-1 [164], and TRIM24 [165], as well as with BRCA1 thereby regulating genes involved in DNA repair [166]. *BRD7* deletions and reduced expression levels have been observed in breast tumors [163] (Table 5.1). In addition, the *BRD7* promoter has also been shown to be silenced by DNA methylation in

nasopharyngeal cancer cell lines and tumors [167]. Mutations in *BAF180/PBRM1* have been recently described in renal clear cell carcinomas [168] and breast tumors (Table 5.1) [169]. BAF57 is required to maintain the proper subunit composition of the PBAF complex and to regulate the transcription of a subset of cell cycle-related genes in Hela cells [170]. Thus far, loss of *BAF57* has only been reported in the breast cancer cell line BT-549 [171]. Thus, aberrant expression of SWI/SNF subunits is a frequent event in a variety of cancer types. Although SWI/SNF complexes control nucleosome positioning, the extent of the changes caused by the mutation of specific subunits remains to be elucidated.

5.3.3.2 INO80 and SWR1

Deregulated expression of the subunits of these complexes may affect H2A.Z deposition and nucleosome dynamics as well as nucleosome position and DNA repair. SRCAP deregulated expression has been found in primary and metastatic prostate cancer, although the mechanisms underlying such dysregulation are unclear [79]. Monoallelic loss of the acetylase Tip60 (a subunit of TRAAP/Tip60/p400) has been reported in lymphomas, and head-and-neck and mammary carcinomas, with decreased mRNA and protein expression levels, suggesting that critical levels of Tip60 are required for normal cell function [172]. Tip60 and P400 expression is also decreased in colorectal tumors compared to normal colon, although no mutations were found in these two genes [173]. Finally, single nucleotide polymorphisms (SNPs) in Tip49a/RUVBL1 have been recently associated with higher risk of serous epithelial ovarian cancer [174].

5.3.3.3 RSF, ACF, CHRAC, WICH, and NURF

To date no mutations in the ATPase subunits of ISWI complexes have been described. However, genomic amplification of bromodomain PHD finger transcription factor (BPTF), a subunit of NURD, has been reported in neuroblastomas and lung cancer cases (Table 5.1) [175, 176]. In addition, increased expression of other subunits of the NURF complex, including Retinoblastoma-related protein 46 (RBBP7/RbAp46), as well as Retinoblastoma-related protein 48 (RBBP4/RbAp48) and hSNF2 have been reported in breast carcinomas [177] and in AML [178], respectively (Table 5.1).

5.3.3.4 NURD

Mutations and loss of expression of the CHD4 ATPase subunit have been recently described in colorectal and gastric cancers (Table 5.1) [179]. MTA1 expression is high in a number of cancer types (Table 5.1) [50]. In contrast, MTA3 expression is lost in advanced breast epithelial carcinoma (Table 5.1) [50].

5.3.3.5 Mutations in Other CHD Proteins

Recent studies have identified the helicase CHD5 as a tumor suppressor involved in the transactivation of *p16Ink4a/p19arf* and deleted or mutated in ovarian and prostate cancer [180, 181], neuroblastomas [182], and hematopoietic malignancies [183]. Silencing of the *CHD5* promoter by DNA hypermethylation has also been observed in various tumor types (Table 5.1) [180, 184–187]. CHD7 plays a role in pluripotency [25] and mutations in CHD7 have been found in more than 50% of the cases of CHARGE syndrome, which is characterized by nonrandom congenital abnormalities in several tissues [188, 189]. In addition, gastric and colorectal cancers also showed mutations in *CHD7* [179].

5.4 Epigenetic Switching

The gene silencing events that take place during tumorigenesis as a consequence of aberrant DNA methylation or histone modification result in a reduction of cellular plasticity. A subset of genes becomes repressed by the action of Pc-G proteins through the establishment of the H3K27me3 mark at their promoters when stem cells differentiate into developmental lineages [2]. After differentiation, this mark and, thus, the repressive state are maintained by the action of EZH2. In cancer cells, H3K27me3 is replaced by de novo DNA methylation likely through the recruitment of DNMTs [114, 190–192]. This process is termed "epigenetic switching" and results in permanent silencing of genes that may be implicated in tumorigenesis by locking nucleosome positions.

5.5 Epigenetic Therapy and Gene Reactivation

Epigenetic therapy aims to reverse epigenetic aberrations that occur in cancer in order to restore a more normal epigenetic state [55]. The first characterized DNA methylation inhibitors, namely 5-Azacitidine (5′-aza-CR, Azacitidine) and 5-aza-2-deoxy-cytidine (5′-aza-CdR, Decitabine) [193], are incorporated into the DNA of proliferating cells during DNA replication and inhibit DNA methylation by trapping DNMTs onto the DNA, leading to their depletion [2, 56]. The resulting DNA hypomethylation causes nucleosome depletion at the promoters of tumor suppressor genes that were silenced during tumorigenesis, leading to gene reactivation and growth arrest [2, 65]. Azacitidine and decitabine have been approved by the FDA for the treatment of myelodysplastic syndromes and have shown great promise in the treatment of AML and myeloid leukemia [194]. Decitabine has also been tested in clinical trials for the treatment of epithelial ovarian cancer, alone or in combination with chemotherapy [195]. These studies have shown that combination therapies are more effective, particularly in patients with platinum resistance, likely due to re-sensitization [195]. Clinical applications for Zebularine, a newer generation

DNMT inhibitor that can be orally administered, are currently under investigation [196]. Alternative approaches include small molecule DNMT inhibitors, such as SGI110, RG108, and MG98, which block DNMT enzyme activity or target regulatory messenger RNA sequences [2].

Loss of histone acetylation at promoter regions occurs concomitant to DNA hypermethylation, and therefore HDAC inhibitors (HDACI) have also been tested as potential therapeutic agents. HDACIs induce growth arrest, apoptosis, cell differentiation, and tumor suppressor gene reactivation. Suberoylanilide hydroxamic acid (SAHA, Vorinostat) has been recently approved for the treatment of T-cell cutaneous lymphoma [197]; however, it was not successful for the treatment of recurrent ovarian cancer [195]. Treatment with another HDACI, belinostat (PDX, 101), has shown to lead to disease stabilization in patients with different malignancies, including sarcoma, renal cancer, thymoma and melanoma, and ovarian cancer [195]. Other HDACIs are currently under investigation [2, 197]. The lysine HMT inhibitors described to date, chaetocin, DZNep, and BIX-01294, have shown some antitumor properties in vitro [197]. Combined epigenetic therapies have also been tested; for instance, chemotherapeutic agents have been successfully used in combination with HDAC, SIRT, DNMT inhibitors [197]. Thus, epigenetic drugs currently in use or under investigation target histone modifiers or DNMTs to restore chromatin plasticity, thereby affecting nucleosome positioning in an indirect manner. Targeting subunits of the ATPase-dependent chromatin remodeler complexes may provide a more efficient and direct way to restore nucleosome position and composition.

5.6 Challenges and Future Prospects

In recent years, high-throughput technologies have been successfully applied to the field of epigenetics allowing for the mapping of histone modifications, proteins binding to DNA, nucleosome positioning, and DNA methylation. The emerging picture is that nucleosome positioning and occupancy is determined by the combined action of DNA sequence, transcription factors, and chromatin remodelers, and that the resulting nucleosome configuration has direct effects in sequence accessibility and gene transcription (Fig. 5.1). Recent studies show that the genes more frequently mutated in various types of cancers encode for subunits of chromatin remodeler complexes [197], further highlighting the relevance of nucleosome positioning in tumorigenesis (Fig. 5.1). As most of these genes regulate multiple cellular processes, they are likely to be important therapeutic targets.

Although the wealth of information generated by epigenomic studies has greatly improved our understanding of chromatin regulation, the integration of epigenetic, genetic, and transcriptional changes will be essential to advance our knowledge of cancer development and progression. Several challenges lay ahead as we explore further the development of epigenetic therapies, although a combinatorial approach holds promise. Key issues to be resolved include type of agent combinations and optimal doses, agent specificity, the sequence of agent delivery, and the method of

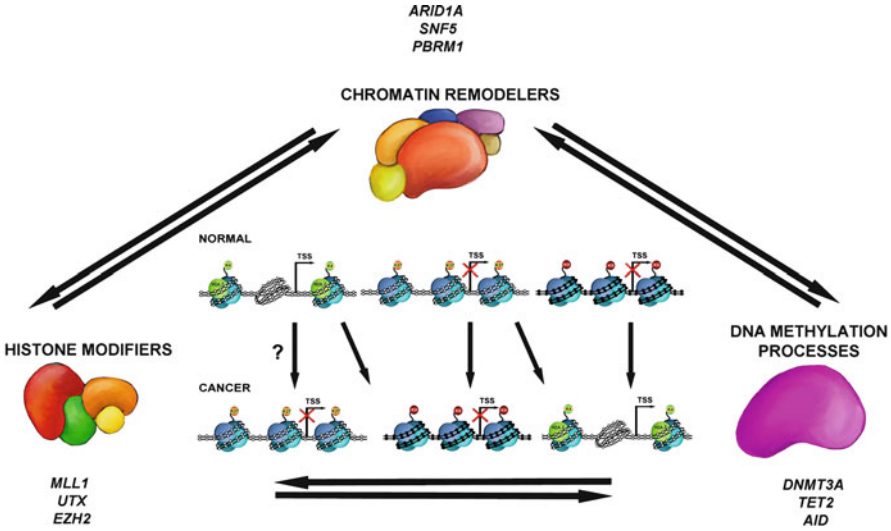

Fig. 5.1 The emerging picture is that nucleosome positioning and occupancy, which is influenced by chromatin remodelers, histone modifiers and DNA methylating enzymes, has direct effects in sequence accessibility and gene transcription and that. In normal cells, gene promoter regions exist in three epigenetic states: open (left), which shows nucleosome depletion at the transcriptional start site (TSS) and contains active histone marks (e.g. H3K4me3) and the histone variant H2A.Z; repressed (center), which shows nucleosome occupancy at the TSS and contains repressive histone marks (e.g. H3K27me3); or silenced (right), which shows nucleosome occupancy at the TSS and DNA methylation, and contains silencing histone marls (e.g.H3K9me3). These epigenetic states correlate with transcriptional activity (left) or lack thereof (center and right). In cancer, epigenetic states are altered, and active promoters may become silenced by DNA methylation, or, potentially, repressed; repressed promoters may become reactivated or silenced by DNA methylation; and silenced promoters may become reactivated, thereby causing profound changes in gene expression patterns. Recent studies show that the genes more frequently mutated in various types of cancers encode for subunits of chromatin remodeler complexes (e.g. *ARID1A, SNF5, PBRM1*), histone modifying enzymes (e.g. *MLL1, UTX, EZH2*) or enzymes involved in the DNA methylation pathway (e.g. *DNMT3A, TET2, AID*). These studies provide evidence for a link between genetic mutation and epigenetic alterations

delivery. Given the current multi-institutional and multinational efforts to map the human epigenome in all cancer types, it is likely that therapeutic development will be significantly advanced in the near future.

References

1. Luger K, Mader AW, Richmond RK, Sargent DF, Richmond TJ (1997) Crystal structure of the nucleosome core particle at 2.8 A resolution. Nature 389:251–260
2. Sharma S, Kelly TK, Jones PA (2010) Epigenetics in cancer. Carcinogenesis 31:27–36
3. Deaton AM, Bird A (2011) CpG islands and the regulation of transcription. Genes Dev 25:1010–1022

4. Segal E, Widom J (2009) What controls nucleosome positions? Trends Genet 25:335–343
5. Sadeh R, Allis CD (2011) Genome-wide "re"-modeling of nucleosome positions. Cell 147:263–266
6. Bettecken T, Frenkel ZM, Trifonov EN (2011) Human nucleosomes: special role of CG dinucleotides and Alu-nucleosomes. BMC Genomics 12:273
7. Tillo D et al (2010) High nucleosome occupancy is encoded at human regulatory sequences. PLoS One 5:e9129
8. Tanaka Y, Yamashita R, Suzuki Y, Nakai K (2010) Effects of Alu elements on global nucleosome positioning in the human genome. BMC Genomics 11:309
9. Valouev A et al (2011) Determinants of nucleosome organization in primary human cells. Nature 474:516–520
10. Zhang Z et al (2011) A packing mechanism for nucleosome organization reconstituted across a eukaryotic genome. Science 332:977–980
11. Han H et al (2011) DNA methylation directly silences genes with non-CpG island promoters and establishes a nucleosome occupied promoter. Hum Mol Genet 20(22):4299–4310
12. Hattori N et al (2004) Epigenetic control of mouse Oct-4 gene expression in embryonic stem cells and trophoblast stem cells. J Biol Chem 279:17063–17069
13. Futscher BW et al (2002) Role for DNA methylation in the control of cell type specific maspin expression. Nat Genet 31:175–179
14. You JS et al (2011) OCT4 establishes and maintains nucleosome-depleted regions that provide additional layers of epigenetic regulation of its target genes. Proc Natl Acad Sci USA 108:14497–14502 [AU4]
15. Taberlay PC, Jones PA (2010) DNA methylation and cancer. Prog Drug Res 67:1–23
16. Wolff EM et al (2010) Hypomethylation of a LINE-1 promoter activates an alternate transcript of the MET oncogene in bladders with cancer. PLoS Genet 6:e1000917
17. Sharma S, De Carvalho DD, Jeong S, Jones PA, Liang G (2011) Nucleosomes containing methylated DNA stabilize DNA methyltransferases 3A/3B and ensure faithful epigenetic inheritance. PLoS Genet 7:e1001286
18. Jones PA, Liang G (2009) Rethinking how DNA methylation patterns are maintained. Nat Rev Genet 10:805–811
19. Chodavarapu RK et al (2010) Relationship between nucleosome positioning and DNA methylation. Nature 466:388–392
20. Jeong S et al (2009) Selective anchoring of DNA methyltransferases 3A and 3B to nucleosomes containing methylated DNA. Mol Cell Biol 29:5366–5376
21. Mills AA (2010) Throwing the cancer switch: reciprocal roles of polycomb and trithorax proteins. Nat Rev Cancer 10:669–682
22. Bell O, Tiwari VK, Thoma NH, Schubeler D (2011) Determinants and dynamics of genome accessibility. Nature reviews 12:554–564
23. Berman BP, Frenkel B, Coetzee GA, Jia L (2010) Androgen receptor responsive enhancers are flanked by consistently-positioned H3-acetylated nucleosomes. Cell Cycle 9:2249–2250
24. Kapoor-Vazirani P, Kagey JD, Powell DR, Vertino PM (2008) Role of hMOF-dependent histone H4 lysine 16 acetylation in the maintenance of TMS1/ASC gene activity. Cancer Res 68:6810–6821
25. Hargreaves DC, Crabtree GR (2011) ATP-dependent chromatin remodeling: genetics, genomics and mechanisms. Cell Res 21:396–420
26. Clapier CR, Cairns BR (2009) The biology of chromatin remodeling complexes. Annu Rev Biochem 78:273–304
27. Wilson BG, Roberts CW (2011) SWI/SNF nucleosome remodellers and cancer. Nat Rev Cancer 11:481–492
28. Reisman D, Glaros S, Thompson EA (2009) The SWI/SNF complex and cancer. Oncogene 28:1653–1668
29. Pal S, Sif S (2007) Interplay between chromatin remodelers and protein arginine methyltransferases. J Cell Physiol 213:306–315

30. Choi HK et al (2007) The functional role of the CARM1-SNF5 complex and its associated HMT activity in transcriptional activation by thyroid hormone receptor. Exp Mol Med 39:544–555
31. Morrison AJ, Shen X (2009) Chromatin remodelling beyond transcription: the INO80 and SWR1 complexes. Nat Rev Mol Cell Biol 10:373–384
32. Hur SK et al (2010) Roles of human INO80 chromatin remodeling enzyme in DNA replication and chromosome segregation suppress genome instability. Cell Mol Life Sci 67: 2283–2296
33. van Attikum H, Gasser SM (2009) Crosstalk between histone modifications during the DNA damage response. Trends Cell Biol 19:207–217
34. Park EJ, Hur SK, Kwon J (2010) Human INO80 chromatin-remodelling complex contributes to DNA double-strand break repair via the expression of Rad54B and XRCC3 genes. Biochem J 431:179–187
35. Kashiwaba S et al (2010) The mammalian INO80 complex is recruited to DNA damage sites in an ARP8 dependent manner. Biochem Biophys Res Commun 402:619–625
36. Ruhl DD et al (2006) Purification of a human SRCAP complex that remodels chromatin by incorporating the histone variant H2A.Z into nucleosomes. Biochemistry 45:5671–5677
37. Wong MM, Cox LK, Chrivia JC (2007) The chromatin remodeling protein, SRCAP, is critical for deposition of the histone variant H2A.Z at promoters. J Biol Chem 282:26132–26139
38. Yang X et al (2012) Gene reactivation by 5-Aza-2'-deoxycytidine requires H2A.z insertion to establish but not to maintain nucleosome depleted regions. PLoS Genet 8:e1002604
39. Xu Y et al (2010) The p400 ATPase regulates nucleosome stability and chromatin ubiquitination during DNA repair. J Cell Biol 191:31–43
40. Gevry N et al (2009) Histone H2A.Z is essential for estrogen receptor signaling. Genes Dev 23:1522–1533
41. Slupianek A, Yerrum S, Safadi FF, Monroy MA (2010) The chromatin remodeling factor SRCAP modulates expression of prostate specific antigen and cellular proliferation in prostate cancer cells. J Cell Physiol 224:369–375
42. Svotelis A, Gevry N, Gaudreau L (2009) Regulation of gene expression and cellular proliferation by histone H2A.Z. Biochem Cell Biol 87:179–188
43. Ong CT, Corces VG (2011) Enhancer function: new insights into the regulation of tissue-specific gene expression. Nature reviews 12:283–293
44. Kelly TK et al (2010) H2A.Z maintenance during mitosis reveals nucleosome shifting on mitotically silenced genes. Mol Cell 39:901–911
45. Valdes-Mora F et al (2011) Acetylation of H2A.Z is a key epigenetic modification associated with gene deregulation and epigenetic remodeling in cancer. Genome Res 20(22):4299–4310
46. Zemach A, McDaniel IE, Silva P, Zilberman D (2010) Genome-wide evolutionary analysis of eukaryotic DNA methylation. Science 328:916–919
47. Zilberman D, Coleman-Derr D, Ballinger T, Henikoff S (2008) Histone H2A.Z and DNA methylation are mutually antagonistic chromatin marks. Nature 456:125–129
48. Li X et al (2011) Chromatin boundaries require functional collaboration between the hSET1 and NURF complexes. Blood 118:1386–1394
49. Sims JK, Wade PA (2011) Mi-2/NuRD complex function is required for normal S phase progression and assembly of pericentric heterochromatin. Mol Biol Cell 22:3094–3102
50. Lai AY, Wade PA (2011) Cancer biology and NuRD: a multifaceted chromatin remodelling complex. Nat Rev Cancer 11:588–596
51. Andreu-Vieyra C et al (2011) Dynamic nucleosome-depleted regions at androgen receptor enhancers in the absence of ligand in prostate cancer cells. Mol Cell Biol 31:4648–4662
52. Lidor Nili E et al (2010) p53 binds preferentially to genomic regions with high DNA-encoded nucleosome occupancy. Genome Res 20:1361–1368
53. Jones PA, Baylin SB (2002) The fundamental role of epigenetic events in cancer. Nature reviews 3:415–428
54. Jones PA, Baylin SB (2007) The epigenomics of cancer. Cell 128:683–692

55. Baylin SB, Jones PA (2011) A decade of exploring the cancer epigenome—biological and translational implications. Nat Rev Cancer 11:726–734
56. Egger G, Liang G, Aparicio A, Jones PA (2004) Epigenetics in human disease and prospects for epigenetic therapy. Nature 429:457–463
57. Jones PA, Laird PW (1999) Cancer epigenetics comes of age. Nat Genet 21:163–167
58. Xu W et al (2004) A methylation-mediator complex in hormone signaling. Genes Dev 18:144–156
59. Geiman TM et al (2004) DNMT3B interacts with hSNF2H chromatin remodeling enzyme, HDACs 1 and 2, and components of the histone methylation system. Biochem Biophys Res Commun 318:544–555
60. Geiman TM et al (2004) Isolation and characterization of a novel DNA methyltransferase complex linking DNMT3B with components of the mitotic chromosome condensation machinery. Nucleic Acids Res 32:2716–2729
61. Sunami E, de Maat M, Vu A, Turner RR, Hoon DS (2011) LINE-1 hypomethylation during primary colon cancer progression. PLoS One 6:e18884
62. Hatziapostolou M, Iliopoulos D (2011) Epigenetic aberrations during oncogenesis. Cell Mol Life Sci 68:1681–1702
63. Xu GL et al (1999) Chromosome instability and immunodeficiency syndrome caused by mutations in a DNA methyltransferase gene. Nature 402:187–191
64. Jelinic P, Shaw P (2007) Loss of imprinting and cancer. J Pathol 211:261–268
65. Lin JC et al (2007) Role of nucleosomal occupancy in the epigenetic silencing of the MLH1 CpG island. Cancer Cell 12:432–444
66. Tsuruta T et al (2011) miR-152 is a tumor suppressor microRNA that is silenced by DNA hypermethylation in endometrial cancer. Cancer Res 71(20):6450–6462
67. Agirre X et al (2009) Epigenetic silencing of the tumor suppressor microRNA Hsa-miR-124a regulates CDK6 expression and confers a poor prognosis in acute lymphoblastic leukemia. Cancer Res 69:4443–4453
68. Balaguer F et al (2010) Epigenetic silencing of miR-137 is an early event in colorectal carcinogenesis. Cancer Res 70:6609–6618
69. Zhang S et al (2011) Downregulation of miR-132 by promoter methylation contributes to pancreatic cancer development. Carcinogenesis 32:1183–1189
70. Huang YW et al (2009) Epigenetic repression of microRNA-129-2 leads to overexpression of SOX4 oncogene in endometrial cancer. Cancer Res 69:9038–9046
71. Wong KY et al (2011) Epigenetic inactivation of the miR-124-1 in haematological malignancies. PLoS One 6:e19027
72. Saito Y et al (2006) Specific activation of microRNA-127 with downregulation of the proto-oncogene BCL6 by chromatin-modifying drugs in human cancer cells. Cancer Cell 9:435–443
73. Dudziec E et al (2011) Hypermethylation of CpG islands and shores around specific microRNAs and mirtrons is associated with the phenotype and presence of bladder cancer. Clin Cancer Res 17:1287–1296
74. Kozaki K, Imoto I, Mogi S, Omura K, Inazawa J (2008) Exploration of tumor-suppressive microRNAs silenced by DNA hypermethylation in oral cancer. Cancer Res 68:2094–2105
75. Suzuki H et al (2010) Methylation-associated silencing of microRNA-34b/c in gastric cancer and its involvement in an epigenetic field defect. Carcinogenesis 31:2066–2073
76. Yan XJ et al (2011) Exome sequencing identifies somatic mutations of DNA methyltransferase gene DNMT3A in acute monocytic leukemia. Nat Genet 43:309–315
77. Tiu RV, Visconte V, Traina F, Schwandt A, Maciejewski JP (2011) Updates in cytogenetics and molecular markers in MDS. Curr Hematol Malig Rep 6:126–135
78. Tan PT, Wei AH (2011) The epigenomics revolution in myelodysplasia: a clinico-pathological perspective. Pathology 43:536–546
79. Bianco-Miotto T et al (2010) Global levels of specific histone modifications and an epigenetic gene signature predict prostate cancer progression and development. Cancer Epidemiol Biomarkers Prev 19:2611–2622

80. Kanai Y, Ushijima S, Nakanishi Y, Sakamoto M, Hirohashi S (2003) Mutation of the DNA methyltransferase (DNMT) 1 gene in human colorectal cancers. Cancer Lett 192:75–82
81. Miremadi A, Oestergaard MZ, Pharoah PD, Caldas C (2007) Cancer genetics of epigenetic genes. Hum Mol Genet 16(Spec No 1):R28–R49
82. Bhutani N et al (2011) Reprogramming towards pluripotency requires AID-dependent DNA demethylation. Nature 463:1042–1047
83. Guo JU, Su Y, Zhong C, Ming GL, Song H (2011) Emerging roles of TET proteins and 5-hydroxymethylcytosines in active DNA demethylation and beyond. Cell Cycle 10:2662–2668
84. Mullighan CG (2009) TET2 mutations in myelodysplasia and myeloid malignancies. Nat Genet 41:766–767
85. Jardin F et al (2011) TET2 and TP53 mutations are frequently observed in blastic plasmacytoid dendritic cell neoplasm. Br J Haematol 153:413–416
86. Quivoron C et al (2011) TET2 inactivation results in pleiotropic hematopoietic abnormalities in mouse and is a recurrent event during human lymphomagenesis. Cancer Cell 20:25–38
87. Bacher U et al (2010) Mutations of the TET2 and CBL genes: novel molecular markers in myeloid malignancies. Ann Hematol 89:643–652
88. Tefferi A et al (2009) Frequent TET2 mutations in systemic mastocytosis: clinical, KITD816V and FIP1L1-PDGFRA correlates. Leukemia 23:900–904
89. Tefferi A et al (2009) Detection of mutant TET2 in myeloid malignancies other than myeloproliferative neoplasms: CMML, MDS, MDS/MPN and AML. Leukemia 23:1343–1345
90. Tefferi A et al (2009) TET2 mutations and their clinical correlates in polycythemia vera, essential thrombocythemia and myelofibrosis. Leukemia 23:905–911
91. Kim YH et al (2011) TET2 promoter methylation in low-grade diffuse gliomas lacking IDH1/2 mutations. J Clin Pathol 64(10):850–852
92. Klemm L et al (2009) The B cell mutator AID promotes B lymphoid blast crisis and drug resistance in chronic myeloid leukemia. Cancer Cell 16:232–245
93. Tan AY, Manley JL (2009) The TET family of proteins: functions and roles in disease. J Mol Cell Biol 1:82–92
94. Esteller M (2007) Cancer epigenomics: DNA methylomes and histone-modification maps. Nature reviews 8:286–298
95. Kondo Y et al (2008) Gene silencing in cancer by histone H3 lysine 27 trimethylation independent of promoter DNA methylation. Nat Genet 40:741–750
96. Choi HN et al (2011) Expression and role of SIRT1 in hepatocellular carcinoma. Oncol Rep 26:503–510
97. Stunkel W et al (2007) Function of the SIRT1 protein deacetylase in cancer. Biotechnol J 2:1360–1368
98. Kim YR, Kim SS, Yoo NJ, Lee SH (2010) Frameshift mutation of SIRT1 gene in gastric and colorectal carcinomas with microsatellite instability. APMIS 118:81–82
99. Ansari KI, Mandal SS (2010) Mixed lineage leukemia: roles in gene expression, hormone signaling and mRNA processing. FEBS J 277:1790–1804
100. Gui Y et al (2011) Frequent mutations of chromatin remodeling genes in transitional cell carcinoma of the bladder. Nat Genet 43:875–878
101. Marschalek R (2010) Mixed lineage leukemia: roles in human malignancies and potential therapy. FEBS J 277:1822–1831
102. Morin RD et al (2011) Frequent mutation of histone-modifying genes in non-Hodgkin lymphoma. Nature 476:298–303
103. Shaknovich R, Melnick A (2011) Epigenetics and B-cell lymphoma. Curr Opin Hematol 18:293–299
104. Ashktorab H et al (2010) Distinct genetic alterations in colorectal cancer. PLoS One 5:e8879
105. Balakrishnan A et al (2007) Novel somatic and germline mutations in cancer candidate genes in glioblastoma, melanoma, and pancreatic carcinoma. Cancer Res 67:3545–3550

106. Wang XX et al (2011) Somatic mutations of the mixed-lineage leukemia 3 (MLL3) gene in primary breast cancers. Pathol Oncol Res 17:429–433
107. Liu H, Westergard TD, Hsieh JJ (2009) MLL5 governs hematopoiesis: a step closer. Blood 113:1395–1396
108. Hodge JC et al (2009) Identifying the molecular signature of the interstitial deletion 7q subgroup of uterine leiomyomata using a paired analysis. Genes Chromosomes Cancer 48:865–885
109. Damm F et al (2011) Prognostic importance of histone methyltransferase MLL5 expression in acute myeloid leukemia. J Clin Oncol 29:682–689
110. Dalgliesh GL et al (2010) Systematic sequencing of renal carcinoma reveals inactivation of histone modifying genes. Nature 463:360–363
111. Portela A, Esteller M (2010) Epigenetic modifications and human disease. Nat Biotechnol 28:1057–1068
112. Tsang DP, Cheng AS (2010) Epigenetic regulation of signaling pathways in cancer: role of the histone methyltransferase EZH2. J Gastroenterol Hepatol 26:19–27
113. Kondo Y et al (2007) Alterations of DNA methylation and histone modifications contribute to gene silencing in hepatocellular carcinomas. Hepatol Res 37:974–983
114. Vire E et al (2006) The Polycomb group protein EZH2 directly controls DNA methylation. Nature 439:871–874
115. Yang GF et al (2010) Intensive expression of Bmi-1 is a new independent predictor of poor outcome in patients with ovarian carcinoma. BMC Cancer 10:133
116. Bea S et al (2001) BMI-1 gene amplification and overexpression in hematological malignancies occur mainly in mantle cell lymphomas. Cancer Res 61:2409–2412
117. Brunner M et al (2008) Expression of VEGF-A/C, VEGF-R2, PDGF-alpha/beta, c-kit, EGFR, Her-2/Neu, Mcl-1 and Bmi-1 in Merkel cell carcinoma. Mod Pathol 21:876–884
118. Jaju RJ et al (2001) A novel gene, NSD1, is fused to NUP98 in the t(5;11)(q35;p15.5) in de novo childhood acute myeloid leukemia. Blood 98:1264–1267
119. Job B et al (2010) Genomic aberrations in lung adenocarcinoma in never smokers. PLoS One 5:e15145
120. Berdasco M et al (2009) Epigenetic inactivation of the Sotos overgrowth syndrome gene histone methyltransferase NSD1 in human neuroblastoma and glioma. Proc Natl Acad Sci USA 106:21830–21835
121. Kahl P et al (2006) Androgen receptor coactivators lysine-specific histone demethylase 1 and four and a half LIM domain protein 2 predict risk of prostate cancer recurrence. Cancer Res 66:11341–11347
122. van Haaften G et al (2009) Somatic mutations of the histone H3K27 demethylase gene UTX in human cancer. Nat Genet 41:521–523
123. Shi Y (2007) Histone lysine demethylases: emerging roles in development, physiology and disease. Nature reviews 8:829–833
124. Liu G et al (2009) Genomic amplification and oncogenic properties of the GASC1 histone demethylase gene in breast cancer. Oncogene 28:4491–4500
125. Xu Y, Yan W, Chen X (2010) SNF5, a core component of the SWI/SNF complex, is necessary for p53 expression and cell survival, in part through eIF4E. Oncogene 29:4090–4100
126. Oruetxebarria I et al (2004) P16INK4a is required for hSNF5 chromatin remodeler-induced cellular senescence in malignant rhabdoid tumor cells. J Biol Chem 279:3807–3816
127. Sansam CG, Roberts CW (2006) Epigenetics and cancer: altered chromatin remodeling via Snf5 loss leads to aberrant cell cycle regulation. Cell Cycle 5:621–624
128. Caramel J, Medjkane S, Quignon F, Delattre O (2008) The requirement for SNF5/INI1 in adipocyte differentiation highlights new features of malignant rhabdoid tumors. Oncogene 27:2035–2044
129. Caramel J, Quignon F, Delattre O (2008) RhoA-dependent regulation of cell migration by the tumor suppressor hSNF5/INI1. Cancer Res 68:6154–6161
130. McKenna ES et al (2008) Loss of the epigenetic tumor suppressor SNF5 leads to cancer without genomic instability. Mol Cell Biol 28:6223–6233

131. Wang X et al (2009) Oncogenesis caused by loss of the SNF5 tumor suppressor is dependent on activity of BRG1, the ATPase of the SWI/SNF chromatin remodeling complex. Cancer Res 69:8094–8101
132. Gadd S, Sredni ST, Huang CC, Perlman EJ (2010) Rhabdoid tumor: gene expression clues to pathogenesis and potential therapeutic targets. Lab Invest 90:724–738
133. Modena P et al (2005) SMARCB1/INI1 tumor suppressor gene is frequently inactivated in epithelioid sarcomas. Cancer Res 65:4012–4019
134. Kreiger PA et al (2009) Loss of INI1 expression defines a unique subset of pediatric undifferentiated soft tissue sarcomas. Mod Pathol 22:142–150
135. Trobaugh-Lotrario AD, Tomlinson GE, Finegold MJ, Gore L, Feusner JH (2009) Small cell undifferentiated variant of hepatoblastoma: adverse clinical and molecular features similar to rhabdoid tumors. Pediatr Blood Cancer 52:328–334
136. Cheng JX et al (2008) Renal medullary carcinoma: rhabdoid features and the absence of INI1 expression as markers of aggressive behavior. Mod Pathol 21:647–652
137. Lin H, Wong RP, Martinka M, Li G (2009) Loss of SNF5 expression correlates with poor patient survival in melanoma. Clin Cancer Res 15:6404–6411
138. Medina PP et al (2005) Transcriptional targets of the chromatin-remodelling factor SMARCA4/BRG1 in lung cancer cells. Hum Mol Genet 14:973–982
139. Wong AK et al (2000) BRG1, a component of the SWI-SNF complex, is mutated in multiple human tumor cell lines. Cancer Res 60:6171–6177
140. Rodriguez-Nieto S, Sanchez-Cespedes M (2009) BRG1 and LKB1: tales of two tumor suppressor genes on chromosome 19p and lung cancer. Carcinogenesis 30:547–554
141. Medina PP et al (2004) Genetic and epigenetic screening for gene alterations of the chromatin-remodeling factor, SMARCA4/BRG1, in lung tumors. Genes Chromosomes Cancer 41:170–177
142. Parsons DW et al (2010) The genetic landscape of the childhood cancer medulloblastoma. Science 331:435–439
143. Schneppenheim R et al (2010) Germline nonsense mutation and somatic inactivation of SMARCA4/BRG1 in a family with rhabdoid tumor predisposition syndrome. Am J Hum Genet 86:279–284
144. Sun A et al (2007) Aberrant expression of SWI/SNF catalytic subunits BRG1/BRM is associated with tumor development and increased invasiveness in prostate cancers. Prostate 67:203–213
145. de Zwaan SE, Haass NK (2010) Genetics of basal cell carcinoma. Australas J Dermatol 51:81–92; quiz 93–94
146. Moloney FJ et al (2009) Hotspot mutation of Brahma in non-melanoma skin cancer. J Invest Dermatol 129:1012–1015
147. Patsialou A, Wilsker D, Moran E (2005) DNA-binding properties of ARID family proteins. Nucleic Acids Res 33:66–80
148. Dallas PB et al (2000) The human SWI-SNF complex protein p270 is an ARID family member with non-sequence-specific DNA binding activity. Mol Cell Biol 20:3137–3146
149. Nagl NG Jr, Zweitzig DR, Thimmapaya B, Beck GR Jr, Moran E (2006) The c-myc gene is a direct target of mammalian SWI/SNF-related complexes during differentiation-associated cell cycle arrest. Cancer Res 66:1289–1293
150. Nagl NG Jr, Wang X, Patsialou A, Van Scoy M, Moran E (2007) Distinct mammalian SWI/SNF chromatin remodeling complexes with opposing roles in cell-cycle control. EMBO J 26:752–763
151. Nagl NG Jr et al (2005) The p270 (ARID1A/SMARCF1) subunit of mammalian SWI/SNF-related complexes is essential for normal cell cycle arrest. Cancer Res 65:9236–9244
152. Jones S et al (2010) Frequent mutations of chromatin remodeling gene ARID1A in ovarian clear cell carcinoma. Science 330:228–231
153. Wiegand KC et al (2010) ARID1A mutations in endometriosis-associated ovarian carcinomas. N Engl J Med 363:1532–1543
154. Maeda D et al (2010) Clinicopathological significance of loss of ARID1A immunoreactivity in ovarian clear cell carcinoma. Int J Mol Sci 11:5120–5128

155. Wiegand KC et al (2011) Loss of BAF250a (ARID1A) is frequent in high-grade endometrial carcinomas. J Pathol 224:328–333
156. Guan B et al (2011) Mutation and loss of expression of ARID1A in uterine low-grade endometrioid carcinoma. Am J Surg Pathol 35:625–632
157. Birnbaum DJ et al (2011) Genome profiling of pancreatic adenocarcinoma. Genes Chromosomes Cancer 50:456–465
158. Zhang X et al (2012) Frequent low expression of chromatin remodeling gene ARID1A in breast cancer and its clinical significance. Cancer Epidemiol 36(3):288–293
159. Wang X et al (2004) Expression of p270 (ARID1A), a component of human SWI/SNF complexes, in human tumors. Int J Cancer 112:636
160. Li XS, Trojer P, Matsumura T, Treisman JE, Tanese N (2010) Mammalian SWI/SNF–a subunit BAF250/ARID1 is an E3 ubiquitin ligase that targets histone H2B. Mol Cell Biol 30:1673–1688
161. Li M et al (2011) Inactivating mutations of the chromatin remodeling gene ARID2 in hepatocellular carcinoma. Nat Genet 43:828–829
162. Burrows AE, Smogorzewska A, Elledge SJ (2010) Polybromo-associated BRG1-associated factor components BRD7 and BAF180 are critical regulators of p53 required for induction of replicative senescence. Proc Natl Acad Sci USA 107:14280–14285
163. Drost J et al (2010) BRD7 is a candidate tumour suppressor gene required for p53 function. Nat Cell Biol 12:380–389
164. Kim S, Lee J, Park J, Chung J (2003) BP75, bromodomain-containing M(r) 75,000 protein, binds dishevelled-1 and enhances Wnt signaling by inactivating glycogen synthase kinase-3 beta. Cancer Res 63:4792–4795
165. Kikuchi M et al (2009) TRIM24 mediates ligand-dependent activation of androgen receptor and is repressed by a bromodomain-containing protein, BRD7, in prostate cancer cells. Biochim Biophys Acta 1793:1828–1836
166. Mantovani F, Drost J, Voorhoeve PM, Del Sal G, Agami R (2010) Gene regulation and tumor suppression by the bromodomain-containing protein BRD7. Cell Cycle 9:2777–2781
167. Liu H et al (2008) Promoter methylation inhibits BRD7 expression in human nasopharyngeal carcinoma cells. BMC Cancer 8:253
168. Varela I et al (2011) Exome sequencing identifies frequent mutation of the SWI/SNF complex gene PBRM1 in renal carcinoma. Nature 469:539–542
169. Xia W et al (2008) BAF180 is a critical regulator of p21 induction and a tumor suppressor mutated in breast cancer. Cancer Res 68:1667–1674
170. Hah N et al (2010) A role for BAF57 in cell cycle-dependent transcriptional regulation by the SWI/SNF chromatin remodeling complex. Cancer Res 70:4402–4411
171. Decristofaro MF et al (2001) Characterization of SWI/SNF protein expression in human breast cancer cell lines and other malignancies. J Cell Physiol 186:136–145
172. Gorrini C et al (2007) Tip60 is a haplo-insufficient tumour suppressor required for an oncogene-induced DNA damage response. Nature 448:1063–1067
173. Mattera L et al (2009) The p400/Tip60 ratio is critical for colorectal cancer cell proliferation through DNA damage response pathways. Oncogene 28:1506–1517
174. Notaridou M et al (2011) Common alleles in candidate susceptibility genes associated with risk and development of epithelial ovarian cancer. Int J Cancer 128:2063–2074
175. Wang CL et al (2009) Discovery of retinoblastoma-associated binding protein 46 as a novel prognostic marker for distant metastasis in nonsmall cell lung cancer by combined analysis of cancer cell secretome and pleural effusion proteome. J Proteome Res 8:4428–4440
176. Buganim Y et al (2010) A novel translocation breakpoint within the BPTF gene is associated with a pre-malignant phenotype. PLoS One 5:e9657
177. Thakur A et al (2007) Aberrant expression of X-linked genes RbAp46, Rsk4, and Cldn2 in breast cancer. Mol Cancer Res 5:171–181
178. Sakhinia E et al (2005) Routine expression profiling of microarray gene signatures in acute leukaemia by real-time PCR of human bone marrow. Br J Haematol 130:233–248
179. Kim MS, Chung NG, Kang MR, Yoo NJ, Lee SH (2011) Genetic and expressional alterations of CHD genes in gastric and colorectal cancers. Histopathology 58:660–668

180. Gorringe KL et al (2008) Mutation and methylation analysis of the chromodomain-helicase-DNA binding 5 gene in ovarian cancer. Neoplasia 10:1253–1258
181. Robbins CM et al (2011) Copy number and targeted mutational analysis reveals novel somatic events in metastatic prostate tumors. Genome Res 21:47–55
182. Fujita T et al (2008) CHD5, a tumor suppressor gene deleted from 1p36.31 in neuroblastomas. J Natl Cancer Inst 100:940–949
183. Bagchi A et al (2007) CHD5 is a tumor suppressor at human 1p36. Cell 128:459–475
184. Mulero-Navarro S, Esteller M (2008) Chromatin remodeling factor CHD5 is silenced by promoter CpG island hypermethylation in human cancer. Epigenetics 3:210–215
185. Wang J et al (2011) The involvement of CHD5 hypermethylation in laryngeal squamous cell carcinoma. Oral Oncol 47:601–608
186. Mokarram P et al (2009) Distinct high-profile methylated genes in colorectal cancer. PLoS One 4:e7012
187. Wang X, Lau KK, So LK, Lam YW (2009) CHD5 is down-regulated through promoter hypermethylation in gastric cancer. J Biomed Sci 16:95
188. Vissers LE et al (2004) Mutations in a new member of the chromodomain gene family cause CHARGE syndrome. Nat Genet 36:955–957
189. Wessels K et al (2010) Novel CHD7 mutations contributing to the mutation spectrum in patients with CHARGE syndrome. Eur J Med Genet 53:280–285
190. Schlesinger Y et al (2007) Polycomb-mediated methylation on Lys27 of histone H3 premarks genes for de novo methylation in cancer. Nat Genet 39:232–236
191. Widschwendter M et al (2007) Epigenetic stem cell signature in cancer. Nat Genet 39:157–158
192. Ohm JE et al (2007) A stem cell-like chromatin pattern may predispose tumor suppressor genes to DNA hypermethylation and heritable silencing. Nat Genet 39:237–242
193. Constantinides PG, Jones PA, Gevers W (1977) Functional striated muscle cells from non-myoblast precursors following 5-azacytidine treatment. Nature 267:364–366
194. Plimack ER, Kantarjian HM, Issa JP (2007) Decitabine and its role in the treatment of hematopoietic malignancies. Leuk Lymphoma 48:1472–1481
195. Matei DE, Nephew KP (2010) Epigenetic therapies for chemoresensitization of epithelial ovarian cancer. Gynecol Oncol 116:195–201
196. Cheng JC et al (2004) Preferential response of cancer cells to zebularine. Cancer Cell 6:151–158
197. Rodriguez-Paredes M, Esteller M (2011) Cancer epigenetics reaches mainstream oncology. Nat Med 17:330–339

Part II
The Impact of Epigenetic Alterations on Cancer Biology

Chapter 6
Epigenetic Regulation of miRNAs in Cancer

Muller Fabbri, Federica Calore, Alessio Paone, Roberta Galli, and George A. Calin

Abstract MicroRNAs (miRNAs) are short noncoding RNAs with gene regulatory functions. It has been demonstrated that the genes encoding for miRNAs undergo the same regulatory epigenetic processes of protein coding genes. In turn, a specific subgroup of miRNAs, called epi-miRNAs, is able to directly target key enzymatic effectors of the epigenetic machinery (such as DNA methyltransferases, histone deacetylases, and polycomb genes), therefore indirectly affecting the expression of epigenetically regulated oncogenes and tumor suppressor genes. Also, several of the epigenetic drugs currently approved as anticancer agents affect the expression of miRNAs and this might explain part of their mechanism of action. This chapter focuses on the tight relationship between epigenetics and miRNAs and provides some insights on the translational implications of these findings, leading to the upcoming introduction of epigenetically related miRNAs in the treatment of cancer.

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6.1 Introduction

MicroRNAs (miRNAs) are small noncoding RNAs (ncRNAs), 19–25 nucleotides (nt) in length, which regulate gene expression. MiRNAs are involved in many biological processes ranging from development, differentiation, and cell cycle regulation to cell senescence and metabolism [1–5]. Mature miRNAs derive from much longer (hundreds nt long) primary transcripts, transcribed by RNA polymerase II as long, capped, polyadenylated precursor-miRNAs (pri-miRNAs) [1]. Then, the double-stranded RNA-specific ribonuclease Drosha, in conjunction with its binding partner DiGeorge syndrome critical region gene 8 (DGCR8, or Pasha), process pri-miRNAs into hairpin RNAs of 60–110 nt known as pre-miRNAs. Translocated from the cell nucleus to the cytoplasm by means of Exportin 5, the pre-miRNA is processed by a ribonuclease III (Dicer) and transactivating response RNA-binding protein (TRBP, which binds human immunodeficiency virus 1) into an 18- to 24-nt duplex. Finally, the duplex interacts with a large protein, RNA-induced silencing complex (RISC), which includes argonaute proteins (AGO1-4 in humans). One strand of the miRNA duplex remains stably associated with RISC and becomes the mature miRNA, which guides the RISC complex mainly (but not exclusively) to the 3′-untranslated region (UTR) of the target messenger RNAs (mRNAs) [1]. Consequently, the translation and/or stability of targeted mRNAs is impaired, causing a reduction in protein expression levels [6]. In addition to this "conventional" mechanism of action, miRNA regulatory effects on gene expression may be more varied than initially proposed. For example, miRNAs can also activate rather than suppress target mRNA expression in particular cell-cycle conditions [7], they can bind also to the coding and the 5′-UTR region of the target mRNAs [8, 9], and they can directly interact with proteins and function as gene promoter regulators [10]. Figure 6.1 summarizes the biogenesis and physiology of miRNAs.

Each miRNA has hundreds or thousands of target genes. We have demonstrated that a specific cluster of two miRNAs (namely, the miR-15a/16-1 cluster) is able to regulate, directly and indirectly, about 14% of the whole genome in a leukemic cell model [11]. Therefore, it is likely that the full coding genome is under the control of miRNAs. The full spectrum of miRNAs expressed in a specific cell type (the miRNome) is different between normal and pathologic tissues [12], and specific signatures of dys-regulated miRNAs harbor diagnostic and prognostic implications [13]. The first link between miRNAs and cancer came from the discovery that these ncRNAs are frequently located in cancer-associated genomic regions, which include minimal regions of amplification, loss of heterozygosity, and common breakpoints in or near oncogenes or tumor suppressor genes (TSGs) and fragile sites (preferential sites of chromatide exchange, deletion, translocation, amplification, or integration of plasmid DNA and tumor-associated viruses) [14]. Since then, myriad studies have investigated aberrations in the miRNome in most types of human cancer (for reviews, see [15–21]). In particular, while some miRNAs act mainly as TSGs, others are frequently overexpressed in human tumors and target TSGs, thereby exerting

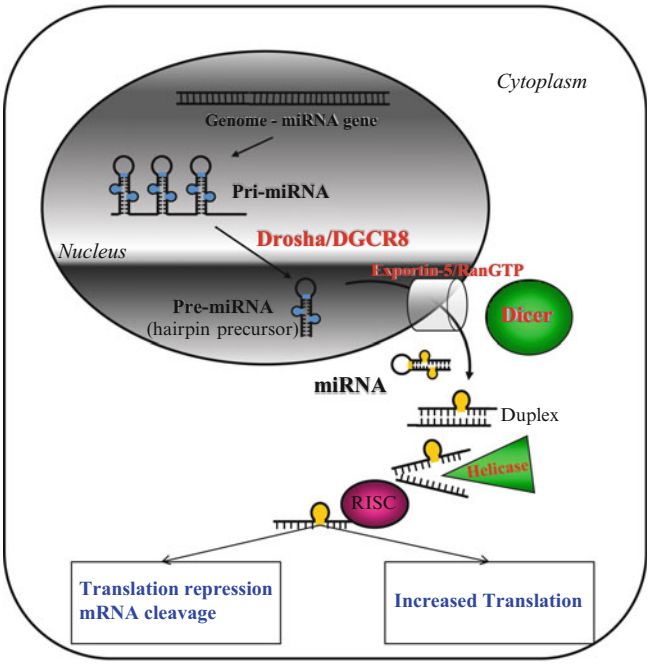

Fig. 6.1 Biogenesis and physiology of miRNAs. MiRNAs are transcribed as pri-miRNAs (in some cases as a cluster of multiple miRs, such as miR-15a and miR-16-1 on the long arm of chromosome 13) and then processed in a hairpin shaped pre-miRNA precursor in the nucleus of the cell. The precursor is then transported in the cytoplasm by means of Exportin 5 and processed until it becomes a single-stranded mature miRNA that eventually binds to a ribonucleoproteic complex (RISC) which directs the miRNA to its target mRNAs. As a result, both translational repression (or mRNA cleavage) and increased target translation can occur (see text for more explanation)

a tumorigenic function. MiRNAs with well-established roles as oncogenes, for instance, include the miR-17-92 cluster, which is transactivated by the *c-MYC* oncogene and dramatically accelerates lymphomagenesis in murine models [22, 23]; miR-155, which induces leukemia in transgenic murine models [24] and has an important function as a regulator of inflammation and the immune response [25–27], and miR-21, which targets important TSGs, such as *PTEN1* [28] and *PDCD4*, in several types of cancer [29–31]. Conversely, the miR-15a/16-1 cluster acts as a TSG in chronic lymphocytic leukemia (CLL) by targeting the antiapoptotic gene *BCL2* [32]. Interestingly, the same miR-15a-16-1 cluster also acts as an oncogene (OG), in CLL, by directly targeting the pro-apoptotic gene *p53* [33], leading to the conclusion that each miRNA should not be labeled exclusively as an OG or as a TSG, since it may have a dual nature (both as OG and TSG) [34], in which the overall effect depends on the specific conditions (tumor type, species specificity, concentration, etc.) in which it operates.

It has been demonstrated that miRNAs, similar to protein coding genes, (PCG), can undergo epigenetic regulation. More recently, it has been shown that a specific

group of miRNAs, called epi-miRNAs, can affect the epigenetic regulation of a given gene by targeting key enzymatic effectors of the epigenetic machinery, such as DNA methyltransferases (DNMTs), histone deacetylases (HDACs), and polycomb genes.

This chapter focuses on the interactions between epigenetics and miRNAs and presents how this intertwined relationship harbors fundamental implications for human carcinogenesis.

6.2 MiRNAs Are Epigenetically Regulated in Cancer

The expression of miRNAs undergoes epigenetic regulation, similarly to PCG. This regulation involves both chromatin modifications and miRNA gene promoter methylation. By treating a breast cancer cell line with the HDAC inhibitor LAQ824, Scott et al. demonstrated that the expression levels of 27 miRNAs are rapidly modified [35], indicating that HDAC and chromatin conformation affects the miRNome in human cancer. Similarly, Saito et al. showed that by treating bladder cancer cells with both a DNA demethylating agent (5-aza-2′-deoxycytidine, 5-AZA) and an HDAC inhibitor (4-phenylbutyric acid, 4-PBA) the expression levels of about 5% of all human miRNAs increased [36]. Among the most strictly epigenetically regulated miRNAs, there is miR-127, an ncRNA embedded in a CpG island and kept epigenetically silenced by both promoter hypermethylation and histone modifications in cancer cells [36]. Interestingly, this miRNA (which belongs to a large cluster that includes miR-136, -431, -432, and -433) is the only member of the cluster whose re-expression was observed when cells were treated with two epigenetic drugs [36]. Moreover, when cells were treated with each drug alone, no variation in miR-127 expression was detected [36], suggesting that miR-127 epigenetic regulation occurs by combined promoter methylation and chromatin histone modifications. Since the *BCL6* oncogene is a direct target of this miRNA [36], miR-127 acts as a TSG, therefore the severe epigenetic control of its expression represents an important mechanism for bladder carcinogenesis.

Using an HCT-116 colorectal cancer cell line with a double knockout (DKO) of DNMT1 (maintenance DNMT) and DNMT3b (de novo DNMT), Lujambio et al. compared miRNA levels of the DKO and wild-type cells. About 6% of the 320 miRNAs analyzed were upregulated in the DKO cells [37]. Among the dys-regulated miRNAs, only miR-124a was embedded in a CpG island, which is densely methylated in this cancer cell line but not in normal tissue. This might suggest that DNMTs act both directly and indirectly in miRNA expression control. MiRNA-124a directly targets CDK6, and restoration of its expression reduces the levels of CDK6 and impacts the phosphorylation status of the CDK6-downstream effector Rb protein [37]. In a group of 353 acute lymphoblastic leukemia (ALL) patients, Roman-Gomez et al. identified a signature of 13 miRNAs, embedded in CpG islands, with high heterochromatic markers (such as high levels of K9H3me2 and/or low levels of K4H3me3) [38, 39]. Treatment with 5-AZA upregulated at least 1 of the 13 miRNAs in 65% of ALLs

[38]. Among these miRNAs, miR-124a was methylated in 59% of ALLs and hypermethylation of its promoter was associated with higher relapse and mortality rates than in the non-hypermethylated cases: multivariate analysis confirmed that miR-124a promoter methylation status is an independent prognostic factor for disease-free and overall survival [39]. Moreover, miR-124a directly silences *CDK6* in ALL patients [39], confirming the impact of miR-124a on the CDK6-Rb pathway. Recently, Ando et al. showed that hypermethylation of the miR-124a promoter is involved in the formation of an epigenetic field defect, a gastric cancer predisposition condition characterized by the accumulation of abnormal DNA methylation in normal-appearing gastric mucosa that is mostly induced by *Helicobacter pylori* infection [40]. These findings reveal that miR-124a promoter hypermethylation is also an early event in gastric carcinogenesis.

In addition to miR-124a, miR-107, another epigenetically controlled miRNA, targets *CDK6* and affects pancreatic carcinogenesis [41]. In HCT-116 cells deficient for *DNMT1* and *DNMT3B*, Bruckner et al. showed increased expression of let-7a-3, an miRNA normally silenced by promoter hypermethylation in the wild-type cell line [42]. In lung adenocarcinoma primary tumors the let-7a-3 promoter was found to be hypomethylated [42], whereas it was found to be hypermethylated in epithelial ovarian cancer. This hypermethylation was associated with low expression of IGF2 (insulin-like growth factor 2) and with a good prognosis [43]. Therefore, DNA methylation could act as a protective mechanism by silencing miRNAs with oncogenic functions. Also miR-1 is epigenetically regulated and frequently silenced by promoter hypermethylation in hepatocellular carcinoma [44]. However, hypomethylation and re-expression of miR-1 were observed in DNMT1-null HCT-116 cells (but not in DNMT3B-null cells) [44], revealing that the maintenance DNMT is specifically and mainly responsible for miR-1 epigenetic regulation. Overall, these studies demonstrate that epigenetic factors can control human carcinogenesis, not only by directly affecting the expression of OGs and TSGs, but also by affecting the expression of miRNAs involved in oncogenic pathways. In addition, epigenetic control of miRNAs may be tissue-specific (since no variation in miRNA expression was observed in lung cancer cells treated with demethylating agents, HDAC inhibitors, or their combination [45]), miRNA-specific (e.g., miR-127 within the cluster it belongs to [36]), and epigenetic-effector-specific (e.g., miR-1 mainly regulated by DNMT1 [44]).

Epigenetically regulated miRNAs are also affecting one of the main aspects of malignancy: the ability to metastasize. Lujambio et al. treated three lymph node–metastatic cell lines with 5-AZA and checked miRNA levels by high-throughput microarray analysis [46]. They identified four miRNAs (namely, miR-148a, miR-34b/c, and miR-9) that showed cancer-specific CpG island hypermethylation. Epigenetic silencing of these miRNAs was also significantly associated with metastasis in human malignancies [46], while the reintroduction of miR-148a and miR-34b/c into cancer cells with epigenetic inactivation inhibited both motility and metastatic potential of the cells in xenograft models. The miR-34b/c cluster is also epigenetically regulated in colorectal cancer (promoter hypermethylation in 90% of primary colorectal cancer tumors vs. normal colon mucosa) [47], whereas

epigenetic silencing of miR-9 and miR-148a (together with miR-152, -124a, and -663) has also been described in breast cancer. In breast cancer cell lines treated with 5-AZA miR-9 was reactivated, while the levels of other aberrantly methylated miRNAs were unchanged [48], further proving that different epigenetic processes can control miRNA levels in different types of cancer.

MiR-342 is located in an intron of the Ena/Vasp-like (*EVL*) gene and represents a good model to study the relationship between miRNAs and the epigenetic regulation of cognate host genes. *EVL* promoter hypermethylation occurs in 86% of colorectal cancers and is present in 67% of adenomas at diagnosis, suggesting that it is an early event in colon carcinogenesis [49]. Treatment with 5-AZA and the HDAC inhibitor trichostatin A restores the synchronized expression of EVL and miR-342 [49]. Another gene, the *EGFL7* gene, which is frequently downregulated in several cancer cell lines and in primary bladder and prostate tumors, hosts miR-126 in one intron. The mature miR-126 can be encoded by three different transcripts of the cognate host gene, each of them with its own promoter. However, miR-126 is concomitantly upregulated with one of the EGFL7 transcripts that has a CpG-island promoter when cancer cell lines are treated with DNA methylation and histone deacetylation inhibitors, indicating that the silencing of intronic miRNAs in cancer may occur by means of epigenetic changes in cognate host genes [50].

Fazi et al. showed that transcription factors can recruit epigenetic effectors to miRNA promoter regions to regulate their expression. The AML1/ETO fusion oncoprotein, the aberrant product of the t(8;21) translocation in acute myeloid leukemia, can bind to the pre-miR-223 region. The oncoprotein recruits epigenetic effectors (i.e., DNMTs, HDAC1, and MeCP2), leading to aberrant hypermethylation of the CpG site near the AML1/ETO binding site and H3-H4 deacetylation of the same chromatin region [51].

In summary, several studies have addressed how epigenetics regulates miRNA expression in human cancer. It has emerged that epigenetic factors account for several of the miRNome aberrancies observed in human cancer, ultimately implicated in both carcinogenesis and in metastasis formation. Therefore, a better understanding of miRNA epigenetic regulation will lead to a better comprehension of the mechanisms responsible for abnormal miRNA levels in cancer and to the development of strategies able to revert these anomalies. Interestingly, miRNAs can also affect the expression of epigenetically regulated PCGs, revealing a further layer of complexity between miRNome and epigenome.

6.3 Epi-miRNAs Affect the Expression of Epigenetically Regulated Genes in Cancer

In addition to being epigenetically regulated, like PCG, miRNAs can also affect the expression of epigenetically regulated genes by targeting key enzymes responsible for epigenetic reactions. We call this group of miRNAs, epi-miRNAs. Some epi-miRNAs are also epigenetically regulated themselves. Our group provided the first

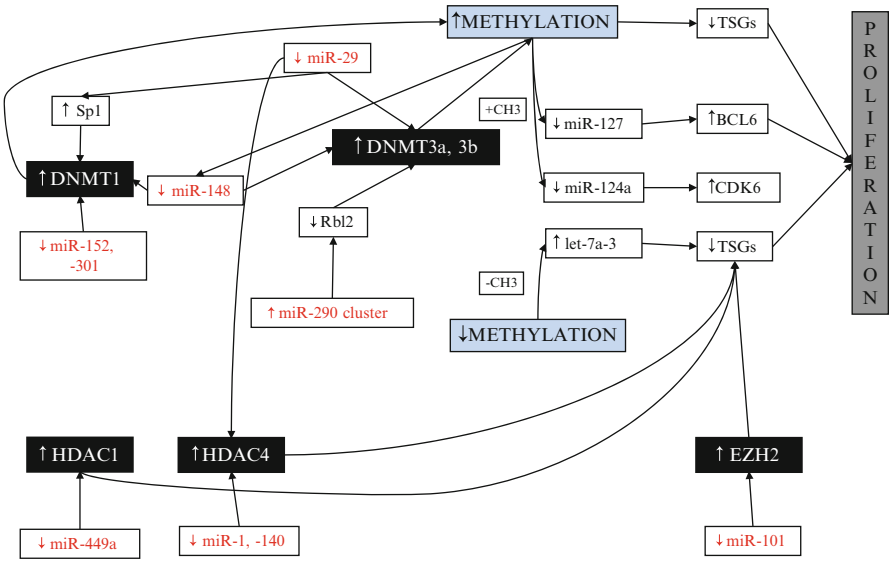

Fig. 6.2 Epi-miRNAs and cancer. Epi-miRNAs (in *red*) directly target effectors of the epigenetic machinery (in *black boxes*) and indirectly affect the expression of epigenetically regulated miRNAs and protein coding genes, ultimately affecting carcinogenesis. *TSGs* tumor suppressor genes; *DNMT* DNA methyltransferase; *HDAC* histone deacetylase; *EZH2* enhancer of zeste homolog 2; *BCL6* B-cell CLL/lymphoma 6; *CDK6* cyclin-dependent kinase 6; *SP1* Sp1 transcription factor; *RBL2* retinoblastoma-like 2 (p130); CH_3 methyl group

evidence that miRNAs can regulate the expression of members of the epigenetic machinery in humans [52]. We demonstrated in both lung cancer cell lines and primary tumors that a family of miRNAs (namely the miR-29 family, composed of miR-29a, -29b, and -29c) directly targets both DNMT3a and DNMT3b, the two key de novo DNMTs. We observed that miR-29 restoration reduces global DNA methylation, induces re-expression of TSGs (such as *WWOX* and *FHIT*, whose expression is silenced by promoter hypermethylation in lung cancer), and exerts an overall antitumoral effect both in vitro and in vivo [52]. The global hypomethylating effect observed in tumor cells upon miR-29 re-expression is the result of a direct targeting effect of these miRNAs on DNMT3a and DNMT3b, and of an indirect silencing effect on DNMT1, occurring through the direct targeting of the DNMT1 transactivating factor SP1 [53]. Figure 6.2 summarizes the relationship between epi-miRNAs and cancer. Duursma et al. [54] have shown that miR-148 also directly targets DNMT3b by binding to a conserved target sequence located in the coding region of the mRNA. Intriguingly, the authors concluded that the targeting of the coding region may play a role in determining the relative abundance of different splice variants of DNMT3b. Furthermore, miRNAs can affect the expression of DNMTs also through an indirect mechanism. Sinkkonen et al. showed that in mouse embryonic stem (ES) cells, members of the miR-290 cluster directly target Rbl2, a factor contributing to the suppression of *DNMT3* genes [55]. By restoring the expression of

the miR-290 cluster, de novo methylation, which had been disrupted in ES Dicer$^{-/-}$ cells, was reestablished, suggesting that DNMTs are indirectly regulated by the miR-290 cluster. These results were confirmed by Benetti et al. [56], who also observed that the aberrant DNA methylation occurring after miR-290 cluster silencing in ES Dicer$^{-/-}$ cells is responsible for increased telomere recombination and aberrant telomere elongation. Notably, the miR-290 Rbl2-mediated regulation of *DNMT3a* and *DNMT3b* was not observed in HEK293 cells with knockdown of Dicer [55], revealing that the described regulatory mechanism might be restricted to ES cells. Moreover, neither of the above-mentioned studies identified the miR-29 family as direct regulators of de novo DNMTs, suggesting that this interaction could also be species-, tumor-, or even histotype-specific.

Epi-miRNAs can also target *DNMT1*. In a study by Braconi et al., it was shown that miR-148a, miR-152, and miR-301 directly target *DNMT1* in cholangiocarcinoma cells [57], resulting in the re-expression of the *RASSF1A* and *p16INK4a* genes, two well-known TSGs that are epigenetically silenced in several malignancies. As previously reported, miR-29b indirectly targets *DNMT1*, by directly silencing its activator SP1 in hematological malignancies [53]. These studies suggest that miR-29b plays a key role in the epigenetic control of human genome.

Epi-miRNAs also regulate the expression of HDACs and PRC genes. *HDAC4* is a direct target of both miR-1, miR-140, and miR-29b [58–60], whereas miR-449a binds to the 3′-UTR region of *HDAC1* [61]. *HDAC1* is upregulated in several types of cancer, and miR-449a re-expression in prostate cancer cells induces cell-cycle arrest, apoptosis, and a senescent-like phenotype by reducing the levels of HDAC1 [61]. EZH2 is the catalytic subunit of PRC2 and is responsible for heterochromatin formation by trimethylating histone H3 on lysine 27 (H3K27me3), leading to the silencing of several TSGs. Varambally et al. showed in prostate cancer cell lines and primary tumors that the level of EZH2 is inversely correlated with the expression of miR-101, which decreases during cancer progression. These findings suggest a role for miR-101 as an epi-miRNA, a hypothesis that was tested and confirmed by showing that miR-101 directly targets EZH2 both in prostate and in bladder cancer models [62, 63]. Moreover, the miR-101-mediated suppression of EZH2 inhibits cancer cell proliferation and colony formation, revealing a role for miR-101 as a TSG that is mediated by its modulatory effects on the cancer epigenome [63].

In summary, an increasing number of epi-miRNAs is being identified and will clarify which epigenetic effectors are involved in the regulation of OGs and TSGs. This knowledge will lead to the development of new strategies to prevent and cure human carcinogenesis by selective modulation of the epi-miRNome.

6.4 Epigenetics and miRNAs: Clinical Implications and Final Remarks

The epigenetics–miRNA relationship harbors several clinical implications. First, some of the demethylating agents (such as 5-AZA or Vidaza) used to show that miRNAs are re-expressed upon demethylation and therefore undergo epigenetic

regulations are drugs, currently approved for the treatment of myelodysplastic syndromes (MDS) [64]. Therefore, part of the observed therapeutic effects of 5-AZA or decitabine might be mediated by their effect on the miRNome. Also, currently available anticancer drugs (such as Bortezomib) induce the expression of miR-29b [65], a key epi-miRNA targeting both DNMTs and HDACs. Moreover, SAHA (suberoylanilide hydroxamic acid), also known as Vorinostat is an HDAC inhibitor currently approved in the treatment of cutaneous T cell lyphomas, may exert an anticancer effect by re-expressing epigenetically regulated miRNAs [66, 67]. Valproic acid (VPA) is also an HDAC inhibitor currently in phase III studies for the treatment of cervical and ovarian cancer, which is able to modulate the expression of miRNAs in human cord blood-derived multipotent stem cells [68].

Overall, while basic research scientists are trying to improve their understanding of the relationship existing between epigenetics and miRNAs, clinicians have started interpreting some of the effects of epigenetic drugs in terms of their effects on the miRNome. This interaction represents an ideal translational setting, capable of bringing novel insights deriving from basic science to the patients. In addition to better understanding the implications and function of currently available epigenetic drugs on the miRNome, it is likely that in the near future this knowledge will assist in the development of miRNA- and epi-miRNA-based therapies. These therapies will be tailored to the specific set of genes that need to be reverted to a physiological expression, in order to achieve an anticancer effect. Therefore, their effect will specifically affect tumor cells, without introducing any major epigenetic perturbation in noncancerous cells, therefore leading to less side effects. These days are not far to come and will provide a new powerful therapeutic tool in the war against cancer.

References

1. Bartel DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116:281–297
2. Pasquinelli AE, Hunter S, Bracht J (2005) MicroRNAs: a developing story. Curr Opin Genet Dev 15:200–205
3. Harfe BD (2005) MicroRNAs: in vertebrate development. Curr Opin Genet Dev 15:410–415
4. Carleton M, Cleary MA, Linsley PS (2007) MicroRNAs and cell cycle regulation. Cell Cycle 6:2127–2132
5. Boehm M, Slack FJ (2006) MicroRNA control of lifespan and metabolism. Cell Cycle 5:837–840
6. Filipowicz W, Bhattacharyya SN, Sonenberg N (2008) Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? Nature Reviews 9:102–114
7. Vasudevan S, Tong Y, Steitz JA (2007) Switching from repression to activation: microRNAs can up-regulate translation. Science 318:1931–1934
8. Tay Y, Zhang J, Thomson AM, Lim B, Rigoutsos I (2008) MicroRNAs to Nanog, Oct4 and Sox2 coding regions modulate embryonic stem cell differentiation. Nature 455:1124–1128
9. Vatolin S, Navaratne K, Weil RJ (2006) A novel method to detect functional microRNA targets. J Mol Biol 358:983–996
10. Eiring AM et al (2010) MiR-328 functions as an RNA decoy to modulate hnRNP E2 regulation of mRNA translation in leukemic blasts. Cell 140:652–665

11. Calin GA et al (2008) MiR-15a and miR-16-1 cluster functions in human leukemia. Proc Natl Acad Sci USA 105:5166–5171
12. Sevignani C, Calin GA, Siracusa LD, Croce CM (2006) Mammalian microRNAs: a small world for fine-tuning gene expression. Mamm Genome 17:189–202
13. Calin GA et al (2005) A microRNA signature associated with prognosis and progression in chronic lymphocytic leukemia. N Engl J Med 353:1793–1801
14. Calin GA et al (2004) Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. Proc Natl Acad Sci USA 101:2999–3004
15. Nelson KM, Weiss GJ (2008) MicroRNAs and cancer: past, present, and potential future. Mol Cancer Ther 7:3655–3660
16. Fabbri M, Croce CM, Calin GA (2009) MicroRNAs in the ontogeny of leukemias and lymphomas. Leuk Lymphoma 50:160–170
17. Fabbri M, Croce CM, Calin GA (2008) MicroRNAs. Cancer J 14:1–6
18. Fabbri M et al (2008) MicroRNAs and noncoding RNAs in hematological malignancies: molecular, clinical and therapeutic implications. Leukemia 22:1095–1105
19. Deng S, Calin GA, Croce CM, Coukos G, Zhang L (2008) Mechanisms of microRNA deregulation in human cancer. Cell Cycle 7:2643–2646
20. Garzon R, Croce CM (2008) MicroRNAs in normal and malignant hematopoiesis. Curr Opin Hematol 15:352–358
21. Croce CM (2009) Causes, and consequences of microRNA dysregulation in cancer. Nat Rev Genet 10:704–714
22. He L et al (2005) A microRNA polycistron as a potential human oncogene. Nature 435: 828–833
23. Mendell JT (2008) miRiad roles for the miR-17-92 cluster in development and disease. Cell 133:217–222
24. Costinean S et al (2006) Pre-B cell proliferation and lymphoblastic leukemia/high-grade lymphoma in E(mu)-miR155 transgenic mice. Proc Natl Acad Sci USA 103:7024–7029
25. Thai TH et al (2007) Regulation of the germinal center response by microRNA-155. Science 316:604–608
26. Rodriguez A et al (2007) Requirement of bic/microRNA-155 for normal immune function. Science 316:608–611
27. Tili E et al (2007) Modulation of miR-155 and miR-125b levels following lipopolysaccharide/TNF-alpha stimulation and their possible roles in regulating the response to endotoxin shock. J Immunol 179:5082–5089
28. Cully M, You H, Levine AJ, Mak TW (2006) Beyond PTEN mutations: the PI3K pathway as an integrator of multiple inputs during tumorigenesis. Nat Rev Cancer 6:184–192
29. Meng F et al (2007) MicroRNA-21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer. Gastroenterology 133:647–658
30. Asangani IA et al (2008) MicroRNA-21 (miR-21) post-transcriptionally downregulates tumor suppressor Pdcd4 and stimulates invasion, intravasation and metastasis in colorectal cancer. Oncogene 27:2128–2136
31. Frankel LB et al (2008) Programmed cell death 4 (PDCD4) is an important functional target of the microRNA miR-21 in breast cancer cells. J Biol Chem 283:1026–1033
32. Cimmino A et al (2005) miR-15 and miR-16 induce apoptosis by targeting BCL2. Proc Natl Acad Sci USA 102:13944–13949
33. Fabbri M et al (2011) Association of a microRNA/TP53 feedback circuitry with pathogenesis and outcome of B-cell chronic lymphocytic leukemia. JAMA 305:59–67
34. Fabbri M, Ivan M, Cimmino A, Negrini M, Calin GA (2007) Regulatory mechanisms of microRNAs involvement in cancer. Expert Opin Biol Ther 7:1009–1019
35. Scott GK, Mattie MD, Berger CE, Benz SC, Benz CC (2006) Rapid alteration of microRNA levels by histone deacetylase inhibition. Cancer Res 66:1277–1281
36. Saito Y et al (2006) Specific activation of microRNA-127 with downregulation of the proto-oncogene BCL6 by chromatin-modifying drugs in human cancer cells. Cancer Cell 9: 435–443

37. Lujambio A et al (2007) Genetic unmasking of an epigenetically silenced microRNA in human cancer cells. Cancer Res 67:1424–1429
38. Roman-Gomez J et al (2009) Epigenetic regulation of microRNAs in acute lymphoblastic leukemia. J Clin Oncol 27:1316–1322
39. Agirre X et al (2009) Epigenetic silencing of the tumor suppressor microRNA Hsa-miR-124a regulates CDK6 expression and confers a poor prognosis in acute lymphoblastic leukemia. Cancer Res 69(10):4443–4453
40. Ando T et al (2009) DNA methylation of microRNA genes in gastric mucosae of gastric cancer patients: its possible involvement in the formation of epigenetic field defect. Int J Cancer 124:2367–2374
41. Lee KH et al (2009) Epigenetic silencing of microRNA miR-107 regulates cyclin-dependent kinase 6 expression in pancreatic cancer. Pancreatology 9:293–301
42. Brueckner B et al (2007) The human let-7a-3 locus contains an epigenetically regulated microRNA gene with oncogenic function. Cancer Res 67:1419–1423
43. Lu L, Katsaros D, de la Longrais IA, Sochirca O, Yu H (2007) Hypermethylation of let-7a-3 in epithelial ovarian cancer is associated with low insulin-like growth factor-II expression and favorable prognosis. Cancer Res 67:10117–10122
44. Datta J et al (2008) Methylation mediated silencing of MicroRNA-1 gene and its role in hepatocellular carcinogenesis. Cancer Res 68:5049–5058
45. Yanaihara N et al (2006) Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. Cancer Cell 9:189–198
46. Lujambio A et al (2008) A microRNA DNA methylation signature for human cancer metastasis. Proc Natl Acad Sci USA 105:13556–13561
47. Toyota M et al (2008) Epigenetic silencing of microRNA-34b/c and B-cell translocation gene 4 is associated with CpG island methylation in colorectal cancer. Cancer Res 68:4123–4132
48. Lehmann U et al (2008) Epigenetic inactivation of microRNA gene hsa-mir-9-1 in human breast cancer. J Pathol 214:17–24
49. Grady WM et al (2008) Epigenetic silencing of the intronic microRNA hsa-miR-342 and its host gene EVL in colorectal cancer. Oncogene 27:3880–3888
50. Saito Y et al (2009) Epigenetic therapy upregulates the tumor suppressor microRNA-126 and its host gene EGFL7 in human cancer cells. Biochem Biophys Res Commun 379:726–731
51. Fazi F et al (2007) Epigenetic silencing of the myelopoiesis regulator microRNA-223 by the AML1/ETO oncoprotein. Cancer Cell 12:457–466
52. Fabbri M et al (2007) MicroRNA-29 family reverts aberrant methylation in lung cancer by targeting DNA methyltransferases 3A and 3B. Proc Natl Acad Sci USA 104:15805–15810
53. Garzon R et al (2009) MicroRNA -29b induces global DNA hypomethylation and tumor suppressor gene re-expression in acute myeloid leukemia by targeting directly DNMT3A and 3B and indirectly DNMT1. Blood 113:6411–6418
54. Duursma AM, Kedde M, Schrier M, le Sage C, Agami R (2008) miR-148 targets human DNMT3b protein coding region. RNA 14:872–877
55. Sinkkonen L et al (2008) MicroRNAs control de novo DNA methylation through regulation of transcriptional repressors in mouse embryonic stem cells. Nat Struct Mol Biol 15:259–267
56. Benetti R et al (2008) A mammalian microRNA cluster controls DNA methylation and telomere recombination via Rbl2-dependent regulation of DNA methyltransferases. Nat Struct Mol Biol 15:268–279
57. Braconi C, Huang N, Patel T (2010) MicroRNA-dependent regulation of DNA methyltransferase-1 and tumor suppressor gene expression by interleukin-6 in human malignant cholangiocytes. Hepatology 51:881–890
58. Chen JF et al (2006) The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation. Nat Genet 38:228–233
59. Tuddenham L et al (2006) The cartilage specific microRNA-140 targets histone deacetylase 4 in mouse cells. FEBS Lett 580:4214–4217
60. Li Z et al (2009) Biological functions of miR-29b contribute to positive regulation of osteoblast differentiation. J Biol Chem 282:15676–15684

61. Noonan EJ et al (2009) miR-449a targets HDAC-1 and induces growth arrest in prostate cancer. Oncogene 28:1714–1724
62. Varambally S et al (2008) Genomic loss of microRNA-101 leads to overexpression of histone methyltransferase EZH2 in cancer. Science 322:1695–1699
63. Friedman JM et al (2009) The putative tumor suppressor microRNA-101 modulates the cancer epigenome by repressing the polycomb group protein EZH2. Cancer Res 69:2623–2629
64. Kaminskas E, Farrell AT, Wang YC, Sridhara R, Pazdur R (2005) FDA drug approval summary: azacitidine (5-azacytidine, Vidaza) for injectable suspension. Oncologist 10:176–182
65. Liu S et al (2010) Sp1/NFkappaB/HDAC/miR-29b regulatory network in KIT-driven myeloid leukemia. Cancer Cell 17:333–347
66. Shin S et al (2009) MicroRNAs that respond to histone deacetylase inhibitor SAHA and p53 in HCT116 human colon carcinoma cells. Int J Oncol 35:1343–1352
67. Kretzner L et al (2011) Combining histone deacetylase inhibitor vorinostat with aurora kinase inhibitors enhances lymphoma cell killing with repression of c-Myc, hTERT, and microRNA levels. Cancer Res 71:3912–3920
68. Lee S et al (2011) Histone deacetylase regulates high mobility group A2-targeting microRNAs in human cord blood-derived multipotent stem cell aging. Cell Mol Life Sci 68:325–336

Chapter 7
DNA Hypomethylation and Activation of Germline-Specific Genes in Cancer

Charles De Smet and Axelle Loriot

Abstract DNA methylation, occurring at cytosines in CpG dinucleotides, is a potent mechanism of transcriptional repression. Proper genomic methylation patterns become profoundly altered in cancer cells: both gains (hypermethylation) and losses (hypomethylation) of methylated sites are observed. Although DNA hypomethylation is detected in a vast majority of human tumors and affects many genomic regions, its role in tumor biology remains elusive. Surprisingly, DNA hypomethylation in cancer was found to cause the aberrant activation of only a limited group of genes. Most of these are normally expressed exclusively in germline cells and were grouped under the term "cancer-germline" (CG) genes. CG genes represent unique examples of genes that rely primarily on DNA methylation for their tissue-specific expression. They are also being exploited to uncover the mechanisms that lead to DNA hypomethylation in tumors. Moreover, as CG genes encode tumor-specific antigens, their activation in cancer highlights a direct link between epigenetic alterations and tumor immunity. As a result, clinical trials combining epigenetic drugs with anti-CG antigen vaccines are being considered.

7.1 Introduction

Although DNA hypomethylation was the first epigenetic alteration to be described in human cancers, its effect on gene expression programs and tumor biology has remained enigmatic. Initial examination of cancer genomes identified most losses of DNA methylation in repeated elements [29]. This is not surprising, since these DNA elements are highly abundant and comprise most of the CpG sites that are normally methylated in healthy somatic tissues. A crucial question was whether

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DNA hypomethylation also affected protein-encoding genes, leading to their aberrant expression in tumor cells. It appeared, however, that genome hypomethylation in tumors is not generally associated with the ectopic activation of a multitude of genes [5]. A plausible explanation for this is that most tissue-specific genes use other regulatory mechanisms, including histone modifications, and that DNA methylation, if present, serves merely as secondary layer of repression. Losses of DNA methylation within such genes would therefore not be sufficient to trigger transcriptional activation.

Later work, aiming at isolating genes that code for tumor-specific antigens, led to the identification of a particular group of genes, which are normally expressed exclusively in germline cells but become aberrantly activated in a wide variety of tumors [86]. Given this expression profile, these genes were termed "cancer-germline" (CG) genes. Interestingly, CG genes were found to rely primarily on DNA methylation for repression in normal somatic tissues, and their activation in tumors was shown to be a direct consequence of genome hypomethylation [22]. These observations highlighted an unexpected link between epigenetic alterations in tumors and cancer immunity. They also provided clear examples of genes that owe their tissue-specific expression to DNA methylation. Moreover, CG genes are being exploited to try to uncover the molecular mechanisms underlying genome hypomethylation in tumors, as this epigenetic process remains largely unexplained.

7.2 Characterization of CG Genes

Human tumors express specific antigens, as evidenced by the existence in the blood of cancer patients of cytolytic T lymphocytes (CTL) that recognize antigens present on their tumor cells but not on normal cells [10]. Using a gene library transfection approach and a CTL clone isolated from a melanoma patient, Boon and colleagues identified the first human tumor antigen-encoding gene [85]. The gene was named melanoma antigen 1 or *MAGE-1* (later renamed *MAGEA1*). *MAGEA1* expression was not found in normal tissues except for testis, but was instead detected in a significant fraction of melanoma samples, as well as in various other tumor types [20, 23]. The same genetic approach led to the identification of other melanoma antigen genes, namely *BAGE*, *GAGE*, and *MAGEA3*, a gene closely related to *MAGEA1* [9, 34, 84]. For these genes too, expression among normal tissues was restricted to testis, and activation in tumors was detected among various cancer types. Additional tumor antigen genes were subsequently identified, using an alternative cloning approach, called SEREX (serological analysis of recombinant tumor cDNA expression libraries), and based on the presence of high titers of antitumor IgGs in the blood of tumor-bearing patients [73]. Again, several of the identified genes, including *SSX2* and *NY-ESO-1*, had their normal expression restricted to testis and were activated in a percentage of different tumor types. Later studies indicated that the normal expression of most isolated genes was confined to the germ cells in both testis and fetal ovary [44, 52, 82].

Together, these findings led to the important notion that specific antigens in tumors arise from the aberrant activation of genes that are normally transcribed exclusively in the germline. From an immunological point of view, this dual expression pattern is understandable. Unlike most somatic cells, germ cells lack MHC class I molecules, which are required to present antigenic peptides at the cell surface [37]. Activation of germline-specific genes in tumor cells therefore leads to the expression of truly tumor-specific antigens, which can be recognized as nonself by the immune system.

Further studies using cDNA subtraction procedures or database mining have permitted the identification of additional genes expressed in germ cells and cancer but not in normal somatic tissues [56, 60, 63, 75]. Some genes identified in this way were subsequently shown to encode tumor-specific antigens recognized by CTLs [86]. Altogether about 50 human genes or gene families were identified, which displayed specific expression in the germline and activation in a significant proportion of cancers [2]. These genes appear to exert a variety of cellular functions, but on the basis of their common expression pattern they were grouped under the term cancer-germline (CG) genes. CG genes are dispersed on several chromosomes, with a marked preference for the X chromosome. In human cancers, CG genes are expressed more frequently in specific tumor types, like for instance lung cancer, head and neck cancer, bladder cancer, and melanoma [76]. Other tumor types like colon cancer, renal cancer, and leukemia only rarely show activation of CG genes. An important feature of CG genes is their frequent co-activation in tumors [74]. It was observed indeed that positive tumors often express several CG genes. Clearly, the widespread and concerted expression of CG genes in tumors indicates that their activation in cancer results from a global gene activation process, rather than stochastic individual events.

7.3 DNA Demethylation in the Activation of CG Genes in Tumors

The marked tendency of CG genes to become co-expressed in tumors suggested that these genes share, at least in part, a common mechanism of transcriptional activation. Initial studies were performed with the *MAGEA1* gene in order to identify essential promoter elements and corresponding transcription factors that may contribute to the cell-type-specific expression of the gene. Surprisingly, however, transfection experiments revealed that all cells, including those that do not express *MAGEA1*, contain transcription factors capable of inducing significant *MAGEA1* promoter activity [24]. Transfection experiments with other CG gene promoter constructs led to similar results [17, 89]. This implied that nonexpressing cells have a repression mechanism, probably operating at the chromatin level that protects CG gene promoters against spurious activation.

The initial observation by Weber and colleagues that *MAGEA1* could be induced in nonexpressing melanoma cell lines following treatment with the DNA

methylation inhibitor 5-aza-2′-deoxycytidine provided a first hint that DNA methylation may contribute to the transcriptional regulation of this gene [91]. This was confirmed by studies showing that the promoter of *MAGEA1* is invariably methylated in all normal somatic tissues and instead unmethylated in germ cells [26]. Likewise, activation of the *MAGEA1* gene in tumors was strictly correlated with demethylation of its promoter [26]. Further studies showed that DNA methylation was similarly involved in the regulation of other CG genes [17, 26, 52, 56, 89]. Altogether, these observations indicated that CG genes rely on DNA methylation for repression in somatic tissues, and that aberrant activation of these genes in tumors results from demethylation of their promoter.

Interestingly, demethylation and activation of CG genes in tumors was found to correlate with global genome hypomethylation [14, 25, 45]. This association was further confirmed by a study on microdissected tumor samples, revealing that intratumor heterogeneity of CG gene expression also correlates with global genome hypomethylation levels [96]. These observations provided therefore the first clear evidence that the process of genome-wide demethylation, common to many cancers, not only affects repeated sequences but also single copy genes, and can lead to aberrant gene activation. The frequent co-activation of CG genes in tumors likely reflects the global process of DNA demethylation, which can simultaneously affect many loci across the cancer genome.

7.4 DNA Methylation in the Regulation of Germline Genes

Considering the potent effect of DNA methylation on transcriptional repression, it was originally proposed that this DNA modification might serve as a general mechanism to control the programmed expression of tissue-specific genes [39, 72]. Evidence, however, indicates that most tissue-specific genes rely on mechanisms other than DNA methylation for repression in nonexpressing cells [8, 88]. This may be ascribed to the distribution of CpG sequences, where cytosine methylation can occur. Vertebrate genomes show a general depletion of CpG dinucleotides, which was attributed to the high mutability of methylated cytosines, and hence the progressive disappearance of this sequence during evolution [7]. Discrete genomic regions however, which appear generally free of CpG methylation, maintained a high density of CpG sites. These so-called CpG islands often overlap gene promoters [19]. Many tissue-specific genes contain a methylation-free CpG island within their promoter and can therefore not rely on DNA methylation for repression in nonexpressing tissues. On the other hand, genes with few CpG sites within their promoter are only little affected by DNA methylation, and often show an inconstant relationship between promoter methylation and transcriptional silencing [12]. It was therefore proposed that DNA methylation in vertebrates is solely involved in the control of retrotransposable elements, monoallelically expressed imprinted genes, and X chromosome inactivation, the only cases where consistent methylation of CpG-rich regions appeared to exist [101].

This view was challenged by the discovery of CG genes, which were found to be characterized by the presence of a high density of CpG sites within their promoter [26]. Yet, unlike classical CpG islands, CpG-rich promoters of CG genes are methylated in all normal somatic tissues. CG gene promoters appear therefore favorably disposed to DNA methylation-mediated regulation. Consistently, transfection experiments with in vitro methylated CG gene constructs indicated that DNA methylation was sufficient to repress transcription, even in cells that express the corresponding endogenous CG gene, and therefore obviously contain appropriate transcriptional activators [17, 26, 27, 78, 89]. This and the above-mentioned observation that unmethylated CG gene promoters are transcriptionally active in nonexpressing cells provided strong evidence that DNA methylation is an essential component of the repression of this group of germline-specific genes in somatic cells.

More recently, genome-wide studies were conducted in order to identify the distribution of differentially methylated CpG sites across the genome of distinct types of human cells [77, 93]. These studies revealed the existence of novel sets of genes with a CpG-rich promoter that was densely methylated in somatic tissues (in addition to the previously characterized CG genes). Remarkably, most of these genes were specifically demethylated and expressed in testis. It appears therefore that DNA methylation has a particular role in the regulation of germline-specific genes.

Why would DNA methylation be particularly suitable for the regulation of genes with specific expression in germline cells rather than in other cell types? A plausible explanation may be that methylation-dependent germline genes have the advantage of being little exposed to the evolutionary loss of methylated CpGs, because they are unmethylated precisely in the cells that transmit their genome to the offspring. As a result, such genes maintain a high density of CpG sites within their promoter and remain therefore fully responsive to DNA methylation.

7.5 Mechanisms Leading to Hypomethylation of CG Genes in Cancer

CG genes have served as model sequences to investigate the distribution and dynamics of methylation losses in tumor genomes. Detailed analysis of the *MAGEA1* locus revealed preferential hypomethylation of a restricted region surrounding the transcription start site of the gene in expressing tumor cells, suggesting that hypomethylated CpG sites are unevenly distributed across cancer genomes [27]. Consistently, recent genome-wide DNA methylation studies confirmed that DNA hypomethylation in tumors adopts mosaic patterns, with defined hypomethylated domains (between one kilobase and several megabases in size) surrounded by normally methylated regions [66, 71, 92]. These observations indicate that certain genomic regions, including CG promoters, are particularly susceptible to DNA hypomethylation in tumors.

The possibility that *MAGEA1*-expressing tumor cells possess a DNA demethylation activity targeted towards the 5′-region of the gene was investigated [27, 58].

Thus, a large genomic fragment comprising the *MAGEA1* gene was methylated in vitro and then stably transfected into several human tumor cell lines, where the endogenous *MAGEA1* gene is hypomethylated and active. The newly integrated *MAGEA1* transgenes did not undergo demethylation, indicating that the process that once led to demethylation of the endogenous *MAGEA1* gene was not preserved in these cells. Remarkably, when unmethylated *MAGEA1* constructs were introduced into such cells, de novo methylation of the transgenes occurred except in a region overlapping the *MAGEA1* promoter [27]. This mechanism of protection against de novo DNA methylation was lost when mutations that impair the *MAGEA1* promoter activity were introduced into the transgene, or when the transgene was transfected into tumor cells that induce only little *MAGEA1* promoter activity. Altogether, these data suggest that site-specific hypomethylation of *MAGEA1* in tumors results from a past event of transient DNA demethylation and is maintained locally by the presence of potent transcriptional activators that prevent remethylation.

In vivo studies, evaluating global genome methylation levels in colon and breast cancers, demonstrated that DNA hypomethylation is present in the early stages of the disease, and does not progress towards later stages, adding support the transient nature of the DNA demethylation process [30, 41]. Other studies, however, reported a higher prevalence of genome hypomethylation and an increased frequency of CG gene activation in more advanced tumor stages [53, 100]. This was interpreted as an indication that DNA demethylation might instead be a continuous process leading to progressive methylation losses with tumor development. Other interpretations for the increased hypomethylation in advanced tumor genomes, which implicate a transient DNA demethylation process, are however possible: (1) transient demethylation would initially produce a mixed population of precancerous cells with varying levels of DNA hypomethylation, and cells with the most hypomethylated genome would later be selected to contribute to the more advanced stages of the disease; or (2) the transient demethylation process could occur at varying time points during tumor progression and would therefore be more likely to have already occurred in late stage tumor samples [22]. Additional support for a transient DNA demethylation process comes from the observation that tumor cell lines with a hypomethylated genome do not show further CpG methylation losses during culturing [32, 55, 94]. Of note, many tumor cells display instead de novo methylation activities [3, 43].

Considering the suggested dynamics of DNA demethylation in tumors, it is reasonable to propose that hypomethylation of CG genes in tumors is mediated by two groups of factors: those that contribute to the transient DNA demethylation process and those that are required to protect the CG gene promoter region against subsequent remethylation.

7.5.1 Process of DNA Demethylation

Factors contributing to the DNA demethylation process during cancer development remain unknown. The apparent transient nature of this process suggests that activation of such demethylation-inducing factors might occur in association with

one (or several) of the multiple steps through which precancerous cells are progressing before acquiring full malignancy. Interestingly, a recent study evaluating genome methylation levels in an isogenic series of human mammary epithelial cell cultures transitioning from normal to malignantly transformed revealed that most losses of DNA methylation occurred at the stage of acquisition of indefinite lifespan [67]. Another study reported that genome hypomethylation and CG gene activation is more prevalent in tumors displaying the alternative telomere (ALT) maintenance phenotype rather than telomerase activation, the two possible mechanisms by which cancer cells stabilize their telomeres and acquire immortality [83]. These observations establish therefore a possible link between DNA demethylation and cellular immortalization. Underlying molecular mechanisms remain, however, to be identified.

Theoretically, DNA demethylation in tumor cells could possibly occur through two distinct processes commonly referred to as active demethylation and passive demethylation [16]. Active demethylation would involve the activation of demethylating enzymes, which can remove methylation marks from the DNA in a replication-independent manner. Enzymes contributing to active DNA demethylation in animal cells are beginning to be characterized [16], but their potential involvement in cancer genome demethylation has not yet been reported. Passive demethylation on the other hand, would rely on the inhibition of DNA methyltransferases, which normally preserve the DNA methylation marks through the successive replication cycles. Three DNA methyltransferases exist in mammals: DNMT1, DNMT3A, and DNMT3B [6]. DNMT1 is primarily involved in DNA methylation maintenance, as it appears to be specialized in copying preexisting methylation sites onto the newly synthesized strand during replication. DNMT3A and DNMT3B instead have de novo DNA methylation activity and are responsible for the establishment of new DNA methylation marks in the developing embryo. For CG genes in particular, studies based on targeted depletion of the distinct DNMTs indicate that DNMT1 is the principal enzyme for methylation maintenance [42, 57]. It is therefore likely that passive DNA demethylation of CG genes in tumors would necessarily involve factors that decrease the amount or impair proper functioning of DNMT1. In certain tumor cells, however, combined depletion of DNMT1 and DNMT3 enzymes was required to obtain efficient demethylation and activation of CG genes [42, 95]. This indicates that de novo methyltransferases can be targeted to these genes, where they might restore lost methylation sites, and underscores the importance of acquiring mechanisms of protection against remethylation for long-term activation.

7.5.2 Factors that Protect Against Remethylation

Studies with the *MAGEA1* promoter suggest that protection of the promoter against DNA remethylation is dependent on the level of transcriptional activation [27]. It is therefore likely that maintenance of CG gene promoter hypomethylation in tumor cells relies on the presence of appropriate transcription factors, as well as on the activation of such factors by upstream signaling pathways.

Several DNA-binding factors have been identified, which appear to induce activation of CG gene promoters. Transcriptional activation of several genes of the *MAGEA* family has been shown to depend on the binding of ETS transcription factors within their promoter [21, 24]. Interestingly, ETS-binding sequences in *MAGEA* promoters contain a CpG site, and it was shown that methylation of this site inhibits binding of the corresponding factor [25]. In the promoter of *MAGEA1*, two ETS-binding sites were shown to be essential to maintain hypomethylation of the promoter in expressing tumor cells, as evidenced by remethylation of transfected *MAGEA1* constructs containing mutations within these two essential promoter elements [27]. The ETS family of transcription factors comprises about 30 members in humans, which all bind a similar DNA motif with a central GGAA/T sequence [68]. The precise member(s) involved in the regulation of *MAGEA* genes remain(s) to be characterized.

SP1 is another transcription factor, which was shown to contribute to the activation of several *MAGEA* genes, as well as the *CTAG1* gene (also termed *NY-ESO-1*) [24, 46]. The ubiquitously expressed SP1 factor acts as a transcriptional activator and recognizes a consensus DNA sequence (GC box element), which includes a CpG site [80]. SP1-binding elements are therefore often present in CG-rich promoter sequences. Binding of SP1 to the *CTAG1* gene was shown to occur only in cells where the promoter is unmethylated [46]. Interestingly, SP1-binding elements were previously shown to be involved in preserving the methylation-free status of classical CpG-island promoters [13, 62]. It is therefore likely that, once bound to the demethylated promoter of CG genes, SP1 proteins contribute to protect the region against remethylation.

BORIS (also known as CTCFL) is a testis-specific paralog of the ubiquitously expressed DNA-binding protein CTCF, which is involved in various aspects of epigenetic regulation, including gene imprinting and X chromosome inactivation [59]. Both proteins share a highly similar central DNA-binding domain, and recognize therefore overlapping DNA sequences, but contain divergent amino- and carboxy-terminal domains. The gene-encoding BORIS belongs to the CG group of genes, as its expression is regulated by DNA methylation and becomes activated in a wide variety of tumors [38, 49, 87, 95]. Remarkably, it has been demonstrated that in expressing tumors cells, BORIS is targeted to the promoters of other CG genes, namely *MAGEA1* and *CTAG1*, where its recruitment coincides with loss of CTCF binding [40, 87]. BORIS exerts transcriptional activation of CG genes, possibly in cooperation with SP1 transcription factors [46, 87]. In one study, forced overexpression of BORIS led to demethylation (albeit only partially) and activation of various CG genes in normal human fibroblasts, suggesting that BORIS activation in tumors might represent a primary triggering event for the epigenetic de-repression of other CG genes [87]. However, similar experiments from other groups did not confirm CG gene demethylation and activation resulting from BORIS overexpression [49, 97]. Moreover, it was found that many tumors display activation of various CG genes in the absence of BORIS expression. It is therefore unlikely that BORIS is a necessary factor for the derepression of other CG genes in tumors. Its presence in certain tumor cells may, however, facilitate maintenance of the hypomethylated and active state of CG gene promoters.

Many more transcription factors involved in CG gene regulation remain to be identified, and it is likely that each particular CG gene is controlled by a distinct combination of transcription factors. Tissue-specific differences in the content of transcription factors probably account for the fact that, while CG genes tend to be co-activated in hypomethylated tumors, some of them nevertheless show preferential activation in specific tumor types [36, 56].

Cell signaling through tyrosine kinase receptors appears to represent an additional level of control of CG gene regulation. A study in mast cell lines reported that signaling through KIT, an oncogenic receptor hyper-activated in several types of cancers, increases transcription of *MAGE* genes [99]. Other studies revealed that signaling through FGFR2, an FGF receptor often down-regulated in thyroid and pituitary cancers, exerts a negative effect on *MAGEA3* and *MAGEA6* transcription [51, 102]. It is therefore possible that particular dysregulations in cancers, such as those affecting cell signaling pathways, increase the activity of transcription factors that target CG genes, and thereby facilitate long-term activation of these genes in hypomethylated tumor cells. This may partially explain the observation that experimental DNA demethylation, by the use of DNMT inhibitors, often induces CG gene activation more efficiently in tumor cells than in normal cells [47].

7.5.3 Histone Modifications

Active CG gene promoters in tumors usually display a hypomethylated region that comprises one to several kilobases [27]. It is therefore likely that the protective influence of transcription factors against DNA remethylation extends beyond their narrow-binding site. Consistently, impaired binding of ETS transcription factors to *MAGEA1* transgenes, as caused by mutations in their recognition sites, resulted in de novo methylation of CpG sites within the entire promoter region, not just those located nearby the mutated ETS-binding sites [27]. This regional, rather than site-specific effect, might be related to the presence of modifications on the chromatin, such as histone modifications, which after being initiated by specific transcription factors often propagate themselves over larger domains [31]. Histone modifications can indeed influence DNA methylation states [15]. Repressive histone marks, such as methylation of lysine 9 and 27 of histone H3 (H3K9 and H3K27), favor local DNA methylation, whereas active marks, such as histone acetylation or methylation of lysine 4 of histone H3 (H3K4), appear to exclude the DNA methylation machinery. Studies from several groups have shown that demethylation and activation of CG genes in tumor cells is always associated with gains in histone acetylation and H3K4 methylation [42, 70]. The repressed state of human CG genes instead has been associated to a certain extent with the presence of H3K27 and H3K9 methylation marks [42, 70]. The exact relationship between histone modifications changes and DNA demethylation in CG gene promoters remains unclear. A crucial question is whether the varying histone modifications in CG gene promoters are a cause or a consequence of DNA methylation alterations. Studies using inhibitors of histone-modifying enzymes showed that these were on their own unable to induce significant

demethylation and activation of CG genes. Only in combination with inhibitors of DNA methylation, did they significantly modulate the level of activation of CG genes [35, 54, 70]. These observations support the notion that DNA methylation exerts a dominant role in the epigenetic repression of CG genes. But it remains possible that histone modifications assume the responsibility of maintaining the active status of the promoter following its demethylation.

7.5.4 Multiple Factors Determining CG Gene Activation in Tumors

Considering the above, it appears that activation of a particular CG gene in a tumor cell will depend on several factors: (1) the extent of CpG methylation losses resulting from the transient DNA demethylation process; (2) the level of de novo DNA methylation activities in the cell, which might induce remethylation of the promoter; (3) the presence of transcriptional activators and histone-modifying enzymes capable of counteracting remethylation activities. The likelihood that a CG gene becomes activated in a tumor cell probably depends on a complex balance between these different factors (Fig. 7.1).

7.6 Oncogenic Function of CG Genes

Activation of CG genes in tumor cells raises the possibility that their proteins might have oncogenic activities. The biological function of most of these genes, which encode very diverse proteins, remains however poorly understood. One extreme possibility is that the main contribution of DNA hypomethylation to tumor progression resides in its repercussions on genomic instability [33], and that the accompanying activation of CG genes is merely a side effect with no impact on malignancy (other than inducing the expression of tumor antigens). Another possibility has been proposed, in which the concerted expression of CG genes in cancer would correspond to the activation of a gametogenic program, thereby bestowing tumor cells with germ cell properties, including the capacity to self-renew (a feature of spermatogonial stem cells) and increased motility (a feature of sperm cells) [79]. Activation of CG genes in tumors is however only partial, making it very unlikely that all genes necessary for inducing a gametogenic program become expressed at the same time. Nevertheless, it remains possible that some CG genes contribute to tumor progression. Several MAGE proteins were found to inhibit p53 transactivation function, thereby exerting antiapoptotic properties [28, 64, 98]. GAGE proteins were also shown to render cells resistant to apoptosis [18]. Other studies reported that MAGEA11 serves as a co-stimulator for the androgen receptor and might therefore contribute to the development of prostate tumors that have become independent of the presence of androgen for their growth [4, 48]. Moreover, it was noted that

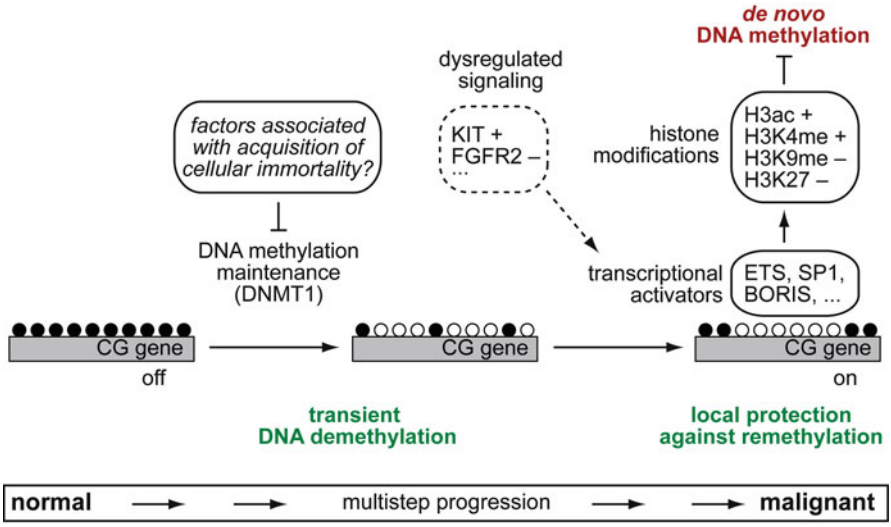

Fig. 7.1 Proposed model of demethylation and activation of CG genes during tumor development. The activation of CG genes in tumors depends on several factors: the extent of the transient DNA demethylation process, occurring at some step of tumor development; the level of counteracting de novo methylation activities in the cell; and the presence of transcriptional activators that protect the CG gene promoter against remethylation, for instance by increasing (+) or decreasing (–) distinct histone marks locally. *Filled circles* represent methylated CpG, *empty circles* unmethylated cytosines

several CG genes, including *BORIS*, *BRDT,* and *ATAD2*, encode nuclear proteins that have a potential impact on chromatin structures and might therefore be involved in the epigenetic alterations commonly affecting cancer genomes [90]. Altogether, these observations support the notion that the activation of several CG genes in tumors, resulting from DNA demethylation, might be associated with the acquisition of oncogenic properties.

Surprisingly, however, two independent studies indicate that *MAGEA4* displays instead tumor-suppressor functions. In one study, MAGEA4 was shown to interact with gankyrin and to inhibit anchorage-independent growth in vitro and tumor formation in mice [65]. In the other study, MAGEA4 was found to promote tumor cell death and to increase their sensitivity to apoptotic stimuli [69]. Clearly, more studies will be required before we can evaluate the full spectrum of consequences of CG gene activation in tumors.

7.7 DNA Hypomethylation in Cancer: An Immunological Paradox

There is now compelling evidence that the immune system is able to identify and destroy tumor cells [81]. This immune surveillance of cancer is believed to provide a barrier to cancer development, even though progressing tumors eventually escape

this obstacle by activating a variety of immune evasion strategies. Evidence for the existence of such surveillance of cancer by the immune system is provided for instance by the observation that solid tumors are often infiltrated by lymphocytes. Not surprisingly, several of these tumor-infiltrating lymphocytes were shown to be directed against antigens encoded by CG genes [50]. This suggests therefore that DNA hypomethylation and the consequent activation of CG genes has, at least at some stage of oncogenesis, a detrimental effect on tumor development. Yet, DNA hypomethylation is observed in most tumors, suggesting that it must otherwise have a strong tumor-promoting effect that outweighs this negative immunogenic effect.

7.8 Epigenetically Assisted Cancer Immunotherapy

Clinical trials of therapeutic vaccination of cancer patients using antigens encoded by CG genes are underway. Noticeable clinical responses were observed, albeit in only a fraction of the treated patients [11]. An interesting possibility to increase vaccination efficiencies would be the use of epigenetic drugs, such as the DNA methylation inhibitor decitabine, which should increase the number of expressed CG genes in the tumors, thereby rendering them more visible to the immune system. Importantly, decitabine is expected to induce reactivation of epigenetically silenced tumor-suppressor genes as well, and hence to reduce the growth rate of the tumors at the same time. Clinical trials combining decitabine and vaccination against antigens encoded by CG gene have been initiated [1].

There are, however, several points concerning the efficiency and safety of such approaches, which remain to be addressed. The first point concerns the specificity of decitabine-induced expression of CG genes in tumor cells rather than normal cells. Although studies have found that tumor cells are more sensitive to decitabine [47], it is obvious that the drug also induces CG genes in normal cell cultures, including fibroblasts and blood lymphocytes [25, 56, 61]. It will therefore be crucial to monitor decitabine/vaccine-treated patients for potential autoimmune reactions directed against their healthy tissues. Another concern relates to the duration of CG gene expression following decitabine treatment. Several studies have shown that CG gene expression in tumor cells was only transient following exposure to decitabine [26, 91]. This may be related to the absence of appropriate transcription factors, and hence lack of protection of the promoters against remethylation. The duration of CG gene expression in tumor cells may be critical to allow complete rejection by the immune cells. In this particular immune context, tumor cells that lose CG gene expression might be strongly selected. Prolonged decitabine treatment or combination with another epigenetic drug favoring protection of CG promoters against remethylation (e.g., drugs affecting histone marks) might be a solution to the problem. Finally, as genome hypomethylation is obviously associated with tumor development, there is a concern that decitabine treatment may generate strongly hypomethylated tumor cells with increased malignancy [33]. This is particularly problematic if it is confirmed that CG genes themselves exert oncogenic functions.

Clearly, a better understanding of the mechanisms of activation and of the biological functions of CG genes should help to resolve these questions, and may help to design the most efficient and safest ways to epigenetically augment tumor immunogenicity, thereby rendering cancer cells more vulnerable to vaccination.

References

1. Akers SN, Odunsi K, Karpf AR (2010) Regulation of cancer germline antigen gene expression: implications for cancer immunotherapy. Future Oncol 6(5):717–732
2. Almeida LG, Sakabe NJ, deOliveira AR, Silva MC, Mundstein AS, Cohen T, Chen YT, Chua R, Gurung S, Gnjatic S, Jungbluth AA, Caballero OL, Bairoch A, Kiesler E, White SL, Simpson AJ, Old LJ, Camargo AA, Vasconcelos AT (2009) CTdatabase: a knowledge-base of high-throughput and curated data on cancer-testis antigens. Nucleic Acids Res 37(Database issue):D816–819
3. Antequera F, Boyes J, Bird A (1990) High levels of de novo methylation and altered chromatin structure at CpG islands in cell lines. Cell 62(3):503–514
4. Bai S, He B, Wilson EM (2005) Melanoma antigen gene protein MAGE-11 regulates androgen receptor function by modulating the interdomain interaction. Mol Cell Biol 25(4):1238–1257
5. Baylin SB, Herman JG, Graff JR, Vertino PM, Issa JP (1998) Alterations in DNA methylation: a fundamental aspect of neoplasia. Adv Cancer Res 72:141–196
6. Bestor TH (2000) The DNA methyltransferases of mammals. Hum Mol Genet 9(16):2395–2402
7. Bird AP (1980) DNA methylation and the frequency of CpG in animal DNA. Nucleic Acids Res 8:1499–1504
8. Bird A (2002) DNA methylation patterns and epigenetic memory. Genes Dev 16(1):6–21
9. Boël P, Wildmann C, Sensi M-L, Brasseur R, Renauld J-C, Coulie P, Boon T, van der Bruggen P (1995) BAGE, a new gene encoding an antigen recognized on human melanomas by cytolytic T lymphocytes. Immunity 2:167–175
10. Boon T, Cerottini J-C, Van den Eynde B, van der Bruggen P, Van Pel A (1994) Tumor antigens recognized by T lymphocytes. Annu Rev Immunol 12:337–365
11. Boon T, Coulie PG, Van den Eynde BJ, van der Bruggen P (2006) Human T cell responses against melanoma. Annu Rev Immunol 24:175–208
12. Boyes J, Bird A (1992) Repression of genes by DNA methylation depends on CpG density and promoter strength: evidence for involvement of a methyl-CpG binding protein. EMBO J 11(1):327–333
13. Brandeis M, Frank D, Keshet I, Siegfried Z, Mendelsohn M, Nemes A, Temper V, Razin A, Cedar H (1994) Sp1 elements protect a CpG island from de novo methylation. Nature 371(6496):435–438
14. Cadieux B, Ching TT, VandenBerg SR, Costello JF (2006) Genome-wide hypomethylation in human glioblastomas associated with specific copy number alteration, methylenetetrahydrofolate reductase allele status, and increased proliferation. Cancer Res 66(17):8469–8476
15. Cedar H, Bergman Y (2009) Linking DNA methylation and histone modification: patterns and paradigms. Nat Rev Genet 10(5):295–304
16. Chen ZX, Riggs AD (2011) DNA methylation and demethylation in mammals. J Biol Chem 286(21):18347–18353
17. Cho B, Lee H, Jeong S, Bang YJ, Lee HJ, Hwang KS, Kim HY, Lee YS, Kang GH, Jeoung DI (2003) Promoter hypomethylation of a novel cancer/testis antigen gene CAGE is correlated with its aberrant expression and is seen in premalignant stage of gastric carcinoma. Biochem Biophys Res Commun 307(1):52–63

18. Cilensek ZM, Yehiely F, Kular RK, Deiss LP (2002) A member of the GAGE family of tumor antigens is an anti-apoptotic gene that confers resistance to Fas/CD95/APO-1, Interferon-gamma, taxol and gamma-irradiation. Cancer Biol Ther 1(4):380–387
19. Cross SH, Bird AP (1995) CpG islands and genes. Curr Opin Genet Dev 5:309–314
20. De Plaen E, Arden K, Traversari C, Gaforio JJ, Szikora J-P, De Smet C, Brasseur F, van der Bruggen P, Lethé B, Lurquin C, Brasseur R, Chomez P, De Backer O, Cavenee W, Boon T (1994) Structure, chromosomal localization and expression of twelve genes of the MAGE family. Immunogenetics 40:360–369
21. De Plaen E, Naerhuyzen B, De Smet C, Szikora J-P, Boon T (1997) Alternative promoters of gene MAGE4a. Genomics 40:305–313
22. De Smet C, Loriot A (2010) DNA hypomethylation in cancer: epigenetic scars of a neoplastic journey. Epigenetics 5(3):206–213
23. De Smet C, Lurquin C, van der Bruggen P, De Plaen E, Brasseur F, Boon T (1994) Sequence and expression pattern of the human MAGE2 gene. Immunogenetics 39:121–129
24. De Smet C, Courtois SJ, Faraoni I, Lurquin C, Szikora JP, De Backer O, Boon T (1995) Involvement of two Ets binding sites in the transcriptional activation of the MAGE1 gene. Immunogenetics 42(4):282–290
25. De Smet C, De Backer O, Faraoni I, Lurquin C, Brasseur F, Boon T (1996) The activation of human gene MAGE-1 in tumor cells is correlated with genome-wide demethylation. Proc Natl Acad Sci USA 93(14):7149–7153
26. De Smet C, Lurquin C, Lethé B, Martelange V, Boon T (1999) DNA methylation is the primary silencing mechanism for a set of germ line- and tumor-specific genes with a CpG-rich promoter. Mol Cell Biol 19:7327–7335
27. De Smet C, Loriot A, Boon T (2004) Promoter-dependent mechanism leading to selective hypomethylation within the 5′ region of gene MAGE-A1 in tumor cells. Mol Cell Biol 24(11):4781–4790
28. Doyle JM, Gao J, Wang J, Yang M, Potts PR (2010) MAGE-RING protein complexes comprise a family of E3 ubiquitin ligases. Mol Cell 39(6):963–974
29. Ehrlich M (2002) DNA methylation in cancer: too much, but also too little. Oncogene 21(35):5400–5413
30. Feinberg AP, Gehrke CW, Kuo KC, Ehrlich M (1988) Reduced genomic 5-methylcytosine content in human colonic neoplasia. Cancer Res 48(5):1159–1161
31. Felsenfeld G, Groudine M (2003) Controlling the double helix. Nature 421(6921):448–453
32. Flatau E, Gonzales FA, Michalowsky LA, Jones PA (1984) DNA methylation in 5-aza-2′-deoxycytidine-resistant variants of C3H 10T1/2C18 cells. Mol Cell Biol 4(10):2098–2102
33. Gaudet F, Hodgson JG, Eden A, Jackson-Grusby L, Dausman J, Gray JW, Leonhardt H, Jaenisch R (2003) Induction of tumors in mice by genomic hypomethylation. Science 300(5618):489–492
34. Gaugler B, Van den Eynde B, van der Bruggen P, Romero P, Gaforio JJ, De Plaen E, Lethé B, Brasseur F, Boon T (1994) Human gene MAGE-3 codes for an antigen recognized on a melanoma by autologous cytolytic T lymphocytes. J Exp Med 179:921–930
35. Goodyear O, Agathanggelou A, Novitzky-Basso I, Siddique S, McSkeane T, Ryan G, Vyas P, Cavenagh J, Stankovic T, Moss P, Craddock C (2010) Induction of a CD8+ T-cell response to the MAGE cancer testis antigen by combined treatment with azacitidine and sodium valproate in patients with acute myeloid leukemia and myelodysplasia. Blood 116(11):1908–1918
36. Grunwald C, Koslowski M, Arsiray T, Dhaene K, Praet M, Victor A, Morresi-Hauf A, Lindner M, Passlick B, Lehr HA, Schafer SC, Seitz G, Huber C, Sahin U, Tureci O (2006) Expression of multiple epigenetically regulated cancer/germline genes in nonsmall cell lung cancer. Int J Cancer 118(10):2522–2528
37. Haas GG Jr, D'Cruz OJ, De Bault LE (1988) Distribution of human leukocyte antigen-ABC and -D/DR antigens in the unfixed human testis. Am J Reprod Immunol Microbiol 18(2):47–51
38. Hoffmann MJ, Muller M, Engers R, Schulz WA (2006) Epigenetic control of CTCFL/BORIS and OCT4 expression in urogenital malignancies. Biochem Pharmacol 72(11):1577–1588

39. Holliday R, Pugh JE (1975) DNA modification mechanisms and gene activity during development. Science 186:226–232
40. Hong JA, Kang Y, Abdullaev Z, Flanagan PT, Pack SD, Fischette MR, Adnani MT, Loukinov DI, Vatolin S, Risinger JI, Custer M, Chen GA, Zhao M, Nguyen DM, Barrett JC, Lobanenkov VV, Schrump DS (2005) Reciprocal binding of CTCF and BORIS to the NY-ESO-1 promoter coincides with derepression of this cancer-testis gene in lung cancer cells. Cancer Res 65(17):7763–7774
41. Jackson K, Yu MC, Arakawa K, Fiala E, Youn B, Fiegl H, Muller-Holzner E, Widschwendter M, Ehrlich M (2004) DNA hypomethylation is prevalent even in low-grade breast cancers. Cancer Biol Ther 3(12):1225–1231
42. James SR, Link PA, Karpf AR (2006) Epigenetic regulation of X-linked cancer/germline antigen genes by DNMT1 and DNMT3b. Oncogene 25(52):6975–6985
43. Jones PA, Wolkowicz MJ, Rideout WM III, Gonzales FA, Marziasz CM, Coetzee GA, Tapscott SJ (1990) De novo methylation of the MyoD1 CpG island during the establishment of immortal cell lines. Proc Natl Acad Sci USA 87(16):6117–6121
44. Jungbluth AA, Chen YT, Stockert E, Busam KJ, Kolb D, Iversen K, Coplan K, Williamson B, Altorki N, Old LJ (2001) Immunohistochemical analysis of NY-ESO-1 antigen expression in normal and malignant human tissues. Int J Cancer 92(6):856–860
45. Kaneda A, Tsukamoto T, Takamura-Enya T, Watanabe N, Kaminishi M, Sugimura T, Tatematsu M, Ushijima T (2004) Frequent hypomethylation in multiple promoter CpG islands is associated with global hypomethylation, but not with frequent promoter hypermethylation. Cancer Sci 95(1):58–64
46. Kang Y, Hong JA, Chen GA, Nguyen DM, Schrump DS (2007) Dynamic transcriptional regulatory complexes including BORIS, CTCF and Sp1 modulate NY-ESO-1 expression in lung cancer cells. Oncogene 26(30):4394–4403
47. Karpf AR, Lasek AW, Ririe TO, Hanks AN, Grossman D, Jones DA (2004) Limited gene activation in tumor and normal epithelial cells treated with the DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine. Mol Pharmacol 65(1):18–27
48. Karpf AR, Bai S, James SR, Mohler JL, Wilson EM (2009) Increased expression of androgen receptor coregulator MAGE-11 in prostate cancer by DNA hypomethylation and cyclic AMP. Mol Cancer Res 7(4):523–535
49. Kholmanskikh O, Loriot A, Brasseur F, De Plaen E, De Smet C (2008) Expression of BORIS in melanoma: lack of association with MAGE-A1 activation. Int J Cancer 122(4):777–784
50. Khong HT, Wang QJ, Rosenberg SA (2004) Identification of multiple antigens recognized by tumor-infiltrating lymphocytes from a single patient: tumor escape by antigen loss and loss of MHC expression. J Immunother 27(3):184–190
51. Kondo T, Zhu X, Asa SL, Ezzat S (2007) The cancer/testis antigen melanoma-associated antigen-A3/A6 is a novel target of fibroblast growth factor receptor 2-IIIb through histone H3 modifications in thyroid cancer. Clin Cancer Res 13(16):4713–4720
52. Koslowski M, Bell C, Seitz G, Lehr HA, Roemer K, Muntefering H, Huber C, Sahin U, Tureci O (2004) Frequent nonrandom activation of germ-line genes in human cancer. Cancer Res 64(17):5988–5993
53. Lin CH, Hsieh SY, Sheen IS, Lee WC, Chen TC, Shyu WC, Liaw YF (2001) Genome-wide hypomethylation in hepatocellular carcinogenesis. Cancer Res 61(10):4238–4243
54. Link PA, Gangisetty O, James SR, Woloszynska-Read A, Tachibana M, Shinkai Y, Karpf AR (2009) Distinct roles for histone methyltransferases G9a and GLP in cancer germ-line antigen gene regulation in human cancer cells and murine embryonic stem cells. Mol Cancer Res 7(6):851–862
55. Lorincz MC, Schubeler D, Goeke SC, Walters M, Groudine M, Martin DI (2000) Dynamic analysis of proviral induction and de novo methylation: implications for a histone deacetylase-independent, methylation density-dependent mechanism of transcriptional repression. Mol Cell Biol 20(3):842–850
56. Loriot A, Boon T, De Smet C (2003) Five new human cancer-germline genes identified among 12 genes expressed in spermatogonia. Int J Cancer 105(3):371–376

57. Loriot A, De Plaen E, Boon T, De Smet C (2006) Transient down-regulation of DNMT1 methyltransferase leads to activation and stable hypomethylation of MAGE-A1 in melanoma cells. J Biol Chem 281(15):10118–10126
58. Loriot A, Sterpin C, De Backer O, De Smet C (2008) Mouse embryonic stem cells induce targeted DNA demethylation within human MAGE-A1 transgenes. Epigenetics 3(1):38–42
59. Loukinov DI, Pugacheva E, Vatolin S, Pack SD, Moon H, Chernukhin I, Mannan P, Larsson E, Kanduri C, Vostrov AA, Cui H, Niemitz EL, Rasko JE, Docquier FM, Kistler M, Breen JJ, Zhuang Z, Quitschke WW, Renkawitz R, Klenova EM, Feinberg AP, Ohlsson R, Morse HC III, Lobanenkov VV (2002) BORIS, a novel male germ-line-specific protein associated with epigenetic reprogramming events, shares the same 11-zinc-finger domain with CTCF, the insulator protein involved in reading imprinting marks in the soma. Proc Natl Acad Sci USA 99(10):6806–6811
60. Lucas S, De Smet C, Arden KC, Viars CS, Lethe B, Lurquin C, Boon T (1998) Identification of a new MAGE gene with tumor-specific expression by representational difference analysis. Cancer Res 58(4):743–752
61. Lurquin C, De Smet C, Brasseur F, Muscatelli F, Martelange V, De Plaen E, Brasseur R, Monaco AP, Boon T (1997) Two members of the human MAGEB gene family located in Xp21.3 are expressed in tumors of various histological origins. Genomics 46(3):397–408
62. Macleod D, Charlton J, Mullins J, Bird AP (1994) Sp1 sites in the mouse aprt gene promoter are required to prevent methylation of the CpG island. Genes Dev 8(19):2282–2292
63. Martelange V, De Smet C, De Plaen E, Lurquin C, Boon T (2000) Identification on a human sarcoma of two new genes with tumor-specific expression. Cancer Res 60(14):3848–3855
64. Monte M, Simonatto M, Peche LY, Bublik DR, Gobessi S, Pierotti MA, Rodolfo M, Schneider C (2006) MAGE-A tumor antigens target p53 transactivation function through histone deacetylase recruitment and confer resistance to chemotherapeutic agents. Proc Natl Acad Sci USA 103(30):11160–11165
65. Nagao T, Higashitsuji H, Nonoguchi K, Sakurai T, Dawson S, Mayer RJ, Itoh K, Fujita J (2003) MAGE-A4 interacts with the liver oncoprotein gankyrin and suppresses its tumorigenic activity. J Biol Chem 278(12):10668–10674
66. Novak P, Jensen T, Oshiro MM, Watts GS, Kim CJ, Futscher BW (2008) Agglomerative epigenetic aberrations are a common event in human breast cancer. Cancer Res 68(20):8616–8625
67. Novak P, Jensen TJ, Garbe JC, Stampfer MR, Futscher BW (2009) Stepwise DNA methylation changes are linked to escape from defined proliferation barriers and mammary epithelial cell immortalization. Cancer Res 69(12):5251–5258
68. Oikawa T, Yamada T (2003) Molecular biology of the Ets family of transcription factors. Gene 303:11–34
69. Peikert T, Specks U, Farver C, Erzurum SC, Comhair SA (2006) Melanoma antigen A4 is expressed in non-small cell lung cancers and promotes apoptosis. Cancer Res 66(9): 4693–4700
70. Rao M, Chinnasamy N, Hong JA, Zhang Y, Zhang M, Xi S, Liu F, Marquez VE, Morgan RA, Schrump DS (2011) Inhibition of histone lysine methylation enhances cancer-testis antigen expression in lung cancer cells: implications for adoptive immunotherapy of cancer. Cancer Res 71(12):4192–4204
71. Rauch TA, Zhong X, Wu X, Wang M, Kernstine KH, Wang Z, Riggs AD, Pfeifer GP (2008) High-resolution mapping of DNA hypermethylation and hypomethylation in lung cancer. Proc Natl Acad Sci USA 105(1):252–257
72. Riggs AD (1989) DNA methylation and cell memory. Cell Biophys 15(1–2):1–13
73. Sahin U, Tureci O, Schmitt H, Cochlovius B, Johannes T, Schmits R, Stenner F, Luo G, Schobert I, Pfreundschuh M (1995) Human neoplasms elicit multiple specific immune responses in the autologous host. Proc Natl Acad Sci USA 92:11810–11813
74. Sahin U, Tureci O, Chen YT, Seitz G, Villena-Heinsen C, Old LJ, Pfreundschuh M (1998) Expression of multiple cancer/testis (CT) antigens in breast cancer and melanoma: basis for polyvalent CT vaccine strategies. Int J Cancer 78(3):387–389

75. Scanlan MJ, Gordon CM, Williamson B, Lee SY, Chen YT, Stockert E, Jungbluth A, Ritter G, Jager D, Jager E, Knuth A, Old LJ (2002) Identification of cancer/testis genes by database mining and mRNA expression analysis. Int J Cancer 98(4):485–492
76. Scanlan MJ, Simpson AJ, Old LJ (2004) The cancer/testis genes: review, standardization, and commentary. Cancer Immun 4:1
77. Shen L, Kondo Y, Guo Y, Zhang J, Zhang L, Ahmed S, Shu J, Chen X, Waterland RA, Issa JP (2007) Genome-wide profiling of DNA methylation reveals a class of normally methylated CpG island promoters. PLoS Genet 3(10):2023–2036
78. Sigalotti L, Coral S, Nardi G, Spessotto A, Cortini E, Cattarossi I, Colizzi F, Altomonte M, Maio M (2002) Promoter methylation controls the expression of MAGE2, 3 and 4 genes in human cutaneous melanoma. J Immunother 25(1):16–26
79. Simpson AJ, Caballero OL, Jungbluth A, Chen YT, Old LJ (2005) Cancer/testis antigens, gametogenesis and cancer. Nat Rev Cancer 5(8):615–625
80. Suske G (1999) The Sp-family of transcription factors. Gene 238(2):291–300
81. Swann JB, Smyth MJ (2007) Immune surveillance of tumors. J Clin Invest 117(5):1137–1146
82. Takahashi K, Shichijo S, Noguchi M, Hirohata M, Itoh K (1995) Identification of MAGE-1 and MAGE-4 proteins in spermatogonia and primary spermatocytes of testis. Cancer Res 55(16):3478–3482
83. Tilman G, Loriot A, Van Beneden A, Arnoult N, Londono-Vallejo JA, De Smet C, Decottignies A (2009) Subtelomeric DNA hypomethylation is not required for telomeric sister chromatid exchanges in ALT cells. Oncogene 28(14):1682–1693
84. Van den Eynde B, Peeters O, De Backer O, Gaugler B, Lucas S, Boon T (1995) A new family of genes coding for an antigen recognized by autologous cytolytic T lymphocytes on a human melanoma. J Exp Med 182:689–698
85. van der Bruggen P, Traversari C, Chomez P, Lurquin C, De Plaen E, Van den Eynde B, Knuth A, Boon T (1991) A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. Science 254(5038):1643–1647
86. Van Der Bruggen P, Zhang Y, Chaux P, Stroobant V, Panichelli C, Schultz ES, Chapiro J, Van Den Eynde BJ, Brasseur F, Boon T (2002) Tumor-specific shared antigenic peptides recognized by human T cells. Immunol Rev 188(1):51–64
87. Vatolin S, Abdullaev Z, Pack SD, Flanagan PT, Custer M, Loukinov DI, Pugacheva E, Hong JA, Morse H III, Schrump DS, Risinger JI, Barrett JC, Lobanenkov VV (2005) Conditional expression of the CTCF-paralogous transcriptional factor BORIS in normal cells results in demethylation and derepression of MAGE-A1 and reactivation of other cancer-testis genes. Cancer Res 65(17):7751–7762
88. Walsh CP, Bestor TH (1999) Cytosine methylation and mammalian development. Genes Dev 13(1):26–34
89. Wang Z, Zhang J, Zhang Y, Lim SH (2006) SPAN-Xb expression in myeloma cells is dependent on promoter hypomethylation and can be upregulated pharmacologically. Int J Cancer 118(6):1436–1444
90. Wang J, Emadali A, Le Bescont A, Callanan M, Rousseaux S, Khochbin S (2011) Induced malignant genome reprogramming in somatic cells by testis-specific factors. Biochim Biophys Acta 1809(4–6):221–225
91. Weber J, Salgaller M, Samid D, Johnson B, Herlyn M, Lassam N, Treisman J, Rosenberg SA (1994) Expression of the MAGE-1 tumor antigen is up-regulated by the demethylating agent 5-aza-2′-deoxycytidine. Cancer Res 54(7):1766–1771
92. Weber M, Davies JJ, Wittig D, Oakeley EJ, Haase M, Lam WL, Schubeler D (2005) Chromosome-wide and promoter-specific analyses identify sites of differential DNA methylation in normal and transformed human cells. Nat Genet 37(8):853–862
93. Weber M, Hellmann I, Stadler MB, Ramos L, Paabo S, Rebhan M, Schubeler D (2007) Distribution, silencing potential and evolutionary impact of promoter DNA methylation in the human genome. Nat Genet 39(4):457–466
94. Wilson VL, Jones PA (1983) DNA methylation decreases in aging but not in immortal cells. Science 220(4601):1055–1057

95. Woloszynska-Read A, James SR, Link PA, Yu J, Odunsi K, Karpf AR (2007) DNA methylation-dependent regulation of BORIS/CTCFL expression in ovarian cancer. Cancer Immun 7:21
96. Woloszynska-Read A, Mhawech-Fauceglia P, Yu J, Odunsi K, Karpf AR (2008) Intertumor and intratumor NY-ESO-1 expression heterogeneity is associated with promoter-specific and global DNA methylation status in ovarian cancer. Clin Cancer Res 14(11):3283–3290
97. Woloszynska-Read A, James SR, Song C, Jin B, Odunsi K, Karpf AR (2010) BORIS/CTCFL expression is insufficient for cancer-germline antigen gene expression and DNA hypomethylation in ovarian cell lines. Cancer Immun 10:6
98. Yang B, O'Herrin SM, Wu J, Reagan-Shaw S, Ma Y, Bhat KM, Gravekamp C, Setaluri V, Peters N, Hoffmann FM, Peng H, Ivanov AV, Simpson AJ, Longley BJ (2007) MAGE-A, mMage-b, and MAGE-C proteins form complexes with KAP1 and suppress p53-dependent apoptosis in MAGE-positive cell lines. Cancer Res 67(20):9954–9962
99. Yang B, Wu J, Maddodi N, Ma Y, Setaluri V, Longley BJ (2007) Epigenetic control of MAGE gene expression by the KIT tyrosine kinase. J Invest Dermatol 127(9):2123–2128
100. Yegnasubramanian S, Haffner MC, Zhang Y, Gurel B, Cornish TC, Wu Z, Irizarry RA, Morgan J, Hicks J, DeWeese TL, Isaacs WB, Bova GS, De Marzo AM, Nelson WG (2008) DNA hypomethylation arises later in prostate cancer progression than CpG island hypermethylation and contributes to metastatic tumor heterogeneity. Cancer Res 68(21):8954–8967
101. Yoder JA, Walsh CP, Bestor TH (1997) Cytosine methylation and the ecology of intragenomic parasites. Trends Genet 13(8):335–340
102. Zhu X, Asa SL, Ezzat S (2008) Fibroblast growth factor 2 and estrogen control the balance of histone 3 modifications targeting MAGE-A3 in pituitary neoplasia. Clin Cancer Res 14(7):1984–1996

Chapter 8
APC and DNA Demethylation in Cell Fate Specification and Intestinal Cancer

Angela Andersen and David A. Jones

Abstract Most cases of colon cancer are initiated by mutation or loss of the tumor suppressor gene adenomatous polyposis coli (*APC*). APC controls many cellular functions including intestinal cell proliferation, differentiation, migration, and polarity. This chapter focuses on the role of APC in regulating a recently identified DNA demethylase system, consisting of a cytidine deaminase and a DNA glycosylase. A global decrease in DNA methylation is known to occur soon after loss of APC; however, how this occurs and its contribution to tumorigenesis has been unclear. In the absence of wild-type *APC*, ectopic expression of the DNA demethylase system leads to the hypomethylation of specific loci, including intestinal cell fating genes, and stabilizes intestinal cells in an undifferentiated state. Further, misregulation of this system may influence the acquisition of subsequent genetic mutations that drive tumorigenesis.

Colon cancer is the second leading cause of cancer-related death in the western world [1]. Truncating mutations in the tumor suppressor gene (TSG) adenomatous polyposis coli (*APC*) underlie 70–80% of sporadic colon cancers, and germ line mutations in *APC* cause familial adenomatous polyposis (FAP) syndrome, which inevitably leads to colon cancer unless the colon is removed [2, 3]. Mutations in *APC* are observed in early intestinal lesions including aberrant crypt foci, and their frequency is similar in benign adenomas and advanced stage carcinomas, suggesting that the loss of *APC* function initiates tumorigenesis [4]. Additional genetic and epigenetic events affect the rate of tumor progression. Changes in DNA methylation are detected in early stage adenomas, and can be classified as drivers or passengers of tumor progression, analogous to genetic mutations [5–8]. Mutations that activate

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the oncogene *KRAS* are infrequent in small polyps but are common in larger, less differentiated adenomas [9]. Loss of p53 function appears to arise even later in tumor progression and is observed mostly in carcinomas [10]. Technological advances in genome and epigenome analyses should facilitate extensive characterization of the spectrum, sequence, and interdependence of the molecular events that promote colon cancer and should also enable the development of more precise, personalized diagnoses and treatments.

8.1 Tumor Suppressor Functions of APC

A well-appreciated role for *APC* in tumor suppression is as a negative regulator of Wnt signaling [11]. In the absence of Wnt signaling, *APC* forms a destruction complex with Axin and two kinases, casein kinase 1 and glycogen syntase kinase 3β, that phosphorylate the transcriptional co-activator β-catenin. Phosphorylated β-catenin is then ubiquitinated and targeted for proteasomal degradation. Wnt signaling inhibits the formation of the destruction complex, thereby stabilizing β-catenin, which subsequently translocates to the nucleus, binds to the transcription factor TCF4, and activates target genes such as *c-myc* and *cyclin D1*. Deleterious mutations in *APC* stabilize β-catenin and are thus thought to trigger ectopic Wnt signaling. This, in turn, affects multiple cellular functions including adhesion, migration, apoptosis, and proliferation. Consistent with this model, stabilizing mutations within the gene encoding β-catenin are sufficient to initiate adenoma formation in transgenic mice and are associated with about 7% of sporadic colon cancers [12–14].

At the same time, a number of studies have suggested that loss of APC function is not sufficient to induce Wnt signaling. For example, tissues lacking functional APC do not always exhibit the predicted nuclear localization of β-catenin associated with activated Wnt signaling [15]. Blaker et al. showed that early adenomas with mild dysplasia displayed elevated levels of β-catenin in the cytoplasm but not the nucleus, whereas β-catenin was nuclear only in late stage adenomas. In addition, Anderson et al. examined grossly uninvolved and adenoma tissues taken from FAP patients and were unable to identify unambiguous staining for nuclear β-catenin in over 90% of the adenomas [16]. Recent advances suggest that Wnt signaling induces posttranslational modifications of β-catenin that regulate its subcellular localization and function as a transcriptional co-activator with TCF4. For instance, β-catenin is upregulated but confined to the cytoplasm in the intestines of homozygous *apc* mutant zebrafish (*apc*^{mcr}) embryos [17]. These mutant zebrafish display a decrease in the number of intestinal epithelial cells, consistent with reduced Wnt signaling and cell proliferation. This study showed that activation of EGF signaling was required to cooperate with loss of APC in order to stimulate nuclear translocation of β-catenin, activate Wnt signaling, and induce proliferation in *apc*^{mcr} mutant fish. The nuclear accumulation of β-catenin depended on Rac1 and Jnk2 activity, extend-

ing previous observations that these kinases are required for canonical Wnt signaling during mouse development [18]. Similarly, the detection of nuclear β-catenin in advanced human colon adenomas is coincident with increased levels of phospho-cJun, an indicator of JNK activity. Thus, loss of APC appears to stabilize β-catenin without necessarily inducing nuclear translocation and activation of target genes. In this model, aberrant Wnt/β-catenin signaling is a distinct event that contributes to tumor progression after loss of APC.

Indeed, the mechanism of tumor initiation following loss of APC activity may involve functions that are independent of β-catenin. For instance, APC binds to microtubules and regulates mitotic spindle dynamics, which in turn may influence many cellular functions, including chromosome segregation, genomic stability, and cell polarity [19–21]. APC was recently shown to promote asymmetric division of intestinal stem cells, possibly by affecting cell shape [22]. In addition, APC also acts as a positive regulator of retinoic acid (RA) biosynthesis, and, as a result, intestinal cell fate specification [23–26]. Retinoic acid is known to play important roles in controlling cell patterning, fate, and differentiation through the binding and activation of specific RA receptors, retinoid A receptors (RARα, RARβ, and RARγ), or retinoid X receptors (RXRα, RXRβ, and RXRγ) [27]. These receptors belong to the nuclear hormone receptor superfamily and are thought to act, following ligand binding, as direct activators or repressors of gene transcription [28]. A number of studies have implicated retinoids in normal colonocyte function and in the development of colon neoplasms. Compelling evidence for retinoic acid in intestinal development comes from previous studies demonstrating that retinol dehydrogenases Rdh1 and Rdh1l were essential for normal development and in intestinal differentiation in zebrafish [25, 26]. Specifically, knockdown of either Rdh1 or Rdh1l function resulted in well-known RA-deficient phenotypes including loss of pectoral fin formation, lack of jaw development, small eyes, absence of differentiated exocrine pancreas, and aberrant intestinal development. Further studies demonstrated a clear genetic connection between loss of APC and impaired retinoic acid biosynthesis. *apc*^{mcr} zebrafish lack rdhs expression and share a number of developmental phenotypes present in rdh-deficient fish. In addition, exogenous retinoic acid can improve developmental abnormalities in APC-deficient zebrafish, including failed intestinal cell differentiation. Despite the data implicating retinoic acid in intestinal cell functions, the direct functions of retinoic acid in this context remained unexplained.

8.2 Aberrant DNA Methylation Is Associated with Colon Cancer Progression

Retinoic acid induces cell differentiation of different cell types in vitro and in vivo and is thus associated with changes in DNA methylation [28–30]. About 4% of cytosines in a vertebrate genome are methylated by the action of DNA methyltransferases (Dnmt) [31]. Methylcytosine can further be converted to hydroxymethylcytosine,

formylmethylcytosine, and carboxymethylcytosine [32–34]. Methylated cytosine usually occurs at CpG dinucleotides, although significant cytosine methylation outside the CpG context is observed in embryonic stem cells and induced pluripotent cells [35]. Methylated CpG sites are enriched within repetitive sequences such as long interspersed nuclear elements (LINEs) and satellites. Dense methylation of these regions contributes to genomic stability by silencing retrotransposons and suppressing recombination. In contrast, CpG islands, which are short CpG-rich regions frequently found within promoters, tend to be unmethylated in normal tissue [36]. CpG island shores, which are regions located outside of gene promoters but within 2 kb of CpG islands, are differentially methylated in pluripotent cells, different tissues and tumors [37, 38]. Methylation of CpG islands and CpG island shores is associated with gene silencing; however, DNA methylation within gene bodies and intergenic regions has been shown to promote transcription [39]. In addition, DNA methylation was recently shown to influence alternative splicing [40]. Thus, specific patterns of DNA methylation throughout the genome regulate genomic stability and cell-type-specific gene expression.

Aberrant DNA methylation occurs soon after loss of APC, and evidence suggests that it promotes cancer progression. Widespread DNA hypomethylation, inferred from a decrease in LINE-1 methylation, is observed in small adenomas as well as late-stage carcinomas. It was recently shown that most of this hypomethylation corresponds to large, discrete blocks encompassing half the genome and consisting of repetitive sequences as well as genes [41]. Genes within these hypomethylated blocks displayed increased expression variability in different cancer samples, but were not expressed in normal samples, and it was postulated that this stochastic gene expression may contribute to tumor heterogeneity and facilitate the survival of cancer cells in different environments. Demethylation is thought to induce genomic instability by activating retrotransposons and by increasing the frequency of recombination events within repetitive heterochromatin. In addition, hypomethylation could contribute to the chromatin restructuring and nuclear disorganization associated with cancer cells. Smaller regions outside of these blocks were also differentially methylated relative to normal tissue. Hypomethylation was typically observed at CpG island shores and correlated with increased gene expression. In contrast, hypermethylation was associated with CpG islands and gene silencing. The genes that were identified as differentially methylated in colon cancer are enriched for those that are normally differentially methylated between tissues and appear to function in pluripotency, differentiation, and cell fate specification.

8.3 APC Regulates DNA Demethylation and Cell Fate Through Retinoic Acid

DNA methylation may be lost passively or actively removed. Passive demethylation occurs when unmethylated cytosine is incorporated into DNA during replication in the absence of maintenance Dnmt activity. In contrast, during active demethylation methylated cytosines are replaced with unmethylated ones by an enzymatic process

independent of DNA replication. Both mechanisms of demethylation likely contribute to the DNA hypomethylation observed in tumors. An age-dependent decrease of methylation has been observed both in normal tissues and in tumors, consistent with errors in methylated cytosine replication fidelity [42]. This passive, gradual loss of DNA methylation could facilitate tumor initiation or progression by triggering genomic instability and changes in gene expression.

Genetic mutations may also lead to aberrant DNA demethylation. Recently, it was shown that homozygous *apc*^{mcr} zebrafish embryos have reduced DNA methylation at the promoters of genes implicated in intestinal cell fate specification and colorectal cancer, such as *hoxd13a* and *pitx2* [43]. Moreover, these APC-deficient embryos had upregulated the components of a DNA demethylase system, including the cytidine deaminases Aid and Apobec2a, the thymine glycosylase Mbd4, and the DNA repair protein Gadd45α [44, 45]. Knockdown of Mbd4 or of the cytosine deaminases in *apc*^{mcr} zebrafish embryos restored methylation levels. In addition, human colon adenoma samples harboring germ line *APC* mutations also showed reduced DNA methylation at the corresponding loci and upregulation of Aid, Mbd4, and Gadd45α. Thus, APC prevents hypomethylation of key intestinal fating and colorectal cancer genes by repressing the demethylase system.

The upregulation of the demethylase system upon loss of APC was shown to be a consequence of loss of RA production, not misregulated Wnt signaling. Treatment of mutant zebrafish embryos with all-trans retinoic acid, which restores RA levels, but not a pharmacological inhibitor of Cox2, which reduces β-catenin levels downstream of activated Wnt signaling, precluded the upregulation of Aid, Mbd4, and Gadd45α. Further, pharmacological inhibition of RA production in wild-type, adult zebrafish also increased the expression of the demethylase genes and reduced cytosine methylation. Together these observations indicated that DNA demethylation and the expression of the demethylase system are regulated by RA production downstream of APC [43].

Genetic or epigenetic deregulation of genes controlling cell fate decisions can lead to tumorigenesis by precluding the differentiation of progenitor cells [43]. Indeed, DNA hypomethylation of *apc*^{mcr} zebrafish embryos is associated with an expansion of intestinal progenitor cells, revealed by the promoter demethylation and increased expression of intestinal cell fating genes and of *aldh1a2*, a marker of colon crypt progenitor cells, and by the decreased expression of a marker for intestinal differentiation, *fabp2*. Knockdown of the demethylase system components induced intestinal differentiation, indicating that hypomethylation is required to stabilize intestinal cells in a progenitor-like state. In addition, increased cell proliferation was observed in the brain of *apc*^{mcr} zebrafish embryos, and this also depended on the demethylase system. Patterning defects were excluded since the mutant embryos expressed primordial brain and intestinal markers. These data support a role for APC in cell fate specification and differentiation through the regulation of RA production and, in turn, DNA methylation. Thus, loss of APC may initiate tumorigenesis in part by hypomethylating and deregulating cell fate genes, resulting in the expansion of proliferative, progenitor-like cells.

The proposed mechanism of demethylation by this system couples enzyme-mediated deamination of methylated cytosine (me-dC), to produce thymine (dT), with

glycosylase-mediated base excision repair to replace the dG:dT mismatch with a dG:dC base pair [45]. Aid, Mbd4, and Gadd45α were shown promote demethylation of a methylated, double-stranded DNA fragment injected into wild-type zebrafish embryos, and also of bulk genomic DNA. The injected DNA fragment is not replicated, excluding a passive mechanism of demethylation arising from rounds of DNA replication without subsequent cytosine methylation of the newly synthesized strand. Further, co-expression of Aid with a catalytic mutant of Mbd4 in zebrafish embryos stabilized the dG:dT mismatches that would be generated by deamination. Indeed, Aid and a related cytosine deaminase Apobec1 have been shown to deaminate me-dC to dT within single-stranded DNA in vitro [46]. Nevertheless, the field awaits biochemical support for the proposed mechanism and insight into how Aid accesses me-dC within duplex DNA. Given that Mbd4 can recognize and extrude me-dC from duplex DNA, this component of the demethylase system could both target the deaminase to me-dC and promote substrate accessibility [47, 48]. Consistent with this model, Mbd4 was required not only for repair of the dG:dT mismatch, but also for Aid-mediated deamination of me-dC in zebrafish embryos. Moreover, Gadd45α appears to stabilize the physical interaction of Mbd4 with Aid [45]. The stable association of a deaminase with a glycosylase may be important not only for targeting demethylation but also for mediating the repair of the dG:dT intermediate.

That APC may suppress tumor formation partly through negative regulation of DNA demethylase components is consistent with previous observations. Mice carrying the APC multiple intestinal neoplasia (Apc^{min}) mutant allele, which produces truncated APC, develop intestinal lesions similar to human FAP and are frequently employed as a mouse model for colon carcinogenesis. Interestingly, genetic deletion of the cytidine deaminase Apobec1 reduced adenoma formation in $Apc^{min/+}$ mice [49]. Apobec1 is highly expressed in the small intestine and targets a number of mRNAs for C to U editing [50]. It had previously been shown that Apobec1 binds and stabilizes cyclooxygenase 2 (Cox2) mRNA in vitro [51]. Adenomas from $Apc^{min/+}$ *Apobec-1*$^{-/-}$ mice displayed decreased expression of Cox2 and it was suggested that this could account for the reduced tumor burden. This model is consistent with previous reports that Cox2 expression is increased in adenomas, and that genetic or pharmacological inhibition of Cox2 also decreases polyp formation in APC mutant mice [52]. However, Apobec1 can also deaminate DNA, and this activity may also promote tumor progression. Deamination of dC or me-dC results in transitions to dT, and Apobec-1 knockout mice would be predicted to have a reduced frequency of these mutations. This in turn could decrease polyp initiation by preventing second-hit mutations. In addition, given that components of the DNA demethylase system are ectopically expressed in the absence of APC, Apobec1 may also cooperate with a thymine glycosylase to promote DNA demethylation, altered gene expression, and the expansion of intestinal progenitor cells in $Apc^{min/+}$ mice. Thus, $Apc^{min/+}$ *Apobec-1*$^{-/-}$ mice may display reduced adenoma formation in part due to reduced transition mutations and to restored DNA methylation patterns and differentiation of intestinal progenitor cells.

In considering the development of APC loss-dependent colorectal cancer, it is plausible to envision a role for DNA demethylation given its role in reprogramming

in other systems. Genome-wide demethylation of the paternal genome in the mammalian zygote occurs within hours after fertilization [53–55]. Later in embryogenesis, during specification of mouse primordial germ cells, the cytosine methylation that underlies parental imprints is erased and pluripotency is reestablished [56, 57]. Interestingly, genome-wide bisulphite sequencing analysis revealed an increase in global DNA methylation levels in PGCs derived from Aid-null embryos relative to wild-type embryos [58]. However, significant demethylation occurred even in the absence of Aid, suggesting that this process may involve other deaminases like Apobec1 [46] or another mechanism. Similarly, reduced levels of DNA demethylation in zebrafish required simultaneous knockdown of Aid and Apobec2 [45], suggesting redundancy among members of the Aid/Apobec family. DNA demethylation is also a rate-limiting step for reprogramming somatic cells to a pluripotent state [59–61]. Indeed, Aid was required for the demethylation and induction of pluripotency genes in heterokaryons generated by fusing mouse embryonic stem (ES) cells with human fibroblasts. Importantly, Aid-mediated DNA demethylation did not require cell proliferation or DNA replication, providing further support for a role for Aid in active DNA demethylation. Prior to cell fusion, Aid is bound to distinct, methylated promoters in each cell type. For instance, Aid associates with the methylated promoters of Oct4 and Nanog in fibroblasts, but not with their unmethylated promoters in ES cells [61]. These observations suggest that cell-type-specific factors stimulate Aid's deaminase activity at methylated target loci. Thus, active DNA demethylation mechanisms employing deaminases stabilize a pluripotent state in different biological contexts.

The misregulation of the demethlyase system in APC-deficient animals may also reconcile some apparent contradictions arising from previous studies. Adenoma formation in $Apc^{min/+}$ mice is suppressed either by pharmacologic inhibition of Dnmt activity with 5-aza-deoxycytidine or by genetic loss of the DNA methyltransferase *Dnmt1* or *Dnmt3b* [62–65]. However, 5-aza-deoxycytidine did not preclude microadenoma formation, nor did it preclude adenoma progression once a polyp had formed, suggesting an irreversible event occurs prior to, and is required for, the transition to a macroadenoma. Microadenomas have lost the wild-type allele of *APC*, indicating that this step is not rate limiting for macroadenoma formation. One explanation for these findings could be that hypermethylation and silencing of TSGs is required for tumor growth, and that reducing Dnmt activity inhibits this step [66, 67]. It has been shown that the CpG islands upstream of some TSGs are methylated in some cells within the normal intestinal mucosa of $Apc^{min/+}$ mice, and that their methylation increases in polyps [62]. Genetic loss of *Dnmt1* reduced the extent of methylation at these sites in both normal mucosa and polyps, and reduced polyp formation, extending the correlation between localized methylation and tumor growth. Although these observations are consistent with a reduction in TSG expression promoting tumor progression, DNA methylation could also contribute to tumorigenesis by affecting the rate and spectrum of genetic mutations [68, 69]. Spontaneous or enzymatic deamination of me-dC yields dT, resulting in a dC to dT transition mutation if it is not repaired prior to replication. Transition mutations at CpG dinucleotides, the target for DNA methylation, contribute significantly to tumorigenesis despite the under-representation of CpG in the genome [70, 71]. Loss of *APC* could increase

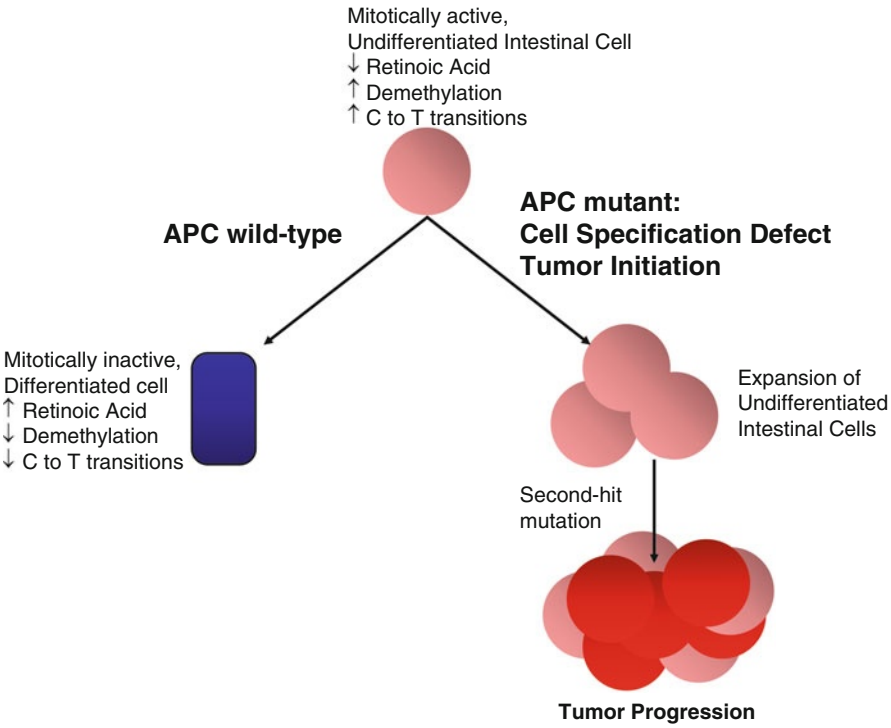

Fig. 8.1 In the intestine, APC promotes differentiation through the production of retinoic acid and the negative regulation of DNA demethylase components. In APC mutants, there is decreased retinoic acid production, maintaining cells in an undifferentiated state due to the continued expression of the demethylase system and of genes controlling cell fate and proliferation. In addition, expression of the demethylase system may promote C to T transition mutations. Both the cell specification defect and accumulation of second-hit mutations upon loss of APC may contribute to tumorigenesis

the rate of dC to dT transitions due to the upregulation of deaminases such as Aid and Apobec2 [43]. Thus, in addition to stabilizing a progenitor-like state, loss of *APC* and deregulation of the DNA demethylase system may separately contribute to tumorigenesis by increasing the likelihood of second-hit transition mutations. In this model, inhibition of Dnmt activity would suppress adenoma formation upon loss of *APC* by reducing the levels of me-dC, a substrate for deamination, which ultimately decreases the frequency of tumor-promoting dC to dT transitions. Similarly, genetic loss of Mbd4, which can repair the dT generated by deamination of me-dC, increased the rate of dC to dT transitions at CpG dinucleotides and accelerated intestinal tumorigenesis in APC mutant mice [72, 73].

The above findings support a new model linking loss of APC, impaired intestinal differentiation, and tumor initiation to RA-mediated control of DNA methylation dynamics. APC serves a critical role in cell fate specification by positive regulation of RA production and, in turn, inhibition of the DNA demethylase system (Fig. 8.1).

In the absence of APC function, there is an expansion of intestinal progenitor cells. Further, the misregulation of deaminases downstream of loss of APC may lead to an increased frequency of second-hit mutations. In this way, loss of APC may both directly and indirectly affect tumor initiation and progression.

References

1. Markowitz SD (2007) Aspirin and colon cancer–targeting prevention? N Engl J Med 356(21):2195–2198
2. Bienz M, Clevers H (2000) Linking colorectal cancer to Wnt signaling. Cell 103(2): 311–320
3. Fearon ER (2011) Molecular genetics of colorectal cancer. Annu Rev Pathol 6:479–507
4. Kinzler KW, Vogelstein B (1996) Lessons from hereditary colorectal cancer. Cell 87(2):159–170
5. Sunami E et al (2011) LINE-1 hypomethylation during primary colon cancer progression. PLoS One 6(4):e18884
6. Feinberg AP et al (1988) Reduced genomic 5-methylcytosine content in human colonic neoplasia. Cancer Res 48(5):1159–1161
7. Cravo M et al (1996) Global DNA hypomethylation occurs in the early stages of intestinal type gastric carcinoma. Gut 39(3):434–438
8. Goelz SE et al (1985) Hypomethylation of DNA from benign and malignant human colon neoplasms. Science 228(4696):187–190
9. Fearon ER, Vogelstein B (1990) A genetic model for colorectal tumorigenesis. Cell 61(5):759–767
10. Baker SJ et al (1990) p53 gene mutations occur in combination with 17p allelic deletions as late events in colorectal tumorigenesis. Cancer Res 50(23):7717–7722
11. Clevers H (2006) Wnt/beta-catenin signaling in development and disease. Cell 127(3):469–480
12. Sparks AB et al (1998) Mutational analysis of the APC/beta-catenin/Tcf pathway in colorectal cancer. Cancer Res 58(6):1130–1134
13. Morin PJ et al (1997) Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC. Science 275(5307):1787–1790
14. Romagnolo B et al (1999) Intestinal dysplasia and adenoma in transgenic mice after overexpression of an activated beta-catenin. Cancer Res 59(16):3875–3879
15. Blaker H et al (2003) Somatic mutations in familial adenomatous polyps. Nuclear translocation of beta-catenin requires more than biallelic APC inactivation. Am J Clin Pathol 120(3):418–423
16. Anderson CB, Neufeld KL, White RL (2002) Subcellular distribution of Wnt pathway proteins in normal and neoplastic colon. Proc Natl Acad Sci USA 99(13):8683–8688
17. Phelps RA et al (2009) A two-step model for colon adenoma initiation and progression caused by APC loss. Cell 137(4):623–634
18. Wu X et al (2008) Rac1 activation controls nuclear localization of beta-catenin during canonical Wnt signaling. Cell 133(2):340–353
19. Caldwell CM, Green RA, Kaplan KB (2007) APC mutations lead to cytokinetic failures in vitro and tetraploid genotypes in Min mice. J Cell Biol 178(7):1109–1120
20. Caldwell CM, Kaplan KB (2009) The role of APC in mitosis and in chromosome instability. Adv Exp Med Biol 656:51–64
21. Green RA, Wollman R, Kaplan KB (2005) APC and EB1 function together in mitosis to regulate spindle dynamics and chromosome alignment. Mol Biol Cell 16(10):4609–4622
22. Quyn AJ et al (2010) Spindle orientation bias in gut epithelial stem cell compartments is lost in precancerous tissue. Cell Stem Cell 6(2):175–181
23. Jette C et al (2004) The tumor suppressor adenomatous polyposis coli and caudal related homeodomain protein regulate expression of retinol dehydrogenase L. J Biol Chem 279(33):34397–34405

24. Nadauld LD et al (2006) Adenomatous polyposis coli control of C-terminal binding protein-1 stability regulates expression of intestinal retinol dehydrogenases. J Biol Chem 281(49): 37828–37835
25. Nadauld LD et al (2004) Adenomatous polyposis coli control of retinoic acid biosynthesis is critical for zebrafish intestinal development and differentiation. J Biol Chem 279(49): 51581–51589
26. Nadauld LD et al (2005) The zebrafish retinol dehydrogenase, rdh1l, is essential for intestinal development and is regulated by the tumor suppressor adenomatous polyposis coli. J Biol Chem 280(34):30490–30495
27. Mark M, Ghyselinck NB, Chambon P (2009) Function of retinoic acid receptors during embryonic development. Nucl Recept Signal 7:e002
28. Duester G (2008) Retinoic acid synthesis and signaling during early organogenesis. Cell 134(6):921–931
29. Deb-Rinker P et al (2005) Sequential DNA methylation of the Nanog and Oct-4 upstream regions in human NT2 cells during neuronal differentiation. J Biol Chem 280(8):6257–6260
30. Fisher CL, Fisher AG (2011) Chromatin states in pluripotent, differentiated, and reprogrammed cells. Curr Opin Genet Dev 21(2):140–146
31. Wild L, Flanagan JM (2010) Genome-wide hypomethylation in cancer may be a passive consequence of transformation. Biochim Biophys Acta 1806(1):50–57
32. Ito S et al (2011) Tet proteins can convert 5-methylcytosine to 5-formylcytosine and 5-carboxylcytosine. Science 333(6047):1300–1303
33. Kriaucionis S, Heintz N (2009) The nuclear DNA base 5-hydroxymethylcytosine is present in Purkinje neurons and the brain. Science 324(5929):929–930
34. Tahiliani M et al (2009) Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. Science 324(5929):930–935
35. Lister R et al (2009) Human DNA methylomes at base resolution show widespread epigenomic differences. Nature 462(7271):315–322
36. Deaton AM, Bird A (2011) CpG islands and the regulation of transcription. Genes Dev 25(10):1010–1022
37. Doi A et al (2009) Differential methylation of tissue- and cancer-specific CpG island shores distinguishes human induced pluripotent stem cells, embryonic stem cells and fibroblasts. Nat Genet 41(12):1350–1353
38. Irizarry RA et al (2009) The human colon cancer methylome shows similar hypo- and hypermethylation at conserved tissue-specific CpG island shores. Nat Genet 41(2):178–186
39. Wu H et al (2010) Dnmt3a-dependent nonpromoter DNA methylation facilitates transcription of neurogenic genes. Science 329(5990):444–448
40. Shukla S et al (2011) CTCF-promoted RNA polymerase II pausing links DNA methylation to splicing. Nature 479(7371):74–79
41. Hansen KD et al (2011) Increased methylation variation in epigenetic domains across cancer types. Nat Genet 43(8):768–775
42. Suzuki K et al (2006) Global DNA demethylation in gastrointestinal cancer is age dependent and precedes genomic damage. Cancer Cell 9(3):199–207
43. Rai K et al (2010) DNA demethylase activity maintains intestinal cells in an undifferentiated state following loss of APC. Cell 142(6):930–942
44. Barreto G et al (2007) Gadd45a promotes epigenetic gene activation by repair-mediated DNA demethylation. Nature 445(7128):671–675
45. Rai K et al (2008) DNA demethylation in zebrafish involves the coupling of a deaminase, a glycosylase, and gadd45. Cell 135(7):1201–1212
46. Morgan HD et al (2004) Activation-induced cytidine deaminase deaminates 5-methylcytosine in DNA and is expressed in pluripotent tissues: implications for epigenetic reprogramming. J Biol Chem 279(50):52353–52360
47. Hendrich B et al (1999) Genomic structure and chromosomal mapping of the murine and human Mbd1, Mbd2, Mbd3, and Mbd4 genes. Mamm Genome 10(9):906–912

48. Wu P et al (2003) Mismatch repair in methylated DNA. Structure and activity of the mismatch-specific thymine glycosylase domain of methyl-CpG-binding protein MBD4. J Biol Chem 278(7):5285–5291
49. Blanc V et al (2007) Deletion of the AU-rich RNA binding protein Apobec-1 reduces intestinal tumor burden in Apc(min) mice. Cancer Res 67(18):8565–8573
50. Rosenberg BR et al (2011) Transcriptome-wide sequencing reveals numerous APOBEC1 mRNA-editing targets in transcript 3′ UTRs. Nat Struct Mol Biol 18(2):230–236
51. Anant S et al (2004) Apobec-1 protects intestine from radiation injury through posttranscriptional regulation of cyclooxygenase-2 expression. Gastroenterology 127(4):1139–1149
52. Oshima M et al (1996) Suppression of intestinal polyposis in Apc delta716 knockout mice by inhibition of cyclooxygenase 2 (COX-2). Cell 87(5):803–809
53. Mayer W et al (2000) Demethylation of the zygotic paternal genome. Nature 403(6769): 501–502
54. Oswald J et al (2000) Active demethylation of the paternal genome in the mouse zygote. Curr Biol 10(8):475–478
55. Santos F et al (2002) Dynamic reprogramming of DNA methylation in the early mouse embryo. Dev Biol 241(1):172–182
56. Hajkova P et al (2002) Epigenetic reprogramming in mouse primordial germ cells. Mech Dev 117(1–2):15–23
57. Feng S, Jacobsen SE, Reik W (2010) Epigenetic reprogramming in plant and animal development. Science 330(6004):622–627
58. Popp C et al (2010) Genome-wide erasure of DNA methylation in mouse primordial germ cells is affected by AID deficiency. Nature 463(7284):1101–1105
59. Simonsson S, Gurdon J (2004) DNA demethylation is necessary for the epigenetic reprogramming of somatic cell nuclei. Nat Cell Biol 6(10):984–990
60. Mikkelsen TS et al (2008) Dissecting direct reprogramming through integrative genomic analysis. Nature 454(7200):49–55
61. Bhutani N et al (2010) Reprogramming towards pluripotency requires AID-dependent DNA demethylation. Nature 463(7284):1042–1047
62. Eads CA, Nickel AE, Laird PW (2002) Complete genetic suppression of polyp formation and reduction of CpG-island hypermethylation in Apc(Min/+) Dnmt1-hypomorphic Mice. Cancer Res 62(5):1296–1299
63. Yamada Y et al (2005) Opposing effects of DNA hypomethylation on intestinal and liver carcinogenesis. Proc Natl Acad Sci USA 102(38):13580–13585
64. Lin H et al (2006) Suppression of intestinal neoplasia by deletion of Dnmt3b. Mol Cell Biol 26(8):2976–2983
65. Laird PW et al (1995) Suppression of intestinal neoplasia by DNA hypomethylation. Cell 81(2):197–205
66. Linhart HG et al (2007) Dnmt3b promotes tumorigenesis in vivo by gene-specific de novo methylation and transcriptional silencing. Genes Dev 21(23):3110–3122
67. Eads CA et al (2000) Fields of aberrant CpG island hypermethylation in Barrett's esophagus and associated adenocarcinoma. Cancer Res 60(18):5021–5026
68. Jones PA et al (1992) Methylation, mutation and cancer. Bioessays 14(1):33–36
69. Laird PW, Jaenisch R (1994) DNA methylation and cancer. Hum Mol Genet 3 Spec No:1487–1495
70. Greenblatt MS et al (1994) Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. Cancer Res 54(18):4855–4878
71. Holliday R, Grigg GW (1993) DNA methylation and mutation. Mutat Res 285(1):61–67
72. Wong E et al (2002) Mbd4 inactivation increases Cright-arrowT transition mutations and promotes gastrointestinal tumor formation. Proc Natl Acad Sci USA 99(23):14937–14942
73. Millar CB et al (2002) Enhanced CpG mutability and tumorigenesis in MBD4-deficient mice. Science 297(5580):403–405

Chapter 9
Epigenetic Changes During Cell Transformation

Bernard W. Futscher

Abstract Malignant cancer emerges from normal healthy cells in a multistep process that involves both genetic and epigenetic lesions. Both genetic and environmental inputs participate in driving the epigenetic changes that occur during human carcinogenesis. The pathologic changes seen in DNA methylation and histone posttranslational modifications are complex, deeply intertwined, and act in concert to produce malignant transformation. To better understand the causes and consequences of the pathoepigenetic changes in cancer formation, a variety of experimentally tractable human cell line model systems that accurately reflect the molecular alterations seen in the clinical disease have been developed. Results from studies using these cell line model systems suggest that early critical epigenetic events occur in a stepwise fashion prior to cell immortalization. These epigenetic steps coincide with the cell's transition through well-defined cell proliferation barriers of stasis and telomere dysfunction. Following cell immortalization, stressors, such as environmental toxicants, can induce malignant transformation in a process in which the epigenetic changes occur in a smoother progressive fashion, in contrast to the stark stepwise epigenetic changes seen prior to cell immortalization. It is hoped that developing a clearer understanding of the identity, timing, and consequences of these epigenetic lesions will prove useful in future clinical applications that range from early disease detection to therapeutic intervention in malignant cancer.

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9.1 Introduction

Malignant cancer cells arise from normal cells via a multistep process that involves both genetic and epigenetic change. Similar to genetic lesions, epigenetic lesions can be diverse in nature, serving to alter the structure and function of the genome thereby participating in a cell's acquisition of limitless uncontrolled growth and the phenotypic hallmarks of the malignant cancer cell. In general, the degree of epigenetic difference between cancer cells and normal cells greatly exceeds the epigenetic differences that are seen between normal cells of different phenotypes and even different germ layers (e.g., fibroblasts and epithelial cells). Since epigenetic mechanisms are a primary determinant governing normal cell identity, this comparison underscores how epigenetically different cancer cells are from normal cells. Mutation and altered expression of proteins involved in the writing or reading of the epigenetic code are two mechanisms that help produce aberrant epigenetic changes seen in not only cancer, but other human diseases as well. The complexity and the frequency of the epigenetic changes seen in cancer cells, however, seem to defy explanations that rely on a single event. Instead, it appears that pathologic epigenetic change during carcinogenesis results from myriad genetic mutations and environmental inputs which perturb the manifold nodes of epigenetic regulation.

Environmental inputs acting on the epigenetic nodes are highly variable and can include contributions from both physiologic and xenobiotic sources such as hormonal status; microenvironmental milieu; nutritional, metabolic, or oxidative state; and toxicant and therapeutic drug exposures. Since the epigenetic state is important in governing cell identity, cellular nodes of epigenetic control acted upon by stimuli will show some variation between different cell types, suggesting that environmental inputs may show cell type selectivity, as well as display activity towards a broad array of cell types. Once these epigenetic changes are "fixed" into the chromatin, they can be vertically transmitted through cell generations. The inherent plasticity of the epigenetic control systems coupled to the cancer cell's limitless replicative potential provides the ability to generate extraordinary phenotypic diversity and rapidly respond to changing environmental stimuli and stresses.

Chromatin is rich in epigenetic marks, and these marks participate in the regulation and control of likely most or all genomic functions. The primary epigenetic mark found on DNA, 5-methylcytosine, is produced via the enzymatic methylation of the C5 position of cytosine through the action of multiple specialized DNA methyltransferases. The patterns and levels of DNA methylation across the genome have been mapped for a variety of normal and cancer cells, with cancer cells showing complex and extensive patterns of DNA methylation derangements. These DNA methylation derangements either participate in or reflect a number of different genomic processes, with its role in the regulation of gene expression being the best understood. Other C5 cytosine modifications have been identified recently, such as 5-hydroxymethylcytosine. It appears that these newly identified modifications are a result of an active DNA demethylation process and it is likely that these DNA epigenetic marks will prove biologically important; however, it has not yet been elucidated how these marks change and participate in the process of malignant transformation.

Posttranslational histone modifications are an additional layer of epigenetic control altered during human carcinogenesis. These posttranslational modifications include acetylation, methylation, phosphorylation, ubiquitylation, and sumoylation, and over 40 different amino acid residues in histones are currently known to undergo one or more of these modifications, especially in histones H3 and H4. Similar to DNA methylation, the histone posttranslational marks participate in a number of different genomic processes. Some histone marks are highly predictive of gene promoter location and transcriptional activity, such as histone H3K4 trimethylation and histone H3 and H4 lysine acetylation, and these modifications show strong negative correlations with DNA methylation levels in a typical genomic region. Other posttranslational histone modifications are linked to a transcriptionally repressed state and display positive correlations with DNA methylation levels, such as H3K9 methylation repressive marks. Still other histone marks, such as H3K27 trimethylation, are closely linked to transcriptional repression, preferentially target developmentally regulated genes and largely appears to be a repressive epigenetic control system that operates independently of the repressive DNA methylation system. Overall, a number of in vitro studies have provided clear mechanistic links between DNA methylation and histone modification state indicating that the control of the DNA methylation and histone modification patterns are deeply intertwined. As such, it is not surprising that, similar to DNA methylation, the normal levels and patterns of histone posttranslational modifications become compromised in human cancer cells.

In a clinical setting, the multistep nature of epithelial cell malignant transformation manifests as hyperplasia, dysplasia, benign tumor, carcinoma in situ, and finally frank malignancy and metastases; analogous pathologic progressions can be seen in some hematologic pathologies, as well, and may very well exist for most or all human cancers. Analysis of clinical specimens has shown that epigenetic aberrations are seen in the earliest stages of this multistep process, although obtaining quantitative information-rich epigenetic data from minute clinical specimens creates unique technical challenges that have slowed the ability to identify pathoepigenetic events that directly translate to clinical impact with respect to the detection, prognostication, treatment, and management of human cancer. For example, technical limitations such as specimen size and quality have hindered success in analyzing the posttranslational modification state of histones in clinical specimens. With respect to DNA methylation analysis, quantitative high resolution approaches for the analysis of the minute clinical cancer specimens typically available have been available for over 20 years in the form of bisulfite sequencing [1, 2], and today comprehensive DNA methylome sequencing approaches have emerged and should attain wide availability over the next few years [3, 4]. In the translational science arena, there are a few early applications where the results indicate DNA methylation analysis may be a useful tool in predicting response to cancer therapy [5, 6]. Results such as these should provide significant optimism and encouragement to investigators that epigenetic analysis will prove useful in the areas of prediction, detection, prognostication, as well as treatment of cancer. While significant progress has been made in understanding the causes, consequences, and temporal sequence of pathologic epigenetic events in cancer, their utility on the clinical management of cancer is largely a promissory note with their potential not yet fully realized.

9.2 Laboratory Model Systems of Cell Transformation

To better discover and understand the pathoepigenetic events that mechanistically participate in the conversion of a normal cell to a malignant cell, there is value in using experimentally tractable models systems that faithfully reflect the in vivo process. To this end, a variety of useful and complementary in vitro human cell line and animal model systems have been developed that recapitulate aspects of clinical multistep carcinogenesis and that allow for detailed analysis of epigenetic/epigenomic events as they unfold during the transformation from the normal to the malignant phenotype. These models have a number of advantages as laboratory tools—certainly the most important being that the genetic and epigenetic changes present in them accurately reflect the known (epi)genetic etiology of the clinical form of the disease, thereby providing a solid platform for the discovery and dissection of new epigenetic events relevant to clinical cancer. These cell line systems also allow for the production of pure and reproducible populations of cells that can be fairly easily generated in large number and at relatively low costs. In our experience, the epigenetic state of the cell line models we have employed does not vary to a significant extent when grown under appropriate and consistent conditions. We routinely verify cell line identity using STR profiling using 13 CODIS markers; reference DNA fingerprinting data for most of the widely used cell lines are available from cell line collections such as the ATCC or from the investigators who developed the models [7, 8].

A majority of the human cell culture model systems that have been developed perhaps best address the final step(s) of malignant human cancer, specifically the steps that follow cell immortalization. Since immortalization through telomerase activation may be a rate limiting step in human carcinogenesis, these models may not be best suited for the identification of the earliest epigenetic events in carcinogenesis. Cell model systems that adequately address the earliest steps in human carcinogenesis, prior to cell immortalization, are more limited. These are discussed later in the chapter. As is always the case, each model system used to evaluate the steps from normal finite life span cell to immortal malignant cancer cell has distinct qualities and limitations. Together, these laboratory models allow for the molecular dissection of epigenetic dysfunction during the pathologic process and help provide new insights that can be used to develop approaches to better detect, prognosticate, treat, and manage the myriad human cancers.

9.3 Immortalization to Malignant Transformation

Cell line systems that model the epigenetic events that occur following epithelial cell immortalization are widespread and provide useful tools to study malignant transformation (meant here as the in vitro assessments of anchorage independent growth and tumor forming ability in immunocompromised mice). These immortal-

ized cell line model systems have generally overcome normal cell proliferation barriers either by (1) direct immortalization of primary cell strains through overexpression of hTERT, (2) selective genetic strategies that inactivate the p16/Rb and p53 pathways, frequently via viral approaches, or (3) establishing cell lines from cultured pathologic specimens that are already immortal, but not fully malignant. A variety of immortalized variants of different epithelial cell models have been generated and examples include, but are not limited to, prostate epithelial cells immortalized by HPV18 (RWPE), bronchial epithelial cells immortalized with SV40 (HBE16, BEAS-2B), keratinocytes that arose spontaneously in culture from primary cells (HaCAT), breast epithelial cells derived from diseased tissue (MCF10A) or non-diseased healthy tissue (HMEC), and urinary bladder cells immortalized with hTERT or SV40 (UROtsa) [9–18]. Although some approaches used to immortalize cells are not themselves etiologic agents involved in clinical human carcinogenesis (e.g., viral inactivation of p53 or the genetic introduction of hTERT), they do provide reproducible approaches that target proteins and pathways known to be critical to the human tumor cell phenotype.

These immortalized cell line systems should not be considered normal cells; however, since they have had perhaps the most dramatic phenotypic shift possible—acquisition of limitless replicative potential. In addition, these cells have often also acquired genetic abnormalities (e.g., deletions, translocations, aneuploidy). It is highly likely that these immortalized cells have undergone changes in the epigenetic state, if compared to its normal finite life span counterpart, although detailed studies to this end are limited. Indeed, the p53 inactivation strategies used in immortalization strategies may instigate epigenetic change itself. Following a cellular stress, activated p53 binds to DNA in a sequence-specific manner while also recruiting coactivators or corepressors to participate in transcriptional regulation. Thus, loss of p53 binding and coactivator/corepressor recruitment may produce long-term epigenetic changes at p53 target loci disrupting their normal transcriptional regulation and altering attendant cellular phenotypes [19–21]. As such, these immortalized models likely provide more limited information regarding the nature of the epigenetic changes that may occur early in multistep carcinogenesis and prior to immortalization. Overall, these models have proven useful in identifying novel epigenetic changes, the molecular mechanisms responsible for these epigenetic changes, and the genetic and/or environmental events that provoke the epigenetic changes.

9.4 Epigenetic Remodeling by Environmental Arsenicals

Our laboratory has been interested in the effect that environmental arsenicals has on the epigenetic state. Arsenic is a widespread environmental toxicant that exists as a number of different molecular species and ranks as the 20th most common element in the earth's crust. Humans may be exposed to arsenicals to varying degrees through water, air, soil, and food. Arsenic may also be the world's most well recognized poison. Acute high dose exposure to arsenic has been used repeatedly throughout

history for murder by intentional poisoning and has earned the moniker, “Poison of Kings and King of Poisons [22].” In contrast, various forms of arsenic have also been used for centuries to treat a wide range of illnesses, including syphilis, malaria, asthma, chorea, eczema, psoriasis, and cancer [23]. Today, one molecular species of arsenic, arsenic trioxide (As_2O_3) is an FDA-approved therapy to treat acute promyelocytic leukemia and also shows promising anticancer activity in laboratory models of other human cancers [24–26]. In the most common setting, however, that of chronic low dose, environmental exposures, arsenicals are associated with a number of human maladies, among them cancer, neurologic disorders, cardiovascular disease, developmental abnormalities, and diabetes [27–30].

Of all the pathologic effects associated with long-term arsenic exposure, cancer is the most widely studied. A number of epidemiological studies have convincingly linked human arsenic exposure with various cancers, especially cancers of the lung, urinary tract, and skin [31]. Arsenicals are classified as a group 1 carcinogen by the International Agency for Research on Cancer (IARC); however, a precise mechanism of arsenical action remains wanting. A few observations suggest that epigenetic remodeling may be important in arsenical-associated cancers. Arsenicals do not appear to cause point mutations and on their own are unable to cause cancer in standard animal assays or immortalize primary human epithelial cells [32, 33]. However, earlier studies showed arsenicals can change DNA methylation levels [34], and long-term nontoxic exposure to arsenicals has been sufficient to reproducibly induce malignant transformation in a variety of immortalized nonmalignant human epithelial cells derived from tissues with known arsenical sensitivity. Examples of cell line models that have been malignantly transformed by arsenicals include HaCaT, BEAS-2B, RWPE, and UROtsa [35–39].

Human transitional carcinoma of the bladder arises from the transformation of urinary bladder epithelial cells, and those tumors that progress clinically to a malignant phenotype generally demonstrate genetic inactivation of the p16/Rb and p53 pathways [40]. In vitro, benign immortalized urothelial cell lines that resemble the earlier stages of clinical bladder cancer can be reproducibly generated from finite life span urothelial cell strains via genetic manipulations that target these pathways for inactivation. In our studies of epigenetic changes that occur during the transition from a benign immortal cell to a malignant cancer cell, we have used the immortalized, non-tumorigenic human urothelial cell line, UROtsa, generated from the urothelial cells of a young female donor and immortalized using a temperature sensitive SV40 large-T antigen construct [14]. Further evaluation of these cells has revealed hypodiploidy, genetic deletion of a small region of chromosome 9 that contains p16, and hTERT expression (unpublished observations).

Malignant transformation of UROtsa cells using long-term nontoxic exposures to environmental toxicants such as arsenic has been successfully performed by multiple independent laboratories [36, 39]. The phenotypic manifestations of the malignant conversion process can first be detected in these cells at approximately 12 weeks of exposure at a faster growth rate. With increased exposure time, the ability to form colonies in an anchorage independent fashion occurs, and finally arsenic-exposed

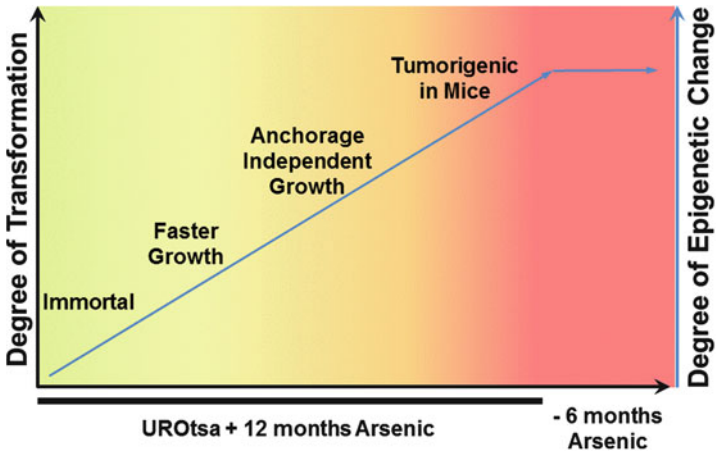

Fig. 9.1 UROtsa cell line model of malignant transformation. The immortalized urothelial cell line UROtsa was exposed to arsenicals for periods of up to a year. Arsenical exposed cells were probed at various time points for markers of malignant transformation. After 3 months there was a significant increase in proliferation rate, after 6 months a significant increase in anchorage independent growth, and after 12 months, arsenic exposed cells formed tumors in immune compromised mice [36, 39]. Progressive epigenetic changes occur during this transition from a benign immortal to malignant phenotype

UROtsa cells acquire the ability to form tumors in immunocompromised mice. Interestingly, the arsenical-induced malignant phenotype is stable, as removal of the toxicant for at least 6 months has not led to the reversion to a more benign phenotype (Fig. 9.1).

Broad epigenetic changes begin to rise in UROtsa cells during exposure to arsenic at concentrations seen in real-world situations, such as can be found in drinking water from wells (5–10 ppb). We examined epigenetic changes in a genome-wide and temporal manner using histone modification-specific chromatin and 5-methylcytosine-specific immunoprecipitations coupled to two-color DNA microarray analysis. We found global changes emerging around 12 weeks after initial exposure. These epigenetic changes appear progressive—the degree of epigenetic change increases at the individual targets with time. The epigenetic changes also are stable—after malignant transformation, the toxicant can be removed, but the malignant phenotype as well as the epigenetic changes remains. Some of the epigenetic changes identified were in genes overtly relevant to the malignant phenotype and have functional roles in cancer in general, and bladder cancer in particular [41], while the roles for most of the changes seen remain enigmatic. It appears unlikely that the observed epigenetic changes seen in UROtsa following arsenical exposure are simply due to the outgrowth or simple selection of a preexisting clone, since the arsenical-transformed cells grow significantly faster (~35%) than the non-malignant parental UROtsa cell line. Rather, it seems possible that (epi)genetic alterations may arise during and as a result of arsenic exposure, and given enough time (cell divisions), which is provided by the cell immortality, and optimal growth

conditions, a faster growing, more malignant population of cells emerges, which are then selected for based on their growth characteristics.

Probing the DNA methylation profile of the arsenical transformed UROtsa cells and comparing them to the non-transformed immortal parental cells revealed that ~3% of the assessed regions were hypermethylated, while ~1% were hypomethylated. The hypermethylation events occurred mostly within gene promoters, whereas the hypomethylation events were more prevalent in repetitive elements spread throughout the genome [42], consistent with what is well established for human cancers. We attempted to assess whether the DNA methylation changes acquired during malignant transformation were specifically or randomly distributed in the genome by analyzing two different arsenical-transformed UROtsa cell lines, created in two different laboratories using two different arsenicals (i.e., sodium arsenite and monomethyl arsenous acid). A statistical analysis of the numerical size of the overlap of aberrantly DNA methylated promoters between these two cell lines indicates that the DNA methylation changes seen are nonrandom and suggest that common epigenetic changes occur in association with arsenical malignant transformation.

The types of DNA methylation changes observed during the arsenical-mediated malignant transformation can be roughly divided into two groups, focal and long range. Focal DNA methylation events refer to DNA differentially methylated regions that cover a single gene promoter and are typically ≤1 kb in size. These types of aberrant DNA hypermethylation events seem to predominate and are closely linked to the silencing of a large number of tumor suppressor genes. In the UROtsa malignant transformation model, several potential tumor suppressor genes were found to be hypermethylated such as DBCCR1 (deleted in bladder cancer chromosome region candidate1); its relevance to bladder cancer having been previously ascertained [41]. Overall, the DNA hypermethylation changes were correlated to corresponding losses in the permissive histone modification marks of histone acetylation and H3K4 methylation and loss of gene expression, although as is often the case, apparent exceptions to the general rules could also be detected.

The DNA differentially methylated regions that cover much larger contiguous regions, along with corresponding changes in histone modifications, are linked to chromatin remodeling of more extended regions of the genome in a process termed long-range epigenetic silencing [43]. This type of epigenetic lesion has been found in a number of human cancer cell lines as well as clinical tumor specimens, suggesting that this type of coordinate epigenetic regulation over large regions may be a common and important event in cancer [43–46]. Interestingly, it appears that the gain of aberrant agglomerative DNA methylation changes and associated long-range epigenetic silencing can be observed over the time course of arsenical-mediated transformation of UROtsa from a benign to a malignant phenotype. Recent studies in the laboratory indicate that the PCDH and HOXC gene clusters undergo extensive aberrant DNA and that these epigenetic lesions are also found in malignant human bladder cancer specimens. Overall, these results suggest that the UROtsa malignant transformation model may be a laboratory tool to discern the molecular underpinnings responsible for long-range epigenetic silencing and identifies a

significant environmental toxicant as a possible etiologic agent of this pathologic epigenetic lesion.

In an initial measure evaluating the commonality of the epigenetic change in arsenical-induced malignant transformation, we sought other human epithelial cell line models of arsenical-mediated malignant transformation. The immortalized human prostate epithelial cell line RWPE-1 was shown to undergo genomic hypomethylation after chronic exposure to AsIII [47, 48], and we have made preliminary comparisons between this model and the UROtsa model. We have found a significant overlap in gene promoters targeted for aberrant DNA methylation in both the UROtsa and RWPE models of arsenical-mediated malignant transformation that is beyond what is expected by random chance. These results suggest that a common ground of epigenetic change occurs in these laboratory models of arsenical exposure and suggests that they may be useful to help identify new epigenetically targeted genes important to malignant transformation and the cellular processes responsible for these epigenetic changes.

Epigenetic regulation resides at a nexus of gene–environment interactions. Together these results suggest that environmental arsenicals may exert their carcinogenic activity by eliciting epigenetic change thereby acting as an epimutagen, an agent whose exposure induces stable and heritable changes to the epigenetic state. The epigenetic changes seen are linked to gene expression changes and coincide with the advent of an increasingly malignant phenotype. Furthermore, results from epigenome-wide analysis suggest that common regions are epigenetically targeted during arsenical-mediated malignant transformation. Importantly, the DNA methylation changes seen in the laboratory models are consistent with what is seen in the relevant in vivo correlates—clinical cancer specimens. These experimentally tractable systems provide a unique opportunity to better discern the causes and consequences of epigenetic change in arsenical-associated cancers.

9.5 Epigenetic Models of Finite Life span to Immortalization (and Beyond)

A cell model we have found particularly useful to study the epigenetics of cell transformation is the human mammary epithelial cell (HMEC) model system developed by Dr. Martha Stampfer during the past 30 years [9, 44, 49, 50]. The utility of this model system for the examination of the early molecular events in human breast carcinogenesis has been demonstrated in a number of studies, both with respect to genetic and epigenetic events [49–53]. In our estimation this isogenic cell model system offers a number of benefits and allows for the temporal analysis of molecular events that occur during the transitions from finite life span through immortalization and on to malignant transformation. This model also allows one to study the effects that directed genetic changes and environmental stressors can have on the epigenetic state.

In this model system, cultured finite life span HMEC must overcome two distinct proliferation barriers in order to achieve immortality and ultimately acquire a malignant phenotype. The first proliferation barrier is termed stasis or stress-induced senescence and is mediated by the Rb protein, characterized by elevated levels of p16INK4A. This first barrier, stasis, has been overcome or bypassed in cultured HMEC by various means, such as exposure to benzo(a)pyrene. The resultant post-stasis cells commonly show p16 inactivation by gene mutation or promoter hypermethylation [50, 54]. Loss of p16 expression due to silencing or mutation is also a common event during in vivo human breast cell transformation [55]. When grown in a serum-free medium, rare HMEC will "spontaneously" silence p16, generating a type of post-stasis HMEC population that has been called post-selection [9, 54]. HMEC that escape the stasis barrier can continue to proliferate for dozens of additional population doublings before encountering a second more stringent proliferation barrier resulting from critically shortened telomeres [49, 56]. When approaching the telomere dysfunction barrier, HMEC exhibit increased chromosomal instability and a DNA damage response. Rare cells that gain telomerase expression may escape this barrier and become immortal, whereby HMEC activates telomerase by as yet undefined, and potentially novel, epigenetic mechanisms. In addition, HMEC systems can acquire immortality through genetic perturbations. For example, under appropriate circumstances direct genetic introduction of constructs that express CMYC, or ZNF217, hTERT can promote HMEC immortalization [57, 58]. Nondirected mutagenesis can also promote HMEC immortalization, as evidenced by the effects of the complete carcinogen benzo(a)pyrene on HMEC. This limitless replicative potential allows for the acquisition and accumulation of additional epigenetic and genetic events that promote the development of additional malignant properties [50, 59–61].

We have used this HMEC model system to begin to develop a timeline of the DNA methylation changes that occurs over the course of multistep breast carcinogenesis, with a particular interest on the earliest stages of the process. Figure 9.2 shows a generalized view of cells we have analyzed, their temporal position in relation to the cellular proliferation barriers, the approximate clinical correlates, and the timing of DNA methylation changes. This figure is an example and not an exhaustive or detailed review of the HMEC strains and cell lines or the multiple treatments and exposures used to create them, and for a more detailed view one can see [62] or visit http://hmec.lbl.gov/mindex.html. In our initial studies using this model system, DNA methylation state was determined using 5-methylcytosine antibody immunopreciptations (MeDIP) coupled to two-color hybridization on a custom 13,500 element human gene promoter microarray and verified using the orthogonal technology of mass spectrometric analysis using Sequenom MassArray [63].

Overall, in this model we observed a stepwise progression of DNA methylation changes with each step coinciding with overcoming a cellular proliferation barrier [62]. In HMEC that overcame stasis produced by stress-inducing serum-free medium, we found, in addition to p16 methylation, hundreds of other differentially methylated regions in the post-stasis cells when compared to pre-stasis cells, representing approximately 2% of all gene promoters on the microarray. These DNA

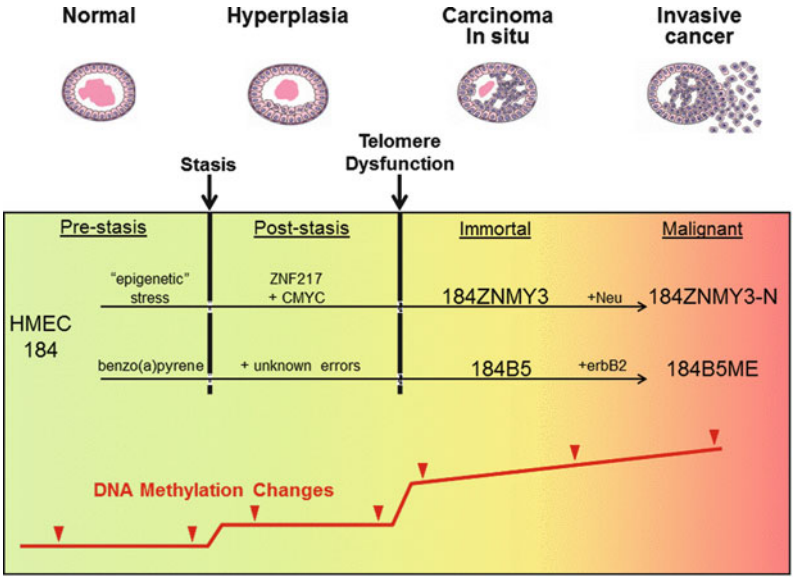

Fig. 9.2 Schematic representation of breast cancer progression and the timing of the underlying DNA methylation changes, with connections between the in vitro HMEC model system and clinical progression based on earlier work [51, 56, 65]. *Top*, the clinical correlates of the HMEC system in relation to the temporal position of the two epithelial cell proliferation barriers of stasis and telomere dysfunction that divides the timeline into pre-stasis, post-stasis, immortal, and malignant epithelial cells. *Middle*, a very simplified view and two examples of HMEC culture models, and the treatment or genetic manipulations used to generate these models. *Bottom*, the timeline of DNA methylation changes identified during the passage of finite life span HMEC through stasis, telomere dysfunction, and culminating in a malignant phenotype. *Arrows* on the DNA methylation changes *curve* show the time points analyzed for DNA methylation state

methylation events were both of the focal and long-range variety. Considering that probably 5–10% of gene promoters in malignant cancer cells show aberrant DNA methylation, a considerable number of DNA methylation changes may occur very early in multistep breast carcinogenesis, and these changes are coincident with overcoming the critical Rb/p16 cell proliferation barrier. Since a majority of the DNA methylation changes seen in the transition of HMEC from pre-stasis to post-stasis in this setting are also seen in malignant breast cancer cell lines and tumor specimens, this transition through the stasis proliferation barrier may represent a critical early event in some pathways of human breast carcinogenesis.

It is worth noting here that current commercial sources of HMEC appear to be of this post-stasis (or post-selection or variant) stage, since these HMEC are produced via the process described above—post-stasis cells that emerge from serum-free media induced stress. As such, the commercially available HMEC may have not only undergone p16 DNA methylation, but are likely to have also acquired hundreds of additional aberrant DNA methylation events [62]. As such, caution should be exercised when evaluating the epigenetic state of primary epithelial cells and considering what is epigenetically "normal."

HMEC that become post-stasis following exposure to the genotoxin and complete carcinogen benzo(a)pyrene showed more than an order of magnitude reduction in DNA differentially methylated regions when compared to the DNA methylation changes induced by stressful serum-free growth conditions. Similarly, HMEC that became post-stasis following genetic knockout of p16 using p16-targeted shRNA have very few DNA methylation changes, underscoring the functional importance of p16 in the first growth barrier. The few DNA methylation changes seen in the benzo(a)pyrene and p16 shRNA-treated cell lines suggest that different pathways through the stasis barrier will have distinct effects on the epigenetic state.

A second step of epigenetic change occurs when telomere dysfunction is overcome and cells acquire immortality. Regardless of the mechanism by which cells pass through telomere dysfunction, hundreds of DNA methylation changes occur. Similar to the DNA methylation changes acquired during the pre-stasis to post-stasis transition, changes that occur during the transition from finite life span to immortal can be focal (≤1 kb) and limited to a single gene or the changes can represent examples of long-range epigenetic silencing and cover extended regions of the genome [64].

These changes seen in the premalignant stages represented by the HMEC model show significant overlap to the DNA methylation changes seen in other human breast cancer cell lines and clinical tumor specimens. Overall, results from the studies using the HMEC model indicate that epigenetic changes occur in a stepwise fashion at critical junctions in the path to cell immortality. These results are consistent with an epigenetic progenitor model where epigenetic changes may occur early, in a stepwise fashion, can precede genetic mutation and allow for an expansion of epigenetically compromised population of cells. The large number of genes affected by epigenetic changes during the transitions through proliferation barriers can provide a foundation for the phenotypic variability and biologic heterogeneity often seen in clinical disease. The DNA methylation changes identified can potentially provide a bank of epigenetic biomarkers for assessing breast cancer risk in premalignant lesions and provide targets for therapeutic interventions.

9.6 Conclusion

In summary, complex and intertwined epigenetic changes occur during multistep carcinogenesis. These changes may be viewed as epigenetic lesions and exist in the genome in a number of forms, from focal to long range. The scope of the epigenetic lesions is likely due to multiple distinct inputs: genetic, such as mutations to chromatin modifier genes; physiologic, such as hormonal and nutritional state; and environmental, such as toxicant exposures. Experimentally tractable laboratory model systems that accurately reflect clinical cancer have been developed and allow for investigations into the causes and consequences of epigenetic change during cell transformation. Results from these systems suggest that early critical epigenetic

events occur prior to cell immortalization and coincide with the transition through well-defined barriers of cell proliferation. Following immortalization, laboratory models suggest that cells can be induced towards malignancy by a variety of stimuli, and that the epigenetic changes arise in a seemingly more progressive smoother fashion, as opposed to the stark stepwise events prior to immortalization. It is hoped that developing a clearer understanding of the identity, timing, and consequences of these epigenetic lesions will prove useful in future clinical applications that range from early disease detection to therapeutic intervention in malignant cancer.

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References

1. Clark SJ et al (1994) High sensitivity mapping of methylated cytosines. Nucleic Acids Res 22(15):2990–2997
2. Frommer M et al (1992) A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. Proc Natl Acad Sci USA 89(5):1827–1831
3. Lister R et al (2009) Human DNA methylomes at base resolution show widespread epigenomic differences. Nature 462(7271):315–322
4. Maunakea AK et al (2010) Conserved role of intragenic DNA methylation in regulating alternative promoters. Nature 466(7303):253–257
5. Drew Y et al (2011) Therapeutic potential of poly(ADP-ribose) polymerase inhibitor AG014699 in human cancers with mutated or methylated BRCA1 or BRCA2. J Natl Cancer Inst 103(4):334–346
6. Hegi ME et al (2005) MGMT gene silencing and benefit from temozolomide in glioblastoma. N Engl J Med 352(10):997–1003
7. Lorenzi PL et al (2009) DNA fingerprinting of the NCI-60 cell line panel. Mol Cancer Ther 8(4):713–724
8. Nims RW et al (2010) Short tandem repeat profiling: part of an overall strategy for reducing the frequency of cell misidentification. In Vitro Cell Dev Biol Anim 46(10):811–819
9. Hammond SL, Ham RG, Stampfer MR (1984) Serum-free growth of human mammary epithelial cells: rapid clonal growth in defined medium and extended serial passage with pituitary extract. Proc Natl Acad Sci USA 81(17):5435–5439
10. Amstad P et al (1988) Neoplastic transformation of a human bronchial epithelial cell line by a recombinant retrovirus encoding viral Harvey ras. Mol Carcinog 1(3):151–160
11. Boukamp P et al (1988) Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. J Cell Biol 106(3):761–771
12. Ke Y et al (1988) Human bronchial epithelial cells with integrated SV40 virus T antigen genes retain the ability to undergo squamous differentiation. Differentiation; research in biological diversity 38(1):60–66
13. Miller FR et al (1993) Xenograft model of progressive human proliferative breast disease. J Natl Cancer Inst 85(21):1725–1732

14. Petzoldt JL et al (1995) Immortalisation of human urothelial cells. Urol Res 23(6):377–380
15. Bello D et al (1997) Androgen responsive adult human prostatic epithelial cell lines immortalized by human papillomavirus 18. Carcinogenesis 18(6):1215–1223
16. Kiyono T et al (1998) Both Rb/p16INK4a inactivation and telomerase activity are required to immortalize human epithelial cells. Nature 396(6706):84–88
17. Dickson MA et al (2000) Human keratinocytes that express hTERT and also bypass a p16(INK4a)-enforced mechanism that limits life span become immortal yet retain normal growth and differentiation characteristics. Mol Cell Biol 20(4):1436–1447
18. Chapman E et al (2006) Expression of hTERT immortalises normal human urothelial cells without inactivation of the p16/Rb pathway. Oncogene 25(36):5037–5045
19. Chang CJ et al (2011) p53 regulates epithelial-mesenchymal transition and stem cell properties through modulating miRNAs. Nat Cell Biol 13(3):317–323
20. Junk DJ et al (2008) Different mutant/wild-type p53 combinations cause a spectrum of increased invasive potential in nonmalignant immortalized human mammary epithelial cells. Neoplasia 10(5):450–461
21. Vrba L et al (2008) p53 induces distinct epigenetic states at its direct target promoters. BMC Genomics 9:486
22. Vahidnia A, van der Voet G, de Wolff F (2007) Arsenic neurotoxicity—a review. Hum Exp Toxicol 10:823–832
23. Rohe G (1896) Arsenic. In: Foster F (ed) Reference-book of practical therapeutics. D. Appleton, New York, p 142
24. Emadi A, Gore SD (2010) Arsenic trioxide—an old drug rediscovered. Blood Rev 24(4–5):191–199
25. Wu D et al (2010) Antitumor effect and mechanisms of arsenic trioxide on subcutaneously implanted human gastric cancer in nude mice. Cancer Genet Cytogenet 2:90–96
26. Yeh K et al (2011) Tumor growth inhibition of metastatic nasopharyngeal carcinoma cell lines by low dose of arsenic trioxide via alteration of cell cycle progression and induction of apoptosis. Head Neck 5:734–742
27. Chen C et al (2007) Arsenic and diabetes and hypertension in human populations: a review. Toxicol Appl Pharmacol 3:298–304
28. Grandjean P, Murata K (2007) Developmental arsenic neurotoxicity in retrospect. Epidemiology 1:25–26
29. Smith A et al (1998) Marked increase in bladder and lung cancer mortality in a region of northern Chile due to arsenic in drinking water. Am J Epidemiol 7:660–669
30. Vahter M (2008) Health effects of early life exposure to arsenic. Basic Clin Pharmacol Toxicol 2:204–211
31. Chen C et al (1992) Cancer potential in liver, lung, bladder and kidney due to ingested inorganic arsenic in drinking water. Br J Cancer 5:888–892
32. IARC Working Group on the Evaluation of Carcinogenic Risks to Humans (2004) Some drinking-water disinfectants and contaminants, including arsenic. IARC Monogr Eval Carcinog Risks Hum 84:1–477
33. Rossman TG et al (1980) Absence of arsenite mutagenicity in *E. coli* and Chinese hamster cells. Environ Mutagen 2(3):371–379
34. Zhao C et al (1997) Association of arsenic-induced malignant transformation with DNA hypomethylation and aberrant gene expression. Proc Natl Acad Sci USA 20:10907–10912
35. Achanzar W et al (2002) Inorganic arsenite-induced malignant transformation of human prostate epithelial cells. J Natl Cancer Inst 24:1888–1891
36. Bredfeldt T et al (2006) Monomethylarsonous acid induces transformation of human bladder cells. Toxicol Appl Pharmacol 1:69–79
37. Chang Q et al (2010) Reduced reactive oxygen species-generating capacity contributes to the enhanced cell growth of arsenic-transformed epithelial cells. Cancer Res 70(12):5127–5135
38. Pi J et al (2008) Arsenic-induced malignant transformation of human keratinocytes: involvement of Nrf2. Free Radic Biol Med 45(5):651–658

39. Sens D et al (2004) Inorganic cadmium- and arsenite-induced malignant transformation of human bladder urothelial cells. Toxicol Sc 1:56–63
40. Dinney CP et al (2004) Focus on bladder cancer. Cancer Cell 6(2):111–116
41. Nishiyama H et al (2001) Negative regulation of G(1)/S transition by the candidate bladder tumour suppressor gene DBCCR1. Oncogene 23:2956–2964
42. Jensen TJ et al (2009) Arsenicals produce stable progressive changes in DNA methylation patterns that are linked to malignant transformation of immortalized urothelial cells. Toxicol Appl Pharmacol 241(2):221–229
43. Frigola J et al (2006) Epigenetic remodeling in colorectal cancer results in coordinate gene suppression across an entire chromosome band. Nat Genet 38(5):540–549
44. Novak P et al (2006) Epigenetic inactivation of the HOXA gene cluster in breast cancer. Cancer Res 66(22):10664–10670
45. Rauch T et al (2007) Homeobox gene methylation in lung cancer studied by genome-wide analysis with a microarray-based methylated CpG island recovery assay. Proc Natl Acad Sci USA 104(13):5527–5532
46. Stransky N et al (2006) Regional copy number-independent deregulation of transcription in cancer. Nat Genet 38(12):1386–1396
47. Benbrahim-Tallaa L et al (2005) Molecular events associated with arsenic-induced malignant transformation of human prostatic epithelial cells: aberrant genomic DNA methylation and K-ras oncogene activation. Toxicol Appl Pharmacol 3:288–298
48. Coppin J, Qu W, Waalkes M (2008) Interplay between cellular methyl metabolism and adaptive efflux during oncogenic transformation from chronic arsenic exposure in human cells. J Biol Chem 28:19342–19350
49. Garbe JC et al (2007) Inactivation of p53 function in cultured human mammary epithelial cells turns the telomere-length dependent senescence barrier from agonescence into crisis. Cell Cycle 6(15):1927–1936
50. Stampfer MR, Bartley JC (1985) Induction of transformation and continuous cell lines from normal human mammary epithelial cells after exposure to benzo[a]pyrene. Proc Natl Acad Sci USA 82(8):2394–2398
51. Chin K et al (2004) In situ analyses of genome instability in breast cancer. Nat Genet 36(9):984–988
52. Holst CR et al (2003) Methylation of p16(INK4a) promoters occurs in vivo in histologically normal human mammary epithelia. Cancer Res 63(7):1596–1601
53. Li Y et al (2007) Transcriptional changes associated with breast cancer occur as normal human mammary epithelial cells overcome senescence barriers and become immortalized. Mol Cancer 6:7
54. Brenner AJ, Stampfer MR, Aldaz CM (1998) Increased p16 expression with first senescence arrest in human mammary epithelial cells and extended growth capacity with p16 inactivation. Oncogene 17(2):199–205
55. Geradts J, Wilson PA (1996) High frequency of aberrant p16(INK4A) expression in human breast cancer. Am J Pathol 149(1):15–20
56. Romanov SR et al (2001) Normal human mammary epithelial cells spontaneously escape senescence and acquire genomic changes. Nature 409(6820):633–637
57. Nonet GH et al (2001) The ZNF217 gene amplified in breast cancers promotes immortalization of human mammary epithelial cells. Cancer Res 61(4):1250–1254
58. Stampfer MR et al (2001) Expression of the telomerase catalytic subunit, hTERT, induces resistance to transforming growth factor beta growth inhibition in p16INK4A(−) human mammary epithelial cells. Proc Natl Acad Sci USA 98(8):4498–4503
59. Clark R et al (1988) Transformation of human mammary epithelial cells by oncogenic retroviruses. Cancer Res 48(16):4689–4694
60. Olsen CL et al (2002) Raf-1-induced growth arrest in human mammary epithelial cells is p16-independent and is overcome in immortal cells during conversion. Oncogene 21(41): 6328–6339

61. Stampfer MR, Yaswen P (2003) Human epithelial cell immortalization as a step in carcinogenesis. Cancer Lett 194(2):199–208
62. Novak P et al (2009) Stepwise DNA methylation changes are linked to escape from defined proliferation barriers and mammary epithelial cell immortalization. Cancer Res 69(12):5251–5258
63. Ehrich M et al (2005) Quantitative high-throughput analysis of DNA methylation patterns by base-specific cleavage and mass spectrometry. Proc Natl Acad Sci USA 102(44): 15785–15790
64. Coolen MW et al (2010) Consolidation of the cancer genome into domains of repressive chromatin by long-range epigenetic silencing (LRES) reduces transcriptional plasticity. Nat Cell Biol 12(3):235–246
65. Walen KH, Stampfer MR (1989) Chromosome analyses of human mammary epithelial cells at stages of chemical-induced transformation progression to immortality. Cancer Genet Cytogenet 37(2):249–261

Chapter 10
Epigenetic Reprogramming of Mesenchymal Stem Cells

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Abstract Mesenchymal stem cells (MSCs) are multipotent stem cells of mesodermal origin that can be isolated from various sources and induced into different cell types. Although MSCs possess immune privilege and are more easily obtained than embryonic stem cells, their propensity to tumorigenesis has not been fully explored. Epigenomic changes in DNA methylation and chromatin structure have been hypothesized to be critical in the determination of lineage-specific differentiation and tumorigenesis of MSCs, but this has not been formally proven. We applied a targeted DNA methylation method to methylate a Polycomb group protein-governed gene, *Trip10*, in MSCs, which accelerated the cell fate determination of MSCs. In addition, targeted methylation of *HIC1* and *RassF1A*, both tumor suppressor genes, transformed MSCs into tumor stem cell-like cells. This new method will allow better control of the differentiation of MSCs and their use in downstream applications.

10.1 Introduction

Mesenchymal stem cells (MSCs) are somatic stem cells that can be isolated from various sources including bone marrow and fat tissue [80, 99]. Although MSCs possess more restricted pluripotency than embryonic stem (ES) cells, MSCs can still be induced to adipocytes, muscles, liver, bones, and neurons in vitro [55, 72, 73], making them a candidate for future cell therapy. From a safety consideration, there are

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debates about whether the MSCs could be transformed in vivo and whether they might be supportive or suppressive to tumoral growth [39, 88, 139]. Therefore, if the application and safety of MSCs could be monitored and well controlled, the application of MSCs will be broadened further.

Epigenetic regulation, including DNA methylation, histone modifications, and microRNAs (miRNAs), possesses the power to maintain the self-renewal or control the differentiation of stem cells [16, 32, 37, 69, 131]. Studies of ES cells have revealed the critical role of epigenetic regulation in controlling cell fate [44, 76, 107, 142]. Because there are almost no coding sequence differences between the ES cells and their derived cells, the differences between these cells are likely to come from differential gene expression [32, 47, 123]. The same rationale has prompted the use of epigenomic modifications as molecular codes to distinguish ES cells, MSCs, and their derived somatic cells. If the rationale were valid and the differences among different cell types originated from the epigenomic modifications, these distinct epigenetic states could represent the "stemness" in MSCs and ES cells, and changes of these epigenetic states might direct/interfere with the MSC differentiation.

Polycomb group proteins such as EZH2 and YY1 regulate part of the bivalent marks that represent the stemness in stem cells [119, 122]. There are loci in ES cells and MSCs associated with both active histone marks like histone 3 lysine 4 trimethylation (H3K4me3) [24, 42, 92] and repressive marks like histone 3 lysine 27 trimethylation (H3K27me3), and these are designated as bivalent loci [43, 114, 133]. These bivalent loci are often silenced [66] but are hypomethylated [134]. Among the histone marks, H3K27me3 is the substrate of Polycomb group proteins and loss of the maintenance of this histone mark is associated with the differentiation of stem cells [1, 21, 53, 70, 86]. These loci can be further activated by the association of active transcription factors and histone modifications like acetylation [61, 71, 94, 111], while their silencing could be further enhanced by DNA methylation in other lineage [5, 31, 48, 79, 87, 108, 109, 111, 118, 128, 138]. The identification of the epigenomic modifications within the bivalent loci could then reveal the ultimate fate of lineage-determining genes.

DNA methylation is one of the most dominant gene silencing mechanisms in cells and changes of methylation states correlate with the switch in cell lineages [58, 110]. It is known that changes in methylation states are inversely correlated with the expressions of corresponding genes, but the changed methylation status may not change cell fate directly. Therefore, a method that can methylate target genes and subsequently change cell fate would be an important demonstration that DNA methylation changes are sufficient to regulate cell fate decisions.

For instance, *Trip10* locus was identified as the target of Polycomb group protein and modified by DNA methylation during MSC differentiation [55]. Methylation of *Trip10* appears to be cell type specific in normal tissues as well as in cancers [55, 57]. This information suggests that *Trip10* methylation might be lineage specific and the targeted methylation of *Trip10* might then be able to direct MSC differentiation. When *Trip10* was methylated in MSCs, the MSC differentiation lineages were limited [55]. The success of the forward evaluation of the cell fate determination by

DNA methylation also opens the gateway to finetune MSC differentiation. On the other hand, the tumor suppressor genes might not have bivalent marks and methylation of these loci may lead to cell transformation. As demonstrated in a recent report, *hypermethylated in cancer* (*HIC1*) and *RassF1A* are two tumor suppressor genes that are not associated with bivalent histone marks and their methylation could transform MSCs [125].

10.2 Mesenchymal Stem Cells

MSCs can be isolated from various sources including bone marrow, adipose tissue, liver, muscle, amniotic fluid, dental pulp, placenta, and umbilical cord blood; the properties among these MSCs seem to vary accordingly [9, 49, 82, 100, 105, 113, 120, 143]. Different cell surface markers identified from different MSCs are reflective of their propensity to differentiate into different cell lineages [19, 34, 98]. Because MSCs can differentiate into different cell types in vitro, it is believed that there are common gene expression repertoires among these MSCs to maintain their stemness, but there are also different gene expression signatures that define the identities and differentiation potentials of different MSCs. Thus understanding the molecular mechanisms underlying the maintenance of cellular identities and determination of cell lineages is critical for the future clinical use of MSCs.

Unregulated differentiation is another reason to decipher the molecular codes that characterize MSCs. Different routes of transplantation make isolated clones of MSCs possess varied degrees of differentiation capacities, and dysregulation of these processes might consequently lead to disease. For example, MSCs together with or without hematopoietic stem cells (HSCs) isolated from bone marrow can be transplanted and differentiated into lung, gut, skin [68], liver and biliary epithelium [68, 97, 126, 127], skeletal myoblast [41, 46], neuroectodermal cells [18, 106], and endothelium [4, 77, 144]. These co-transplantation results imply that there are molecular machineries that might be used to define the MSCs and their derived lineages. These molecular codes also respond to neighboring cells and/or microenvironment of MSCs to maintain or differentiate cell fates. The importance of interplay with the environment is also evident by the reports that MSCs can either inhibit or support tumor growth in a cell setting-specific manner [67, 116]. The other controversy is that MSCs are proposed to both boost the immune system and suppress it [105, 129]; thus, the clinical safety of MSCs remains to be clarified.

10.3 Epigenetic Regulation and the Maintenance of MSC

Stemness needs to be maintained when the stem cells are self-renewing [50, 109, 121]. Since the coding sequences are all the same within ES cells, MSCs, and the differentiated somatic cells, there ought to be other somatic inheritable marks that

could represent the maintenance of stemness. Epigenetic marks are somatically inheritable modifications that regulate gene expression but do not change the associated gene sequence. These cellular epigenetic marks, while they can be reshaped by the environmental factors like diet and growth factors, in general are faithfully passed on to the descended lineage of cells. These properties make the epigenomic marks good candidates for the control of cellular stemness.

Bivalent loci in the stem cells are associated with both active and repressive epigenetic marks and are critical for cellular differentiation [6, 16, 32, 91]. Interactions between different epigenetic modifications can lead the governed genes to become permanently silenced or activated. The Polycomb group proteins, and associated histone modifications like H3K27me3, are one of the representative markers that are associated with stemness [20, 28, 45, 101]. Polycomb group proteins are reported to mediate the transition between the transcriptional silencing and active states of the associated gene [95] and their transitional regulatory role is evidenced by the co-existence of enhancer and suppressor genetic modifier phenotypes when the Polycomb group proteins lost their functions [85]. H3K27me3-associated loci can be further silenced by other epigenetic modifications including DNA methylation and the formation of heterochromatin [6, 62, 84, 137, 141]. On the other hand, the repressive trimethylation can be demethylated and the associated genes can then be reactivated. Loss of maintenance of these trimethylation states leads to differentiation of stem cells, which strongly suggests that maintaining these bivalent marks is critical for the maintenance of stemness [1, 21, 53, 70, 86].

Bivalent loci have been profiled in ES cells, tumors, differentiated cells, and MSCs [6]. Because the identified bivalent loci are different among these cells, the data support the hypothesis that these bivalent loci represent the unique stemness state in different cell types. From a direct comparison, there are more shared bivalent marks between ES cells and tumors than between the differentiated tissues and tumors, suggesting that tumors might be evolved from cells with more stem-like marks, and inappropriate maintenance of these marks could cause devious cell fate changes [23, 96, 132].

The bivalent loci in MSCs also mark developmentally important genes and can be further modified epigenetically [55]. The epigenetic marks on the MSC bivalent loci are distinct from those in the ES cells and differentiated cells. The bivalent loci that reside within the MSCs are often low in DNA methylation (hypomethylated) and can be further methylated or activated. The number and function of these bivalent genes might limit the lineages into which the MSCs can differentiate. It has been reported that undifferentiated MSCs contain both repressive and active chromatin marks on β-catenin-bound *c-myc* and *cyclin D* promoters [15, 35, 36]. When these MSCs became lineage committed, e.g., osteogenic, H3K4me3 was lost. This example indicates that epigenetic modifications regulate the Wnt signaling pathway in MSC, and similar epigenetic modifications are found in ES cells as well. We identified the H3K27me3-associated loci in MSCs that are differentially methylated when the MSCs are differentially induced into hepatocytes or adipocytes [55]. Loci that are not associated with DNA methylation association protein, MeCP2, were

considered hypomethylated. We found more than 383 of these bivalent loci are further associated with MeCP2 and proved to be methylated in either MSC-derived hepatocytes or adipocytes [55]. Therefore, these bivalent loci in MSCs might mark the lineages into which the MSCs are differentiated, and the later-added DNA methylation might further strengthen the cell fate evolution.

10.4 DNA Methylation and the Differentiation of MSC

DNA methylation is one of the most dominant silencing epigenetic modifications and occurs at the CpG dinucleotide in the human genome. A high frequency of CpG dinucleotides is often found at the promoter and/or first exon of genes and are named CpG islands [10, 12, 33]. Up to now, almost all the identified DNA methylation at the CpG islands silence the associated genes [11, 13, 124]. DNA methylation is a reversible event [8, 29, 60], and the removal of the silencing mark is critical for the activation of the associated genes [74, 75]. Compared with histone deacetylation inhibitors that cause less significant gene activation, demethylation induced by 5-aza-2′-deoxycytidine (5-Aza), a DNA methylation inhibitor, often causes a greater extent of restoration of gene expression [22]. Our previous results also indicated that when the estrogen receptor (ER)-targeted genes were silenced long term by DNA methylation, adding estrogen and/or overexpression of ER was insufficient to reactivate the ER target genes. Only after the DNA methylation was removed, could the expression of ER target genes be restored by the stimuli of estrogen [75]. Also, global demethylation results in global reactivation of the expression of these genes [74]. These observations all indicate that DNA methylation is a dominant silencing mark; its appearance leads to the silenced locus and the changes in methylation states reflect the changes in cellular physiology.

Altered DNA methylation status often correlates with the normal differentiation or the onset of diseases like cancer. DNA methylation is now considered a reliable biomarker and the profiling of methylation changes can be used to probe cellular or pathological events. Environmental factors relay their influence into the cells through specific signaling pathways. These influences are then recorded as epigenetic marks like DNA methylation during cell passages and are further selected in the descended population of cells. For example, when ER was knocked down by siRNA in a breast cancer cell line that once expressed ER, the downstream ER target/regulated genes were silenced gradually by various epigenetic marks, and later by DNA methylation [75]. DNA methylation also was accumulated within the ER target loci when the ER-expressing breast cancer cells were cultured long term in an estrogen-deprived environment. The recruitment and accumulation of DNA methylation within the estrogen signaling pathway left specific marks for us to track cell lineage which previously encountered the changed cellular environment. Evidence from genetic models also indicates that the environmental factors work through different signaling pathways and leave different but traceable patterns of DNA methylation. When signals like MYC or P53 were genetically manipulated, specific sets

of genes were methylated in the descended mice [93]. Therefore, the accumulated DNA methylation does not appear to occur at random.

Methylation changes caused by environmental changes like diet can be inherited and may influence cellular physiology as well as the onset of disease. The cofactor for DNA methylation reactions, *S*-adenosyl-methionine (SAM), is produced from dietary folate, and this provides the opportunity for diet to influence DNA methylation [25, 65, 104, 115, 117]. Mammals go through two genomic methylation revolutions during their development: one is during their formation of gametes, the other is directly after the fertilization is complete [64, 102, 112]. DNA methylation is erased during these two stages and re-established according to their paternal or maternal origins [136]. An elegant experiment in which pregnant mice were fed with various concentrations of food that could be converted into corresponding concentrations of SAM caused varied degrees of methylation. The newborn mice showed different degrees of fur color according to the concentration of methyl-supplemented diet consumed by the mothers, and these patterns of color lasted throughout their lives [38, 83, 89, 135]. In this example, environmental factors influenced methylation memories and changed the phenotype of the individuals in a somatically heritable way.

There is evidence indicating that changes in DNA methylation might be involved with the cell fate changes in MSCs as well. The methylation states within somatic stem/progenitor cells are different from the ones in ES cells and differentiated cells. For example, the promoter regions of *OCT4*, *NANOG,* and *SOX2* in adipose-derived MSCs display a greater extent of DNA methylation than in ES cells [6]. This methylation difference also provides an explanation for the fact that MSCs have lower differentiation capacity than the ES cells. Also, there are methylation differences within the promoters of tissue-specific genes between the bone- and adipose-derived MSCs; they correlate with their differences in lineage differentiation potential [63]. Osteoblast-specific genes such as *RUNX2* and *BGLAP* are hypermethylated in adipose-derived MSCs as compared to the bone-derived MSCs, whereas *PPARγ2*, the adipocyte-specific gene, is hypomethylated in adipose-derived MSCs [63]. Our previous data also identified a panel of genes that are differentially methylated within the differentiated hepatocytes or adipocytes when compared to the bone marrow-derived MSCs [55]. Taken together, DNA methylation status could represent the cellular identities and differentiation potentials of MSCs. It has been reported that global DNA methylation was changed in long-term cultured MSCs that might correlate with their altered differentiation capacity [17]. Changes in global methylation caused by demethylation agents have been documented to accelerate the osteogenic [3] or neuronal cell-like [2] differentiation of MSCs. However, it is unclear whether DNA methylation changes are sufficient to set the stage for MSC cell fate changes. It has been reported that predeposited DNA methylation within different isolated MSCs defined the oncogenic SYT-SSX1 fusion protein expression and limited its function in MSCs [30]. On the other hand, methylation profiling of adipogenic promoters from freshly cultured adipose stem cells to the senescence state did not correlate with their reduced differentiation potential [90, 91]. The absence of a targeted methylation method has hindered our understanding of how DNA methylation

determines the cell fate of MSCs. A solution is to find a way to methylate a bivalent gene in MSC and observe if the cell fate changed after targeting.

10.5 TRIP10 as a Model

Trip10 (also known as *CIP4*) encodes Cdc42-interacting protein 4, which was identified to be associated with Cdc42 and to regulate the cytoskeleton and membrane trafficking. Trip10 interacts with the Rho family GTPase TC-10 in adipocytes to regulate the translocation of insulin-stimulated glucose transporter 4 (Glu4) to the plasma membrane and finally to increase the uptake of glucose [26, 81]. In the brain of human Huntington's disease (HD) [52], Trip10 is reported to be a modulator of cell survival in the adjustment of DNA damage [140]. To guard against DNA damage, *Trip10* expression is decreased in hepatocyte growth factor/scatter factor (HGF/SF)-mediated cell protection, but *Trip10* level is significantly increased during hyperbaric oxygen-induced neuroprotection [51]. Overexpression of Trip10 was also observed in human HD brain striatum and the neuronal Trip10 immunoreactivity increased with neuropathological severity in the neostriatum of HD patients [52]. In addition, increased cell death was found in rat striatal neurons transfected with Trip10 [52], suggesting that Trip10 is toxic to striatal neurons. These data suggest that the effect of Trip10 in cell survival and growth is tissue specific. These diverse and sometimes contrary roles of Trip10 could be attributed in part to its splicing variants; equally important is the fact that they are the outcomes between Trip10 interaction with distinct signaling components in different cell settings.

In human bone marrow-derived MSCs, *Trip10* is hypomethylated in the undifferentiated stage and becomes hypermethylated during MSC-to-liver differentiation, but remains hypomethylated during MSC-to-adipocyte differentiation. Therefore, the methylation state of *Trip10* varies in different tissues and becomes a candidate biomarker to track MSC differentiation [55, 57]. We reasoned that the stemness state of Trip10 is maintained by the Polycomb group protein in the MSCs and that changes of chromatin structure, especially by DNA methylation, could restrict the cell lineages of MSCs. The differentiation or death of MSCs was thus predicted to be affected by *Trip10* methylation, and this model could be tested using targeted *Trip10* methylation.

10.6 Targeted DNA Methylation and MSC Differentiation

It has been hypothesized that DNA methylation within certain loci is sufficient to transform or differentiate cells, but this hypothesis had not been proved since there was no method to directly methylate specific loci [54]. Normal or abnormal methylation changes have been identified during cellular differentiation or transformation, but it remains to be elucidated whether all or any of the detected methylation changes

can affect cell fate. Moreover, if we can determine whether DNA methylation within certain loci is sufficient to determine the cell fate, this will provide additional information to evaluate the target genes that control cellular differentiation and transformation.

DNA methylation is initiated and maintained by DNA methyltransferase (DNMT) in mammalian cells [27, 40, 130]. As illustrated in Fig. 10.1a, during the cellular replication, DNMTs are recruited by the semimethylated old template and methylate the newly synthesized strand of DNAs [103]. The newly synthesized strand will then possess the same DNA methylation as the old strand. We reasoned that, by providing a methylated strand of DNA that is complementary with target loci, we might be able to recruit DNMT to the target loci and initiate targeted DNA methylation in the cell (Fig. 10.1b, [55, 56, 78]). A stretch of cloned *Trip10* promoter was in vitro methylated using commercial bacterial methylase, *Sss*I. These methylated inserts were then purified, denatured, and used to transfect MSCs. Unmethylated inserts served as the negative control; they did not induce any methylation at the *Trip10* promoter. Liposome-based transfection agents that were conjugated with florescent compounds were used for transfection in order to calculate the transfection efficiency. Also, the methylated/unmethylated inserts were labeled with Cy-dyes to track if the inserts entered the cell nuclei, because the denatured inserts need to be present and docked in the nuclei for the recruitment of DNMTs. Repeated transfection was needed to ensure the targeted DNA methylation. The promoter insert from another gene like *Casp8AP2* was used as a specificity control, as the methylated *Casp8AP2* inserts did not induce methylation at the *Trip10* promoter [55].

Targeted *Trip10* methylation was detected by semiquantitative methylation-specific PCR and bisulfite sequencing and the reduced *Trip10* expression was determined by RT-PCR and visualized by immunostaining. A two-component reporter gene system was established to validate the methylation-induced silencing at the transcription level and visualize the onset of DNA methylation in live cells. The two-component reporter system [55, 56] consists of two parts: (1) a cloned *Trip10* promoter that is linked with and regulates the expression of the *Tet* repressor (*TetR*) gene; and (2) a CMV promoter that is linked with, and regulates the expression of, the reporter gene enhanced green florescent protein (*EGFP*), with an intervening TetR binding site, $TetO_2$. Both constructs were transfected into a cell line simultaneously and colonies of cells that possess both inserted constructs were selected. Colonies of selected cells were then transfected with in vitro methylated or unmethylated *Trip10* inserts. The unmethylated *Trip10* promoter within the first construct will continue to express *TetR* that in turn represses the expression of *EGFP*. In contrast, targeted DNA methylation at the exogenous *Trip10* promoter silences the *TetR* expression which leads to the expression of the EGFP reporter. This induced EGFP expression could be reversed by adding of 5-Aza, suggesting that the original expression was caused by DNA methylation. With this reporter system, the targeted DNA methylation can be visualized in live cells.

During neuronal induction of MSCs, *Trip10* expression was greatly reduced and its distribution was confined to the peri-nuclei region in these induced cells [57]. Similar to the neuronal induction, targeted *Trip10* DNA methylation caused reduced *Trip10* expression and re-distribution and prompted the MSC-to-neuron

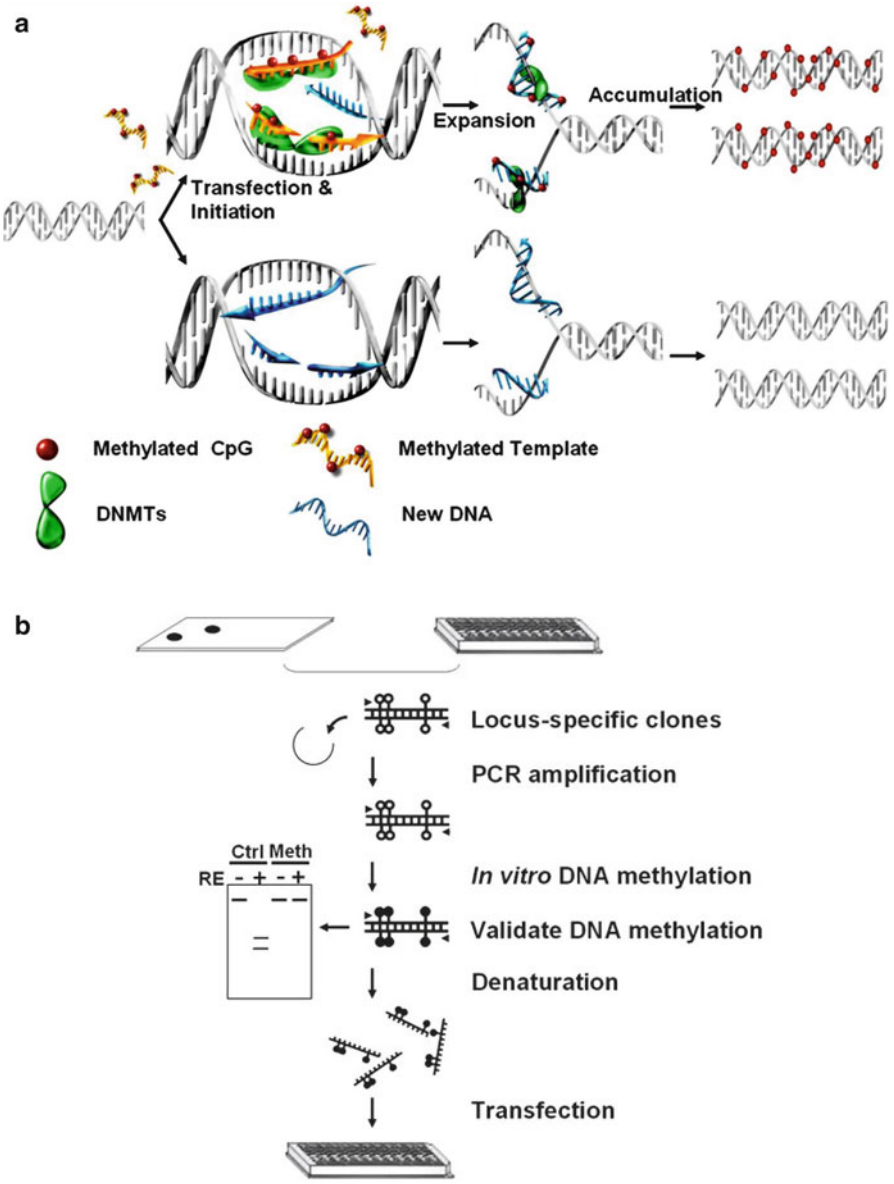

Fig. 10.1 Targeted DNA methylation. (**a**) Illustration of targeted DNA methylation. DNA methylation is maintained by DNMTs during cellular replication. *Upper*, the original unmethylated locus like *Trip10* will not recruit DNMTs to the newly synthesized strands of DNA; therefore, they remain hypomethylated. If the original strand was methylated, then the old template of DNA will recruit DNMTs to the newly synthesizing DNAs and add the methyl group to the new strand of DNAs. Targeted DNA methylation method transfects the cells with a denatured, in vitro methylated DNA with its sequence complemented to the target loci (*upper*). The provided methylated DNAs will pair with the old templates and recruit DNMTs to the newly synthesizing sites and methylate the new strands of DNAs. The seeded DNA methylation then will be spread and maintained during the following replications. (**b**) Flow of targeted DNA methylation (details in text). Templates of targeted DNA methylation could be from the CpG island library or cloned from the cultured cells. RE: methylation sensitive restriction enzymes like *Hpa*II and *BstU*I, etc

differentiation. This preferential cellular differentiation is specific since the same *Trip10* targeted DNA methylation prevented the MSC-to-adipocyte induction (Fig. 10.2a) [55]. These data indicate that DNA methylation within one of the bivalent loci is sufficient to control cellular differentiation.

10.7 DNA Methylation and Tumorigenesis of MSC

It is generally accepted that abnormal hypermethylation of tumor suppressor genes can transform normal cells [7, 12]. To support this theory, *HIC1* and *RassF1A*, two tumor suppressor genes that are methylated in several cancers but are not associated with Polycomb group protein in MSC, were in vitro methylated and then transfected into MSCs. Targeted methylation of *HIC1* or *RassF1A* alone is insufficient to transform the MSCs but concurrent *HIC1* and *RassF1A* methylation transforms the MSCs [125]. However, methylation of nine genes within the Salvador–Warts–Hippo pathway (including *RassF1A*) is insufficient to transform the MSCs [125], indicating that the *HIC1* and *RassF1A* methylation-caused transformation is not random. The transformed MSCs (named *me-H&R* MSCs) can still be differentiated into different cells including osteocytes, neurons, and adipocytes. Immunodeficient mice inoculated with a low number of *me-H&R* MSCs rapidly developed tumors. The developed tumors consisted of several clones of cells that express different cell surface markers, including mesenchymal and epithelial ones. 5-Aza treatment reversed the transformation and the tumoral properties of *me-H&R* MSCs, demonstrating that the transformation was caused by DNA methylation. Taken together, these findings suggest that the *me-H&R* MSCs become cancer stem cell (CSC)-like since they possess both tumoral and stem cell characters [125]. These results also imply that mal-maintained DNA methylation directly contributes to tumorigenesis.

10.8 Application of the Targeted DNA Methylation Technique

Epigenomic profiling in diverse cells including MSCs has revealed many cellular physiologies that are versatile and even personal [14, 17, 55, 59, 116]. Targeted DNA methylation is a direct validation of the profiling results and proves that epigenetic changes like DNA methylation are sufficient to direct MSC differentiation and tumorigenesis. As illustrated in Fig. 10.2a, MSCs could be differentiated into osteocyte, adipocyte, neuron, etc. Targeted *Trip10* methylation limits the differentiation potency of MSCs and accelerates their neural and osteogenic differentiation. On the other hand, targeted *HIC1* and *RassF1A* methylation transforms MSCs into CSC-like cells; targeted DNA methylation within nine loci in the Salvador–Warts–Hippo pathway cannot transform the MSCs but can keep the MSCs proliferating. These results indicate that CSC-like cells might arise from somatic stem cells-like MSCs (Fig. 10.2b), and the tumorigenesis and the immortalization could be dissected by the epigenetic modifications. In summary, using targeted DNA methylation, the differentiation

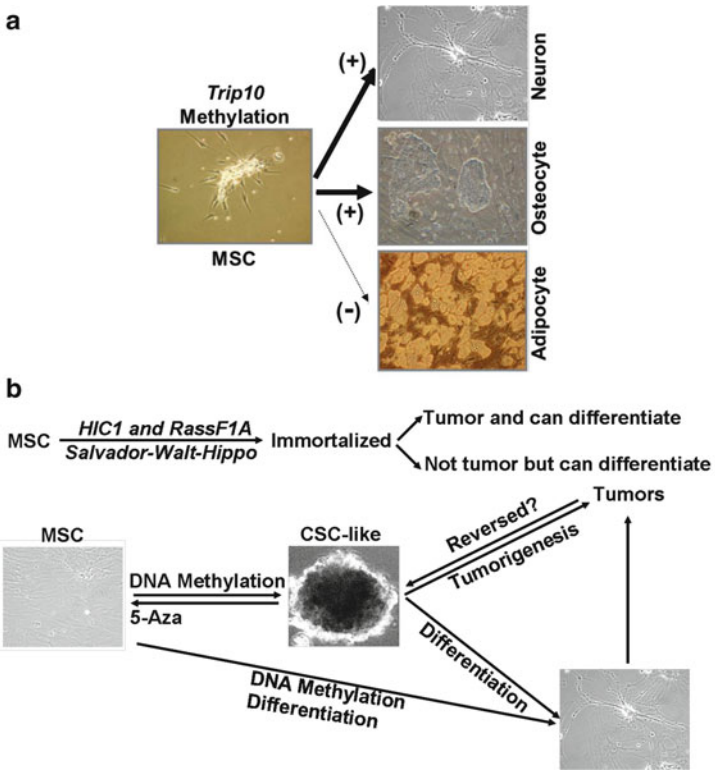

Fig. 10.2 Reprogramming of MSC. (**a**) *Trip10* methylation accelerates MSCs differentiation. Targeted DNA methylation within the *Trip10* promoter accelerates the MSCs to neuron or osteocyte differentiation but blocks their differentiation into adipocytes. (**b**) Summary of MSCs reprogramming. After methylation within the *HIC1* and *RassF1A*, the MSCs became tumors and still can differentiate. After the targeted methylation of nine genes in the Salvador–Warts–Hippo signaling pathway, the MSCs can be stably passaged and differentiated but they are not tumorigenic (*top*). Targeted methylation of *HIC1* and *RassF1A* caused the MSCs to become CSC-like (*bottom*) and this process can be reversed by 5-Aza. The CSC-like MSCs can be further developed into tumor in immunodeficient mice or differentiated into different cells like neurons. Thus, the differentiation, proliferation, and tumorigenic state of MSCs can be controlled by DNA methylation

(*Trip10*), proliferation (Salvador–Warts–Hippo), and tumorigenic (*HIC1* and *RassF1A*) characteristics of MSCs could be revealed.

References

1. Agger K, Cloos PA et al (2007) UTX and JMJD3 are histone H3K27 demethylases involved in HOX gene regulation and development. Nature 449:731–734
2. Alexanian AR (2007) Epigenetic modifiers promote efficient generation of neural-like cells from bone marrow-derived mesenchymal cells grown in neural environment. J Cell Biochem 100:362–371

3. Arnsdorf EJ, Tummala P et al (2010) The epigenetic mechanism of mechanically induced osteogenic differentiation. J Biomech 43:2881–2886
4. Asahara T, Masuda H et al (1999) Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. Circ Res 85:221–228
5. Balch C, Nephew KP et al (2007) Epigenetic "bivalently marked" process of cancer stem cell-driven tumorigenesis. Bioessays 29:842–845
6. Barrand S, Andersen IS et al (2010) Promoter-exon relationship of H3 lysine 9, 27, 36 and 79 methylation on pluripotency-associated genes. Biochem Biophys Res Commun 401:611–617
7. Baylin SB (2005) DNA methylation and gene silencing in cancer. Nat Clin Pract Oncol 2:S4–S11
8. Bender CM, Pao MM et al (1998) Inhibition of DNA methylation by 5-aza-2′-deoxycytidine suppresses the growth of human tumor cell lines. Cancer Res 58:95–101
9. Bianco P, Gehron Robey P (2000) Marrow stromal stem cells. J Clin Invest 105:1663–1668
10. Bird AP (1980) DNA methylation and the frequency of CpG in animal DNA. Nucleic Acids Res 8:1499–1504
11. Bird A (1999) DNA methylation de novo. Science 286:2287–2288
12. Bird A (2002) DNA methylation patterns and epigenetic memory. Genes Dev 16:6–21
13. Bird A, Macleod D (2004) Reading the DNA methylation signal. Cold Spring Harb Symp Quant Biol 69:113–118
14. Bloushtain-Qimron N, Yao J et al (2009) Epigenetic patterns of embryonic and adult stem cells. Cell Cycle 8:809–817
15. Boland GM, Perkins G et al (2004) Wnt 3a promotes proliferation and suppresses osteogenic differentiation of adult human mesenchymal stem cells. J Cell Biochem 93:1210–1230
16. Boquest AC, Noer A et al (2006) Epigenetic programming of mesenchymal stem cells from human adipose tissue. Stem Cell Rev 2:319–329
17. Bork S, Pfister S et al (2010) DNA methylation pattern changes upon long-term culture and aging of human mesenchymal stromal cells. Aging Cell 9:54–63
18. Brazelton TR, Rossi FM et al (2000) From marrow to brain: expression of neuronal phenotypes in adult mice. Science 290:1775–1779
19. Buhring HJ, Battula VL et al (2007) Novel markers for the prospective isolation of human MSC. Ann N Y Acad Sci 1106:262–271
20. Burdach S, Plehm S et al (2009) Epigenetic maintenance of stemness and malignancy in peripheral neuroectodermal tumors by EZH2. Cell Cycle 8(13):1991–1996
21. Burgold T, Spreafico F et al (2008) The histone H3 lysine 27-specific demethylase Jmjd3 is required for neural commitment. PLoS One 3(8):e3034
22. Cameron EE, Bachman KE et al (1999) Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. Nat Genet 21:103–107
23. Cao Q, Yu J et al (2008) Repression of E-cadherin by the polycomb group protein EZH2 in cancer. Oncogene 27:7274–7284
24. Carvin CD, Kladde MP (2004) Effectors of lysine 4 methylation of histone H3 in Saccharomyces cerevisiae are negative regulators of PHO5 and GAL1-10. J Biol Chem 279:33057–33062
25. Caudill MA, Wang JC et al (2001) Intracellular S-adenosylhomocysteine concentrations predict global DNA hypomethylation in tissues of methyl-deficient cystathionine beta-synthase heterozygous mice. J Nutr 131:2811–2818
26. Chang L, Adams RD et al (2002) The TC10-interacting protein CIP4/2 is required for insulin-stimulated Glut4 translocation in 3T3L1 adipocytes. Proc Natl Acad Sci USA 99:12835–12840
27. Cheng X, Blumenthal RM (2008) Mammalian DNA methyltransferases: a structural perspective. Structure 16:341–350
28. Christophersen NS, Helin K (2010) Epigenetic control of embryonic stem cell fate. J Exp Med 207:2287–2295

29. Chuang JC, Warner SL et al (2010) S110, a 5-Aza-2′-deoxycytidine-containing dinucleotide, is an effective DNA methylation inhibitor in vivo and can reduce tumor growth. Mol Cancer Ther 9:1443–1450
30. Cironi L, Provero P et al (2009) Epigenetic features of human mesenchymal stem cells determine their permissiveness for induction of relevant transcriptional changes by SYT-SSX1. PLoS One 4:e7904
31. Cohen NM, Dighe V et al (2009) DNA methylation programming and reprogramming in primate embryonic stem cells. Genome Res 19:2193–2201
32. Collas P (2009) Epigenetic states in stem cells. Biochim Biophys Acta 1790:900–905
33. Cross SH, Bird AP (1995) CpG islands and genes. Curr Opin Genet Dev 5:309–314
34. da Silva ML, Chagastelles PC et al (2006) Mesenchymal stem cells reside in virtually all post-natal organs and tissues. J Cell Sci 119:2204–2213
35. de Boer J, Siddappa R et al (2004) Wnt signaling inhibits osteogenic differentiation of human mesenchymal stem cells. Bone 34:818–826
36. De Boer J, Wang HJ et al (2004) Effects of Wnt signaling on proliferation and differentiation of human mesenchymal stem cells. Tissue Eng 10:393–401
37. De Miguel MP, Fuentes-Julian S et al (2010) Pluripotent stem cells: origin, maintenance and induction. Stem Cell Rev 6:633–649
38. Dolinoy DC, Weidman JR et al (2006) Maternal genistein alters coat color and protects Avy mouse offspring from obesity by modifying the fetal epigenome. Environ Health Perspect 114:567–572
39. Dwyer RM, Kerin MJ (2010) Mesenchymal stem cells and cancer: tumor-specific delivery vehicles or therapeutic targets? Hum Gene Ther 21:1506–1512
40. El-Osta A (2003) DNMT cooperativity—the developing links between methylation, chromatin structure and cancer. Bioessays 25:1071–1084
41. Ferrari G, Cusella-De Angelis G et al (1998) Muscle regeneration by bone marrow-derived myogenic progenitors. Science 279:1528–1530
42. Fingerman IM, Wu CL et al (2005) Global loss of Set1-mediated H3 Lys4 trimethylation is associated with silencing defects in Saccharomyces cerevisiae. J Biol Chem 280:28761–28765
43. Gan Q, Yoshida T et al (2007) Concise review: epigenetic mechanisms contribute to pluripotency and cell lineage determination of embryonic stem cells. Stem Cells 25:2–9
44. Gangaraju VK, Lin H (2009) MicroRNAs: key regulators of stem cells. Nat Rev Mol Cell Biol 10:116–125
45. Glinsky GV (2008) "Stemness" genomics law governs clinical behavior of human cancer: implications for decision making in disease management. J Clin Oncol 26:2846–2853
46. Gussoni E, Soneoka Y et al (1999) Dystrophin expression in the mdx mouse restored by stem cell transplantation. Nature 401:390–394
47. Hanna JH, Saha K et al (2010) Pluripotency and cellular reprogramming: facts, hypotheses, unresolved issues. Cell 143:508–525
48. Hansen KH, Bracken AP et al (2008) A model for transmission of the H3K27me3 epigenetic mark. Nat Cell Biol 10:1291–1300
49. Hematti P (2011) Human embryonic stem cell-derived mesenchymal progenitors: an overview. Methods Mol Biol 690:163–174
50. Hemberger M, Dean W et al (2009) Epigenetic dynamics of stem cells and cell lineage commitment: digging Waddington's canal. Nat Rev Mol Cell Biol 10:526–537
51. Hirata T, Cui YJ et al (2007) The temporal profile of genomic responses and protein synthesis in ischemic tolerance of the rat brain induced by repeated hyperbaric oxygen. Brain Res 1130:214–222
52. Holbert S, Dedeoglu A et al (2003) Cdc42-interacting protein 4 binds to huntingtin: neuropathologic and biological evidence for a role in Huntington's disease. Proc Natl Acad Sci USA 100:2712–2717
53. Hong S, Cho YW et al (2007) Identification of JmjC domain-containing UTX and JMJD3 as histone H3 lysine 27 demethylases. Proc Natl Acad Sci USA 104:18439–18444

54. Hsiao SH, Huang TH et al (2009) Excavating relics of DNA methylation changes during the development of neoplasia. Semin Cancer Biol 19:198–208
55. Hsiao SH, Lee KD et al (2010) DNA methylation of the Trip10 promoter accelerates mesenchymal stem cell lineage determination. Biochem Biophys Res Commun 400:305–312
56. Hsu CC, Li HP et al (2010) Targeted methylation of CMV and E1A viral promoters. Biochem Biophys Res Commun 402:228–234
57. Hsu CC, Leu YW et al (2011) Functional characterization of Trip10 in cancer cell growth and survival. J Biomed Sci 18:12
58. Ji H, Ehrlich LI et al (2010) Comprehensive methylome map of lineage commitment from haematopoietic progenitors. Nature 467:338–342
59. Jones PA, Martienssen R (2005) A blueprint for a Human Epigenome Project: the AACR Human Epigenome Workshop. Cancer Res 65:11241–11246
60. Jones PA, Taylor SM et al (1983) Inhibition of DNA methylation by 5-azacytidine. Recent Results Cancer Res 84:202–211
61. Jung JW, Lee S et al (2010) Histone deacetylase controls adult stem cell aging by balancing the expression of polycomb genes and jumonji domain containing 3. Cell Mol Life Sci 67:1165–1176
62. Kalari S, Pfeifer GP (2010) Identification of driver and passenger DNA methylation in cancer by epigenomic analysis. Adv Genet 70:277–308
63. Kang TH, Lee JH et al (2007) Epigallocatechin-3-gallate enhances CD8+ T cell-mediated antitumor immunity induced by DNA vaccination. Cancer Res 67:802–811
64. Kierszenbaum AL (2002) Genomic imprinting and epigenetic reprogramming: unearthing the garden of forking paths. Mol Reprod Dev 63:269–272
65. Kim D, Yang JY et al (2009) Overexpression of alpha-catenin increases osteoblastic differentiation in mouse mesenchymal C3H10T1/2 cells. Biochem Biophys Res Commun 382:745–750
66. Kirmizis A, Bartley SM et al (2004) Silencing of human polycomb target genes is associated with methylation of histone H3 Lys 27. Genes Dev 18:1592–1605
67. Klopp AH, Gupta A et al (2011) Concise review: dissecting a discrepancy in the literature: do mesenchymal stem cells support or suppress tumor growth? Stem Cells 29:11–19
68. Krause DS, Theise ND et al (2001) Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. Cell 105:369–377
69. Lakshmipathy U, Hart RP (2008) Concise review: microRNA expression in multipotent mesenchymal stromal cells. Stem Cells 26:356–363
70. Lan F, Bayliss PE et al (2007) A histone H3 lysine 27 demethylase regulates animal posterior development. Nature 449:689–694
71. Lau PN, Cheung P (2011) Histone code pathway involving H3 S28 phosphorylation and K27 acetylation activates transcription and antagonizes polycomb silencing. Proc Natl Acad Sci USA 108:2801–2806
72. Lee KD, Kuo TK et al (2004) In vitro hepatic differentiation of human mesenchymal stem cells. Hepatology 40:1275–1284
73. Lee OK, Kuo TK et al (2004) Isolation of multipotent mesenchymal stem cells from umbilical cord blood. Blood 103:1669–1675
74. Leu YW, Rahmatpanah F et al (2003) Double RNA interference of DNMT3b and DNMT1 enhances DNA demethylation and gene reactivation. Cancer Res 63:6110–6115
75. Leu YW, Yan PS et al (2004) Loss of estrogen receptor signaling triggers epigenetic silencing of downstream targets in breast cancer. Cancer Res 64:8184–8192
76. Li E (2002) Chromatin modification and epigenetic reprogramming in mammalian development. Nat Rev Genet 3:662–673
77. Lin Y, Weisdorf DJ et al (2000) Origins of circulating endothelial cells and endothelial outgrowth from blood. J Clin Invest 105:71–77
78. Lin YS, Shaw AY et al (2011) Identification of novel DNA methylation inhibitors via a two-component reporter gene system. J Biomed Sci 18:3

79. Liu YZ, Shao Z et al (2010) Prediction of Polycomb target genes in mouse embryonic stem cells. Genomics 96:17–26
80. Locke M, Feisst V et al (2011) Concise review: human adipose-derived stem cells (ASC): separating promise from clinical need. Stem Cells 29:404–411
81. Lodhi IJ, Chiang SH et al (2007) Gapex-5, a Rab31 guanine nucleotide exchange factor that regulates Glut4 trafficking in adipocytes. Cell Metab 5:59–72
82. Lopez MJ, Spencer ND (2011) In vitro adult rat adipose tissue-derived stromal cell isolation and differentiation. Methods Mol Biol 702:37–46
83. Martin DI, Cropley JE et al (2008) Environmental influence on epigenetic inheritance at the Avy allele. Nutr Rev 66:S12–S14
84. Mathieu O, Probst AV et al (2005) Distinct regulation of histone H3 methylation at lysines 27 and 9 by CpG methylation in Arabidopsis. EMBO J 24:2783–2791
85. Messmer S, Franke A et al (1992) Analysis of the functional role of the Polycomb chromo domain in Drosophila melanogaster. Genes Dev 6:1241–1254
86. Miller SA, Mohn SE et al (2010) Jmjd3 and UTX play a demethylase-independent role in chromatin remodeling to regulate T-box family member-dependent gene expression. Mol Cell 40:594–605
87. Mohn F, Weber M et al (2008) Lineage-specific polycomb targets and de novo DNA methylation define restriction and potential of neuronal progenitors. Mol Cell 30:755–766
88. Momin EN, Vela G et al (2010) The oncogenic potential of mesenchymal stem cells in the treatment of cancer: directions for future research. Curr Immunol Rev 6:137–148
89. Morgan HD, Sutherland HG et al (1999) Epigenetic inheritance at the agouti locus in the mouse. Nat Genet 23:314–318
90. Noer A, Boquest AC et al (2007) Dynamics of adipogenic promoter DNA methylation during clonal culture of human adipose stem cells to senescence. BMC Cell Biol 8:18
91. Noer A, Lindeman LC et al (2009) Histone H3 modifications associated with differentiation and long-term culture of mesenchymal adipose stem cells. Stem Cells Dev 18:725–736
92. Okitsu CY, Hsieh JC et al (2010) Transcriptional activity affects the H3K4me3 level and distribution in the coding region. Mol Cell Biol 30:2933–2946
93. Opavsky R, Wang SH et al (2007) CpG island methylation in a mouse model of lymphoma is driven by the genetic configuration of tumor cells. PLoS Genet 3:1757–1769
94. Pacini S, Carnicelli V et al (2010) Constitutive expression of pluripotency-associated genes in mesodermal progenitor cells (MPCs). PLoS One 5:e9861
95. Papp B, Muller J (2006) Histone trimethylation and the maintenance of transcriptional ON and OFF states by trxG and PcG proteins. Genes Dev 20:2041–2054
96. Pasini D, Malatesta M et al (2010) Characterization of an antagonistic switch between histone H3 lysine 27 methylation and acetylation in the transcriptional regulation of Polycomb group target genes. Nucleic Acids Res 38:4958–4969
97. Petersen BE, Bowen WC et al (1999) Bone marrow as a potential source of hepatic oval cells. Science 284:1168–1170
98. Pittenger MF, Mackay AM et al (1999) Multilineage potential of adult human mesenchymal stem cells. Science 284:143–147
99. Pontikoglou C, Deschaseaux F et al (2011) Bone marrow mesenchymal stem cells: biological properties and their role in hematopoiesis and hematopoietic stem cell transplantation. Stem Cell Rev 7:569–589
100. Prockop DJ (1997) Marrow stromal cells as stem cells for nonhematopoietic tissues. Science 276:71–74
101. Rajasekhar VK, Begemann M (2007) Concise review: roles of polycomb group proteins in development and disease: a stem cell perspective. Stem Cells 25:2498–2510
102. Reik W (2007) Stability and flexibility of epigenetic gene regulation in mammalian development. Nature 447:425–432
103. Robert MF, Morin S et al (2003) DNMT1 is required to maintain CpG methylation and aberrant gene silencing in human cancer cells. Nat Genet 33:61–65

104. Ross SA (2003) Diet and DNA methylation interactions in cancer prevention. Ann N Y Acad Sci 983:197–207
105. Salem HK, Thiemermann C (2010) Mesenchymal stromal cells: current understanding and clinical status. Stem Cells 28:585–596
106. Sanchez-Ramos J, Song S et al (2000) Adult bone marrow stromal cells differentiate into neural cells in vitro. Exp Neurol 164:247–256
107. Sasaki H, Matsui Y (2008) Epigenetic events in mammalian germ-cell development: reprogramming and beyond. Nat Rev Genet 9:129–140
108. Sawarkar R, Paro R (2010) Interpretation of developmental signaling at chromatin: the Polycomb perspective. Dev Cell 19:651–661
109. Schlesinger Y, Straussman R et al (2007) Polycomb-mediated methylation on Lys27 of histone H3 pre-marks genes for de novo methylation in cancer. Nat Genet 39:232–236
110. Schubeler D (2009) Epigenomics: methylation matters. Nature 462:296–297
111. Schwartz YB, Kahn TG et al (2010) Alternative epigenetic chromatin states of polycomb target genes. PLoS Genet 6:e1000805
112. Seki Y, Yamaji M et al (2007) Cellular dynamics associated with the genome-wide epigenetic reprogramming in migrating primordial germ cells in mice. Development 134:2627–2638
113. Sethe S, Scutt A et al (2006) Aging of mesenchymal stem cells. Ageing Res Rev 5:91–116
114. Shafa M, Krawetz R et al (2010) Returning to the stem state: epigenetics of recapitulating pre-differentiation chromatin structure. Bioessays 32:791–799
115. Sibani S, Melnyk S et al (2002) Studies of methionine cycle intermediates (SAM, SAH), DNA methylation and the impact of folate deficiency on tumor numbers in Min mice. Carcinogenesis 23:61–65
116. Siddiqi S, Mills J et al (2010) Epigenetic remodeling of chromatin architecture: exploring tumor differentiation therapies in mesenchymal stem cells and sarcomas. Curr Stem Cell Res Ther 5:63–73
117. Simile MM, Pascale R et al (1994) Correlation between S-adenosyl-L-methionine content and production of c-myc, c-Ha-ras, and c-Ki-ras mRNA transcripts in the early stages of rat liver carcinogenesis. Cancer Lett 79:9–16
118. Simon JA, Kingston RE (2009) Mechanisms of polycomb gene silencing: knowns and unknowns. Nat Rev Mol Cell Biol 10:697–708
119. Sparmann A, van Lohuizen M (2006) Polycomb silencers control cell fate, development and cancer. Nat Rev Cancer 6:846–856
120. Spencer ND, Lopez MJ (2011) In vitro adult canine adipose tissue-derived stromal cell growth characteristics. Methods Mol Biol 702:47–60
121. Spivakov M, Fisher AG (2007) Epigenetic signatures of stem-cell identity. Nat Rev Genet 8:263–271
122. Su Y, Deng B et al (2011) Polycomb group genes in stem cell self-renewal: a double-edged sword. Epigenetics 6:16–19
123. Surani MA, Hayashi K et al (2007) Genetic and epigenetic regulators of pluripotency. Cell 128:747–762
124. Tate PH, Bird AP (1993) Effects of DNA methylation on DNA-binding proteins and gene expression. Curr Opin Genet Dev 3:226–231
125. Teng IW, Hou PC et al (2011) Targeted methylation of two tumor suppressor genes is sufficient to transform mesenchymal stem cells into cancer stem/initiating cells. Cancer Res 71:4653–4663
126. Theise ND, Badve S et al (2000) Derivation of hepatocytes from bone marrow cells in mice after radiation-induced myeloablation. Hepatology 31:235–240
127. Theise ND, Nimmakayalu M et al (2000) Liver from bone marrow in humans. Hepatology 32:11–16
128. Tiwari VK, McGarvey KM et al (2008) PcG proteins, DNA methylation, and gene repression by chromatin looping. PLoS Biol 6:2911–2927
129. Trento C, Dazzi F (2010) Mesenchymal stem cells and innate tolerance: biology and clinical applications. Swiss Med Wkly 140:w13121

130. Turek-Plewa J, Jagodzinski PP (2005) The role of mammalian DNA methyltransferases in the regulation of gene expression. Cell Mol Biol Lett 10:631–647
131. Vincent A, Van Seuningen I (2009) Epigenetics, stem cells and epithelial cell fate. Differentiation 78:99–107
132. Wei Y, Xia W et al (2008) Loss of trimethylation at lysine 27 of histone H3 is a predictor of poor outcome in breast, ovarian, and pancreatic cancers. Mol Carcinog 47:701–706
133. Wei G, Wei L et al (2009) Global mapping of H3K4me3 and H3K27me3 reveals specificity and plasticity in lineage fate determination of differentiating CD4+ T cells. Immunity 30:155–167
134. Weinhofer I, Hehenberger E et al (2010) H3K27me3 profiling of the endosperm implies exclusion of polycomb group protein targeting by DNA methylation. PLoS Genet 6:e1001152
135. Wolff GL, Kodell RL et al (1998) Maternal epigenetics and methyl supplements affect agouti gene expression in Avy/a mice. FASEB J 12:949–957
136. Wu SC, Zhang Y (2010) Active DNA demethylation: many roads lead to Rome. Nat Rev Mol Cell Biol 11:607–620
137. Yamada Y, Watanabe A (2010) Epigenetic codes in stem cells and cancer stem cells. Adv Genet 70:177–199
138. Yamasaki-Ishizaki Y, Kayashima T et al (2007) Role of DNA methylation and histone H3 lysine 27 methylation in tissue-specific imprinting of mouse Grb10. Mol Cell Biol 27:732–742
139. Yang XF (2007) Immunology of stem cells and cancer stem cells. Cell Mol Immunol 4:161–171
140. Yuan R, Fan S et al (2001) Altered gene expression pattern in cultured human breast cancer cells treated with hepatocyte growth factor/scatter factor in the setting of DNA damage. Cancer Res 61:8022–8031
141. Zager RA, Johnson AC (2010) Progressive histone alterations and proinflammatory gene activation: consequences of heme protein/iron-mediated proximal tubule injury. Am J Physiol Renal Physiol 298:F827–F837
142. Zeng X (2007) Human embryonic stem cells: mechanisms to escape replicative senescence? Stem Cell Rev 3:270–279
143. Zheng C, Yang S et al (2009) Human multipotent mesenchymal stromal cells from fetal lung expressing pluripotent markers and differentiating into cell types of three germ layers. Cell Transplant 18:1093–1109
144. Jackson KA, Majka SM et al (2001) Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells. JCI 107:1395–1402

Part III
Clinical Implications and Analysis Methods

Chapter 11
Environmental Toxicants, Epigenetics, and Cancer

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Abstract Tumorigenesis, a complex and multifactorial progressive process of transformation of normal cells into malignant cells, is characterized by the accumulation of multiple cancer-specific heritable phenotypes triggered by the mutational and/or non-mutational (i.e., epigenetic) events. Accumulating evidence suggests that environmental and occupational exposures to natural substances, as well as man-made chemical and physical agents, play a causative role in human cancer. In a broad sense, carcinogenesis may be induced through either genotoxic or non-genotoxic mechanisms; however, both genotoxic and non-genotoxic carcinogens also cause prominent epigenetic changes. This review presents current evidence of the epigenetic alterations induced by various chemical carcinogens, including arsenic, 1,3-butadine, and pharmaceutical and biological agents, and highlights the potential for epigenetic changes to serve as markers for carcinogen exposure and cancer risk assessment.

11.1 Introduction

Tumorigenesis is a complex and multifactorial progressive process of transformation of normal cells into malignant ones. It is characterized by the accumulation of multiple cancer-specific heritable phenotypes, including persistent proliferative

Note: The views expressed in this chapter do not necessarily represent those of the U.S. Food and Drug Administration.

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signaling, resistance to cell death, evasion of growth suppression, replicative immortality, inflammatory response, deregulation of energy metabolism, genomic instability, induction of angiogenesis, and activation of invasion ultimately resulting in metastases [1]. The acquisition of these cancer-specific alterations may be triggered by the mutational and/or non-mutational (i.e., epigenetic) events in the genome which, in turn, affect gene expression and the downstream phenotypes listed above [1, 2]. Furthermore, it has been suggested that epigenetic alterations may play as important or even more prominent role in tumor development [3].

Epigenetic events, most prominently manifested by stable and heritable changes in gene expression that are not due to any alteration in the primary DNA sequence [4], signify the fundamental molecular principles in which genetic information is organized and read [5]. Epigenetic modifications include change in methylation patterns of cytosines in DNA [6, 7], modifications of the proteins that bind to DNA [8, 9], and the nucleosome positioning along DNA [4]. These epigenetic marks are tightly and interdependently connected and are essential for the normal development and the maintenance of cellular homeostasis and functions in adult organisms, particularly for X-chromosome inactivation in females, genomic imprinting, silencing of repetitive DNA elements, regulation of chromatin structure, and proper expression of genetic information [10]. The epigenetic status is well-balanced in normal cells, but may be altered in many ways in cancer cells. Additionally, growing evidence indicates that a number of lifestyle and environmental factors may disrupt this epigenetic balance and compromise the stability of the epigenome in normal cells leading to the development of a wide range of pathologies, including cancer.

11.2 Epigenetic Alterations in Cancer Cells

The unifying molecular feature of neoplastic cells is a profoundly reshaped genome characterized by global genomic *hypo*-methylation, gene-specific *hyper*- or *hypo*-methylation, and altered histone modification patterns [2, 11].

DNA demethylation signifies one of the two major DNA methylation states and refers to a state in which there is a decrease in the number of methylated cytosine bases from the "normal" methylation level. Demethylation of DNA can be achieved either passively or actively. Passive loss of methylated marks in the genome may be a consequence of limited availability of the universal methyl donor S-adenosyl-L-methionine (SAM), compromised integrity of DNA, and altered expression and/or activity of DNA methyltransferases [12]. Until recently, evidence for existence of an active replication-independent DNA demethylation process was controversial and inconclusive [7, 13]. However, recent studies provide compelling experimental evidence that active loss of DNA methylation is associated with the function of DNA repair machinery [14–17].

Global hypomethylation of DNA was the first epigenetic abnormality identified in cancer more than a quarter of century ago [18, 19]. It continues to be one of the

most common molecular alterations found in all human cancers [20, 21]; however, the molecular mechanisms behind cancer-linked global demethylation of the genome remain largely unknown. The loss of DNA methylation in cancer primarily affects stable, methylated areas of the genome composed predominantly of repetitive elements, genes, and intergenic regions [22].

There are several molecular consequences of global demethylation of DNA that may contribute to tumorigenesis. First, genomic hypomethylation causes significant elevation in mutation rates [23], activation of normally silenced tumor-promoting genes [24], and loss of imprinting [25]. Second, demethylation of the repetitive DNA sequences, such as long interspersed nucleotide elements (LINE)-1 and short interspersed nucleotide elements (SINE), retroviral intracisternal A particle (IAP), and Alu elements located at centromeric, pericentromeric, and subtelomeric chromosomal regions induces their activation and transposition leading to chromosomal instability [26–29]. For example, recent findings have demonstrated that DNA hypomethylation causes permissive transcriptional activity at the centromere [28]. Subsequently, the accumulation of small minor satellite transcripts that impair centromeric architecture and function is observed. Likewise, hypomethylation of the repetitive elements at the subtelomeric regions is associated with enhanced transcription of the telomeres [29].

Gene-specific loss of DNA methylation is also a finding for oncogenes and imprinted genes. In addition, many genes that are normally well-methylated, particularly cancer-germline genes, including B melanoma antigen family (*BAGE*), cancer testis antigen (*CAGE*), melanoma antigen family *A* (*MAGE-A*), X antigen family (*XAGE*), and other single-copy genes, including S100 calcium binding protein A4 (*S100A4*), flap endonuclease 1 (*FEN1*), and synuclein-gamma (*SNCG*), undergo progressive hypomethylation, which is accompanied by their increased expression, in human cancers [12, 21].

Despite the large body of evidence indicating that cancer-associated DNA demethylation is an important early event in tumor development, it is still less clear if the loss of DNA methylation is a cause, or a consequence of the malignant transformation [30]. The notion that DNA hypomethylation is playing a role in causation and/or promotion of cancer is based on the results of studies with nutritional "lipogenic methyl-deficient diet" [31–33], genetically engineered *Dnmt*- and *Lsh*-deficient mice [34, 35], and several models of chemical carcinogenesis [36]. In contrast, there is also evidence that cancer-linked DNA hypomethylation may be a passive inconsequential side effect of carcinogenesis [30, 37]. The latter is evidenced by facts that not all tumors exhibit DNA hypomethylation and not all carcinogenic processes are accompanied by the loss of DNA methylation [38]. Indeed, it is highly unlikely to expect that development and progression of diverse types of tumors are all associated with DNA hypomethylation. Furthermore, there is growing evidence that DNA hypomethylation suppresses development of certain tumor types, especially intestinal, gastric, and prostate carcinomas [39–41].

DNA hypermethylation is the state where the methylation of normally undermethylated DNA domains, those that predominantly consist of CpG islands [22], increases. CpG islands are defined as the genomic regions that contain the high

G + C content, have high frequency of CpG dinucleotides, are at least 400–500 bp long, and can be located either at intragenic and intergenic, or at the 5′ ends of genes [42–44]. However, only CpG islands that span 5′ promoters are mainly unmethylated. For instance, less than 3% of CpG islands in gene promoters are methylated [44].

It is well-established that hypermethylation of promoter-located CpG islands causes permanent and stable transcriptional silencing of a range of protein-coding genes [45], which, along with DNA hypomethylation, plays a critical role in cancer development [2, 11]. One of the most compelling examples of the link between DNA hypermethylation and carcinogenesis is epigenetic silencing of critical tumor-suppressor genes, including cyclin-dependent kinase inhibitor 2A (*CDKN2A*; $p16^{INK4A}$), secreted frizzled-related protein (*SFRPs*) genes, adenomatous polyposis coli (*APC*), and GATA binding protein 4 (*GATA4*). The aberrant silencing of these genes allows for survival and clonal expansion of the initiated cells. Additionally, hypermethylation of several DNA repair genes, including O^6-methylguanine-DNA methyltransferase (*MGMT*), xeroderma pigmentosum group C (*XPC*), MutL homolog 1 (*MLH1*), and breast cancer 1 and 2 (*BRCA1* and *BRCA2*) genes results in insufficient DNA repair leading to reduction in genomic stability and various genetic aberrations, particularly, the elevation of mutation rates in critical cancer-related genes [46, 47]. For example, the epigenetic silencing of *MGMT* leads to a greater mutation rate in *K-RAS* and *p53* genes during human colorectal carcinogenesis [48, 49]. Likewise, transcriptional inactivation of the *BRCA1* and *MLH1* genes caused by promoter hypermethylation results in elevated *p53* gene mutation frequency in human sporadic breast cancer [50] and microsatellite instability in sporadic colorectal cancer [51], respectively.

In addition to the vital role that DNA methylation state may play in the etiology and pathogenesis of cancer, it has been shown that disruption of normal patterns of covalent histone modifications is an epigenetic change frequently found in tumor cells. Histones are evolutionary conserved proteins that have globular carboxy-terminal domains critical to nucleosome formation, and flexible amino-terminal tails that protrude from the nucleosome core and contact adjacent nucleosomes to form higher order chromatin structures. At least eight different classes of post-translational modifications, including methylation, acetylation, phosphorylation, ubiquitination, sumoylation, biotinylation, and ADP-ribosylation have been identified on the core histones H2A, H2B, H3, H4, and the H1 family of linker histones [8, 9]. These histone marks are essential for organizing chromatin, maintaining genome stability, silencing repetitive DNA elements, regulating cell cycle progression, recognizing DNA damage sites and repair, and maintenance of proper expression of genetic information.

Accumulating evidence clearly indicates that cancer cells are characterized by a profoundly disturbed pattern of global and/or gene-specific histone modifications accompanied by alterations in the functioning of enzymes that are associated with those marks. There are various combinations of cancer-linked histone modifications that differ according to tumor type; however, one of the most characteristic examples of global changes in histone modifications is loss of histone H4 lysine 20

trimethylation and H4 lysine 16 acetylation, which is a common hallmark of human cancers [52].

Additionally, extensive studies in the past decade have indicated the existence and importance of another epigenetic mechanism of regulation of gene function by means of small non-coding microRNAs (miRNAs). Currently, miRNAs are recognized as one of the major regulatory gatekeepers of protein-coding genes in human genome [53, 54]. MiRNAs are small 16–29 nucleotide-long non-coding RNAs that primarily function as negative gene regulators at the post-transcriptional level [55]. MiRNAs are generated by RNA polymerase II or RNA polymerase III as long primary transcripts, primary miRNAs. Following transcription, primary miRNAs form a stem-loop structure, which is recognized and processed by the RNase III-type enzyme Drosha creating precursor miRNAs. These precursor miRNAs are transported from the nucleus to the cytoplasm by Exportin-5. In the cytoplasm, the pre-miRNAs are further processed by Dicer, an RNase III enzyme, generating miRNA:miRNA hybrids. After unwinding, one strand of the duplex is degraded, and another strand becomes a mature miRNA. MiRNAs can induce mRNA cleavage if complementary to 3′-untranslated region of targets is perfect or translational repression if complementarity is imperfect [53].

Currently there are more than 700 mammalian miRNAs that can potentially target up to one-third of protein-coding genes involved in the development, cell differentiation, metabolic regulation, signal-transduction, cell proliferation, and apoptosis. As the deregulation of these very same biological processes is a hallmark of cancer [1], it has been suggested that changes in miRNA expression might have significance in cancer [56–58]. In tumors, aberrant expression of miRNAs inhibits tumor suppressor genes or inappropriately activates oncogenes have been experimentally associated with most aspects of tumor biology, including tumor progression, invasiveness, metastasis, and acquisition of resistance of malignant cells to various chemotherapeutic agents [58]. This leads to the suggestion that altered expression of miRNAs is an important mechanism of carcinogenesis [57, 59].

11.3 Role of Epigenetic Alterations in Chemical Carcinogenesis

Many environmental and occupational exposures to natural substances, man-made chemical and physical agents are considered to be causative of human cancer [60–62]. In a broad sense, carcinogenesis may be induced through either genotoxic or non-genotoxic mechanisms. Genotoxic carcinogens are agents that interact directly or after metabolic activation with DNA, causing mutations and leading to tumor formation. Non-genotoxic carcinogens are a diverse group of chemical compounds that are known to cause tumors by mechanisms other than direct damage to DNA. The emphasis in carcinogenesis research, until recently, has focused mainly on the investigation of various molecular signaling events, DNA damage, DNA adduct repair, and genetic aberrations, despite the fact that the importance of epigenetic mechanisms in carcinogenic process was first suggested by Miller in 1970 [63].

Accumulating evidence suggests that regardless of the mechanism of action, both genotoxic and non-genotoxic carcinogens may also lead to prominent epigenetic abnormalities in tissues that are susceptible to carcinogenesis as a result of exposure [64–68]. The following sections present an overview of the epigenetic alterations induced by several carcinogens.

11.3.1 Arsenic

Arsenic is a naturally occurring element and a ubiquitous environmental contaminant which is a public health issue world-wide [69]. The major source of human exposure to arsenic is contaminated food and drinking water. Inorganic arsenic was one of the earliest identified human carcinogens [69, 70]. It is widely accepted that exposure to arsenic is associated with skin, lung, and bladder cancers [71]. Additionally, accumulating evidence indicates that long-term exposure to arsenic causes development of liver tumors [72].

Arsenic was classified as a known human carcinogen by the International Agency for Research on Cancer (IARC) in 2004, when sufficient evidence for human carcinogenicity became available [71]; even though limited evidence for animal carcinogenicity of arsenic existed. This may be explained mainly by the absence of adequate relevant animal models to study arsenic carcinogenesis. However, the experiments in transgenic mice, e.g., v-Ha-ras (Tg.AC), keratin VI/ornithine decarboxylase (K6/ODC), and $p53^{+/-}$, or inbred mouse strains that are prone to spontaneous cancer development provided evidence for the carcinogenicity of arsenic in animal studies. For instance, administration of arsenic to A/J mice, a strain that exhibits a susceptibility to different pulmonary pathological states including lung cancer, enhances lung tumor multiplicity and size [70, 73]. Similarly, in utero arsenic exposure of C3H/HeJ mice, which are prone to hepatocarcinogenesis, resulted in increased incidence and multiplicity of hepatocellular carcinomas in adults [74]. The most convincing evidence for the carcinogenicity of arsenic in animals has been presented in a recent report by Tokar et al. [75] that demonstrated that “whole-life” exposure of CD1 mice to arsenic causes induction of various tumors, including lung and liver.

The molecular mechanisms behind the cancer-inducing property of arsenic are not fully elucidated and remain a subject of debate. Several potential mechanisms have been proposed to explain arsenic-induced carcinogenesis, including induction of oxidative stress, DNA–protein crosslinking, chromosomal aberrations [70], disruption of signaling pathways, and epigenetic dysregulation, particularly DNA demethylation [76]. The first evidence demonstrating an association between arsenic tumorigenicity and global DNA hypomethylation was reported by Zhao et al. [77] who showed that exposure of rat liver epithelial TRL-1215 cells to arsenic in vitro led to their malignant transformation and was paralleled by global DNA demethylation. Importantly, the extent of DNA hypomethylation in the transformed cells was positively correlated with the tumorigenicity of the cells upon inoculation

into nude mice, suggesting that loss of DNA methylation may be a causative factor in arsenic-induced carcinogenesis [77]. Since then, a large amount of data has documented a substantial target organ-specific loss of global DNA methylation and repetitive element and gene-specific methylation in various in vitro and in vivo models of arsenic-induced tumorigenesis [78–80].

Several possible explanations exist for the mechanism of DNA demethylation after exposure to arsenic. First, arsenic-induced DNA hypomethylation can be explained by the absolute requirement of SAM for the biomethylation of inorganic arsenic and DNA methylation reactions [76, 81]. Therefore, the biomethylation of inorganic arsenic reduces availability of SAM for DNA and histone methylation. Second, arsenic exposure increases generation of reactive oxygen species that may cause direct damage to DNA [82, 83]. The presence of oxidative lesions in DNA (e.g., 8-oxodeoxyguanosine and 5-hydroxymethylcytosine) severely compromises the ability of DNA methyltransferases to methylate the target cytosine and leads to passive demethylation of DNA [84]. In addition, activation of DNA repair pathway promotes active demethylation of DNA [14–17]. Third, arsenic-induced oxidative stress causes depletion of the level of intracellular reduced glutathione. This consequently leads to the enhanced glutathione biosynthesis in a transsulfuration pathway, which impairs SAM biosynthesis and perturbs DNA and histone methylation reactions [85].

In addition to global and gene-specific DNA hypomethylation, arsenic exposure causes concurrent methylation-induced transcriptional silencing of a number of tumor suppressor genes, including *p53*, *CDKN2A* (*$p16^{INK4A}$*), Ras association domain family member 1 (*RASSF1A*), and death-associated protein kinase (*DAPK*) [73, 86, 87], various histone modification changes [88], and alterations in miRNA expression [89].

It is of note that growing evidence suggests that carcinogenesis induced by an environmental chronic exposure to other metals, such as nickel, chromium, cadmium, and mercury, may also involve molecular epigenetic alterations caused by the ability of these metals to induce damage to DNA and strongly influence intracellular molecular and metabolic alterations [90, 91].

11.3.2 1,3-Butadiene

The gaseous olefin 1,3-butadiene is a major industrial chemical monomer widely used in production of synthetic rubber, resins, and plastic. Additionally, this highly volatile agent is present in industrial and automobile exhaust, cigarette smoke, and ambient air in urban locations and industrial complexes [92]. Based on the results of numerous comprehensive epidemiological studies, the IARC has classified 1,3-butadiene as a known human carcinogen [92–94]. In rodents, it causes tumor formation at several target sites, including the hematopoietic system, lungs, heart, and liver [93]. Importantly, the hematopoietic system, lungs and liver are the most common sites of 1,3-butadiene-induced tumor formation in both humans and mice [93].

It is well-established that the mechanism of tumor induction caused by 1,3-butadiene-exposure is due to genotoxic reactivity of its metabolic epoxides: 1,2-epoxy-3-butene, 1,2:3,4-diepoxybutane, and 3,4-epoxy-1,2-butanediol that interact directly with DNA to form mutagenic DNA adducts [94]. However, recent evidence demonstrates that short-term inhalational exposure of C57BL/6J mice to 1,3-butadiene, in addition to DNA adduct formation, also causes extensive concurrent epigenetic changes. These include a marked reduction of global DNA and repetitive element methylation and a profound loss of histone H3K9, H3K27, and H4K20 trimethylation in the livers of C57BL/6J mice [95].

It is well-established that methylation of lysine residues 9 and 27 at histone H3 and lysine 20 at histone H4 plays a fundamental role in the formation of a condensed heterochromatin structure and transcriptional repression [96–98]. Hence, loss of H3K9 and H4K20 trimethylation induced by 1,3-butadiene-exposure may compromise genomic stability via chromatin relaxation and activation of mobile repetitive elements. Indeed, a recent report showing decondensation of chromatin and activation of main repetitive elements in the livers of 1,3-butadiene-exposed C57BL/6J mice support this suggestion [99]. Additionally, an open chromatin structure may increase further vulnerability of DNA to the genotoxicity of reactive 1,3-butadiene metabolites.

The elucidation of the mechanisms of carcinogenicity is usually carried out in genetically homogeneous in vivo models in order to fix as many variables as possible. This provides information in a single strain, yet the extrapolation of such data to the population effects is constrained by the inference from a single genome to model complex human phenotypes. To overcome this important limitation, panels of genetically defined animals may be used to determine genetic causes of inter-individual variability in cancer susceptibility [100]. In a recent study, Koturbash et al. [99] have demonstrated substantial differences in hepatic genetic and epigenetic response among mouse strains to short-term inhalational exposure to 1,3-butadiene. More importantly, the strain differences were associated with alterations in chromatin structure, mainly in the variability in histone H3K9, H3K27, and H4K20 methylation.

11.3.3 Pharmaceuticals

Diethylstilbestrol is a synthetic non-steroidal estrogen that was widely used to prevent potential miscarriages and as emergency contraceptive (morning-after pill) [101]. Currently, diethylstilbestrol is classified by the IARC as a known human carcinogen [101]. Breast is the main target organ for diethylstilbestrol-induced carcinogenesis in women who were exposed during pregnancy. Additionally, diethylstilbestrol also causes development of adenocarcinoma in the uterus and cervix of women who were exposed in utero.

In addition to the established mechanistic genotoxic and estrogen receptor-mediated carcinogenic events, epigenetic programming also plays a substantial role.

Perinatal exposure to diethylstilbestrol causes persistent demethylation and transcriptional activation of several critical cancer-related genes in the mouse uterus, including lactoferrin (*Lf*), nucleosomal binding protein 1 (*Nsbp1*), and c-*fos* [102–104]. The mechanism of these demethylation events is associated with the ability of diethylstilbestrol to inhibit expression of the maintenance (*Dnmt1*) and de novo (*Dnmt3a* and *Dnmt3b*) DNA methyltransferases in the mouse uterus [105]. Additionally, recent evidence indicates that diethylstilbestrol exposure causes epigenetically induced down-regulation of miRNA-9 in human breast epithelial cells [106], one of the frequently down-regulated miRNAs in human breast cancer [107].

Tamoxifen, a selective non-steroidal anti-estrogen, is a widely used drug for chemotherapy and for chemoprevention of breast cancer worldwide [108]. However, recently the IARC classified tamoxifen as a known human carcinogen based on evidence for endometrial cancer [101]. One of the possible mechanisms of carcinogenic effects of tamoxifen in the uterus is tamoxifen-induced gene expression changes [109], particularly, hypomethylation-linked activation of paired box 2 (*PAX2*) gene [110].

Additionally, a number of studies have demonstrated that tamoxifen is a potent hepatocarcinogen in rats with both tumor initiating and promoting properties [111]. The mechanism of tamoxifen-induced hepatocarcinogenesis is associated with its genotoxic [112, 113] and epigenetic effects [114]. These non-genotoxic epigenetic alterations include demethylation of the entire genome and the repetitive elements, loss of global histone H4 lysine 20 trimethylation [114, 115], and altered expression of miRNAs [116]. The results of these studies further emphasize the importance of non-genotoxic mechanisms in chemical carcinogenesis induced by genotoxic carcinogens.

Phenobarbital, the most widely used anticonvulsant worldwide, is a well-established mitogenic non-genotoxic rodent liver carcinogen. It is known to increase cell proliferation, alter cell cycle checkpoint control, including delaying and attenuating the G1 checkpoint, inhibit the induction of p53, thereby resulting in accumulation of DNA damage, and induce extensive epigenetic abnormalities. Treatment with phenobarbital leads to rapid and progressive accumulation of altered DNA methylation regions in the livers of C57BL/6 and B6C3F1 mice [117]. These changes were more pronounced in livers of tumor-prone B6C3F1 and CAR (constitutive androstane receptor) wild-type mice [118]. Interestingly, the number of hypermethylated regions was noticeably smaller than hypomethylated regions, among which cytochrome P450, family 2, subfamily b, polypeptide 10 (*Cyp2b10)* gene is concomitantly hypomethylated and transcriptionally activated early after phenobarbital treatment [119].

Oxazepam is widely used as a sedative-hypnotic and antianxiety drug. Chronic exposure of B6C3F1 mice to oxazepam induces development of hepatoblastoma and hepatocellular carcinoma in mice [120]. Interestingly, oxazepam, similar to phenobarbital, causes induction of *Cyp2b10* gene in the livers of B6C3F1 mice [121, 122]. Also, oxazepam-induced tumors display a decreased expression of *Apc* and phosphatase and tensin (*Pten*) homolog tumor suppressor genes and genes involved in regulation of DNA methylation and histone modification [122].

11.3.4 Biological Agents

Mycotoxins are a structurally diverse class of molecules of fungal origin that are common contaminants of the human and animal food products [123]. Three of the most ubiquitous mycotoxins, aflatoxin B_1, fumonisin B1, and ochratoxin, are classified by the IARC as known and possible human carcinogens [124, 125]. It is well-established that aflatoxin B_1, fumonisin B1, and ochratoxin A are genotoxic carcinogens [123, 126, 127]; however, accumulating evidence indicates that their carcinogenicity involves also a complex network of epigenetic alterations [128–134].

Aflatoxin B_1 induces several epigenetic abnormalities that may induce and promote tumor development. Specifically, exposure to aflatoxin B_1 causes methylation-induced transcriptional silencing of *MGMT, p16^{INK4A}*, and *RASSF1A* genes, a fundamental epigenetic event in liver carcinogenesis [128–130]. Conversely, aflatoxin B_1 is a strong inducer of epigenetically regulated *SNCG* gene [131]. Additionally, a study conducted by Hu et al. [134] has demonstrated that cytosine methylation at the CpG site at codon 14 of the *K-ras* gene is the major reason for preferential aflatoxin B_1-induced DNA-adduct formation at this codon in normal human bronchial epithelial cells.

Fumonisin B_1, in addition to various genotoxic and non-genotoxic alterations, increases the level of 5-methylcytosine in genomic DNA from 5 to 9% in human intestinal Caco-2 cells [132].

Helicobacter pylori infection is associated with development of gastric cancer, one of the most prevalent human cancers worldwide [135]. The results of several comprehensive studies indicate that *H. pylori* infection causes marked DNA methylation changes in infected normal or preneoplastic gastric mucosa. *H. pylori* infection causes significant aberrant DNA methylation in a number of the promoter CpG island-containing genes, including *p16^{INK4A}*, lipoxygenase (*LOX*), heart and neural crest derivatives expressed 1 (*HAND1*), thrombomodulin (*THBD*), and actin related protein 2/3 complex, subunit p41 (*p41ARC*) gastric cancer-associated genes in gastric mucosa [136–139]. Importantly, hypermethylation of some genes, e.g., *THBD* persisted in gastric mucosa after *H. pylori* eradication [140].

11.4 Epigenetic Alterations and the Evaluation of Cancer Risk

Recognition of the fundamental role of epigenetic alterations in cancer has resulted in the identification of numerous epigenetic abnormalities that may be used as potential biomarkers for the molecular diagnosis of cancer and prognosis of survival or treatment outcomes. Despite a lack of conclusive information to clarify whether or not epigenetic changes are involved directly in neoplastic cell transformation, evidence highlighted above suggests that epigenetic alterations may be used as early indicators of carcinogenesis for both genotoxic and non-genotoxic carcinogens. Importantly, several research groups have argued that epigenetic alterations may be

used as biomarkers in the evaluation of the carcinogenic potential of the environmental factors [5, 67, 68, 141].

Incorporation of the epigenetic biomarkers into the studies on cancer risk of exposures holds a number of advantages over traditionally used methods, such as evaluation of the carcinogen-induced DNA damage, DNA adduct formation, or bacterial mutagenicity. Specifically, we reason that the following features are in favor of greater integration of epigenetic biomarkers in studies of the carcinogenic potential of the environmental exposures: (1) early appearance; (2) stability; (3) target tissue-specificity; (4) relatively low cost of the assays needed to detect these changes; (5) applicability to both genotoxic and non-genotoxic agents; and, more importantly, (6) a greater number of detectable epigenetic changes as compared to the genetic alterations after exposure.

Also, the incorporation of epigenetic technologies into the studies of cancer risk promises to enhance substantially the efficiency of carcinogenicity testing. More importantly, the reversibility of epigenetic alterations opens novel mechanism-based approaches not only to cancer treatment but also to the timely prevention of cancer [142]. However, despite a very promising outlook on the benefits of epigenetic biomarkers, additional studies are still needed to better define the nature and mechanisms of epigenetic abnormalities with respect to carcinogenic processes [60, 143, 144]. Although extensive studies have identified a number of cancer-related epigenetic abnormalities that are associated with carcinogen exposure, there is no consensus on the role of changes in tumorigenesis.

Additionally, it is possible that not all these aberrant epigenetic events are equally important for the tumorigenic process. It is highly unlikely that all of these epigenetic changes play a causative role in tumorigenesis. For example, some epigenetic changes may drive other epigenetic events that contribute to the formation of a transformed phenotype, while others may be passenger epigenetic events that accompany the transformation process [145]. In this respect, the identification of those epigenetic events that drive cell transformation is crucially important for understanding mechanisms of tumorigenesis and for cancer prevention.

References

1. Hanahan D, Weinberg RA (2011) Hallmarks of cancer. Cell 144:646–674
2. Jones PA, Baylin SB (2007) The epigenomics of cancer. Cell 128:683–692
3. Ushijima T, Asada K (2010) Aberrant DNA methylation in contrast with mutations. Cancer Sci 101:300–305
4. Sharma S, Kelly TK, Jones PA (2010) Epigenetics in cancer. Carcinogenesis 31:27–36
5. Marlowe J, Teo SS, Chibout SD, Pognan F, Moggs JJ (2009) Mapping the epigenome—impact for toxicology. EXS 99:259–288
6. Kim JK, Samaranayake M, Pradhan S (2009) Epigenetic mechanisms in mammals. Cell Mol Life Sci 66:596–612
7. Ooi SK, O'Donnell AH, Bestor TH (2009) Mammalian cytosine methylation at a glance. J Cell Sci 122:2787–2791

8. Chen ZX, Riggs AD (2011) DNA methylation and demethylation in mammals. J Biol Chem 286:18347–18353
9. Jenuwein T, Allis CD (2001) Translating the histone code. Science 293:1074–1080
10. Kouzarides T (2007) Chromatin modifications and their function. Cell 128:693–705
11. Esteller M (2008) Epigenetics in cancer. N Engl J Med 358:1148–1159
12. Pogribny IP, Beland FA (2009) DNA hypomethylation in the origin and pathogenesis of human diseases. Cell Mol Life Sci 66:2249–2261
13. Ooi SK, Bestor TH (2008) The colorful history of active DNA demethylation. Cell 133:1145–1148
14. Ma DK, Guo JU, Ming GL, Song H (2009) DNA excision repair proteins and Gadd45 as molecular players for active DNA demethylation. Cell Cycle 8:1526–1531
15. He YF, Li BZ, Li Z, Wang Y, Tang Q, Ding J, Jiz Y, Chen Z, Li L, Sun Y, Li X, Dai Q, Song CX, Zhang K, He C, Xu GL (2011) Tet-mediated formation of 5-hydroxylcytosine and its excision by TDG in mammalian DNA. Science 333:1303–1307
16. Ito S, Shen L, Dai Q, Wu SC, Collins LB, Swenberg JA, He C, Zhang Y (2011) Tet proteins can convert 5-methylcytosine to 5-formylcytosine and 5-carboxylcytosine. Science 333: 1300–1333
17. Cortellino S, Xu J, Sannai M, Moore R, Caretti E, Cigliano A, Le Coz M, Devarajan K, Wessels A, Soprano D, Abramowitz LK, Bartolomei MS, Rambow F, Bassi MR, Bruno T, Fanciulli M, Renner C, Klein-Szanto AJ, Matsumoto Y, Kobi D, Davidson I, Alberti C, Larue L, Bellacosa A (2011) Thymine DNA glycosylase is essential for active DNA demethylation by linked deamination-base excision repair. Cell 146:67–79
18. Feinberg AP, Tycko B (2004) The history of cancer epigenetics. Nat Rev Cancer 4:143–153
19. Ehrlich M (2009) DNA hypomethylation in cancer cells. Epigenomics 1:239–259
20. Wilson AS, Power BE, Molloy PL (2007) DNA hypomethylation and human diseases. Biochim Biophys Acta 1775:138–162
21. De Smet C, Loriot A (2010) DNA hypomethylation in cancer: Epigenetic scars of a neoplastic journey. Epigenetics 5:206–213
22. Rollins RA, Haghighi F, Edwards JR, Das R, Zhang MQ, Ju J, Bestor TH (2006) Large-scale structure of genomic methylation patterns. Genome Res 16:157–163
23. Chen RZ, Pettersson U, Beard C, Jackson-Grusby L, Jaenisch R (1998) DNA hypomethylation leads to elevated mutation rates. Nature 395:89–93
24. Ehrlich M (2002) DNA methylation in cancer: too much, but also too little. Oncogene 21:5400–5413
25. Feinberg AP, Cui H, Ohlsson R (2002) DNA methylation and genomic imprinting: insights from cancer into epigenetic mechanisms. Semin Cancer Biol 12:389–398
26. Howard G, Eiges R, Gaudet F, Jaenisch R, Eden A (2008) Activation and transposition of endogenous retroviral elements in hypomethylation induced tumors in mice. Oncogene 27:404–408
27. Eden A, Gaudet F, Waghmare A, Jaenisch R (2003) Chromosomal instability and tumors promoted by DNA hypomethylation. Science 300:455
28. Wong NC, Wong LH, Quach JM, Canham P, Craig JM, Song IZ, Clark SJ, Choo KH (2006) Permissive transcriptional activity at the centromere through pockets of DNA hypomethylation. PLoS Genet 2:e17
29. Vera E, Canela A, Fraga MF, Esteller M, Blasco MA (2008) Epigenetic regulation of telomeres in human cancer. Oncogene 27:6817–6833
30. Wild L, Flanagan JM (2010) Genome-wide hypomethylation in cancer may be a passive consequence of transformation. Biochim Biophys Acta 1806:50–57
31. Wainfan E, Poirier LA (1992) Methyl groups in carcinogenesis: effects on DNA methylation and gene expression. Cancer Res 52:2071s–2077s
32. Christman JK (1995) Dietary effects on DNA methylation: do they account for hepatocarcinogenic properties of lipotrope diets? Adv Exp Med Biol 369:141–154
33. Pogribny IP, James SJ, Jernigan S, Pogribna M (2004) Genomic hypomethylation is specific for preneoplastic liver in folate/methyl deficient rats and does not occur in non-target tissues. Mutat Res 548:53–59

34. Gaudet F, Hodgson JG, Eden A, Jackson-Grusby L, Dausman J, Gray JW, Leonhardt H, Jaenisch R (2003) Induction of tumors in mice by genomic hypomethylation. Science 300:489–492
35. Fan T, Schmidtmann A, Xi S, Briones V, Zhu H, Suh HC, Gooya J, Keller JR, Xu H, Roayaei J, Anver M, Ruscetti S, Muegge K (2008) DNA hypomethylation caused by Lsh deletion promotes erythroleukemia development. Epigenetics 3:134–142
36. Counts JL, Goodman JI (1994) Hypomethylation of DNA: an epigenetic mechanism involved in tumor promotion. Mol Carcinog 11:185–188
37. Wild L, Funes JM, Boshoff C, Flanagan JM (2010) In vitro transformation of mesenchymal stem cells induces gradual genomic hypomethylation. Carcinogenesis 31:1854–1862
38. Bagnyukova TV, Tryndyak VP, Montgomery B, Churchwell MI, Karpf AR, James SR, Muskhelishvili L, Beland FA (2008) Genetic and epigenetic changes in rat preneoplastic liver tissue induced by 2-acetylaminofluorene. Carcinogenesis 29:638–646
39. Yamada Y, Jackson-Grusby L, Linhart H, Meissner A, Eden A, Lin H, Jaenish R (2005) Opposing effects of DNA hypomethylation on intestinal and liver carcinogenesis. Proc Natl Acad Sci USA 102:13580–13585
40. Tomita H, Hirata A, Yamada Y, Hata K, Oyama T, Mori H, Yamashita S, Ushijima T, Hara A (2010) Suppressive effect of global DNA hypomethylation on gastric carcinogenesis. Carcinogenesis 31:1627–1633
41. Kinney SR, Moser MT, Pascual M, Greally JM, Foster BA, Karpf AR (2010) Opposing roles of Dnmt1 in early- and late-stage murine prostate cancer. Mol Cell Biol 30:4159–4174
42. Maunakea AK, Nagarajan RP, Bilenky M, Ballinger TJ, D'Souza C, Fouse SD, Johnson BE, Hong C, Nielsen C, Zhao Y, Turecki G, Delaney A, Varhol R, Thiessen N, Shchors K, Heine VM, Rowitch DH, Xing X, Fiore C, Schillebeeckx M, Jones SJM, Haussler D, Marra MA, Hirst M, Wang T, Costello JF (2010) Conserved role of intragenic DNA methylation in regulating alternative promoters. Nature 466:253–257
43. Takai D, Jones PA (2002) Comprehensive analysis of CpG islands in human chromosomes 21 and 22. Proc Natl Acad Sci USA 99:3740–3745
44. Illingworth RS, Bird AP (2009) CpG islands—"a rough guide". FEBS Lett 583:1713–1720
45. Deaton AM, Bird A (2011) CpG islands and the regulation of transcription. Genes Dev 25:1010–1022
46. Jacinto FV, Esteller M (2007) Mutator pathways unleashed by epigenetic silencing in human cancer. Mutagenesis 22:247–253
47. Lahtz C, Pfeifer GP (2011) Epigenetic changes of DNA repair genes in cancer. J Mol Cell Biol 3:51–58
48. Esteller M, Toyota M, Sanchez-Cespedes M, Capella G, Peinado MA, Watkins DN, Issa JP, Sidransky D, Baylin SB, Herman JG (2000) Inactivation of the DNA repair gene O6-methylguanine-DNA methyltransferase by promoter hypermethylation is associated with G to A mutations in K-ras in colorectal tumorigenesis. Cancer Res 60:2368–2371
49. Esteller M, Riques RA, Toyota M, Capella G, Moreno V, Peinado MA, Baylin SB, Herman JG (2001) Promoter hypermethylation of the DNA repair gene O6-methylguanine-DNA methyltransferase is associated with the presence G:C to A:T transition mutations in p53 in human colorectal tumorigenesis. Cancer Res 61:4689–4692
50. Birgisdottir V, Stefansson OA, Bodvarsdottir SK, Hilmarsdottir H, Jonasson JG, Eyfjorf JE (2006) Epigenetic silencing and deletion of the BRCA1 gene in sporadic breast cancer. Breast Cancer Res 8:R38
51. Herman JG, Umar A, Polyak K, Graff JR, Ahuja N, Issa JP, Markowitz S, Willson JK, Hamilton SR, Kinzler KW, Kane MF, Kolodner RD, Vogelstein B, Kunkel TA, Baylin SB (1998) Incidence and functional consequences of hMLH1 promoter hypermethylation in colorectal carcinoma. Proc Natl Acad Sci USA 95:6870–6875
52. Fraga MF, Ballestar E, Villar-Garea A, Boix-Chornet M, Espada J, Schotta G, Bonaldi T, Haydon C, Ropero S, Petrie K, Lyer NG, Pérez-Rosado A, Calvo E, Lopez JA, Cano A, Calasanz MJ, Colomer D, Piris MA, Ahn N, Imhof A, Caldas C, Jenuwein T, Esteller M (2005) Loss of acetylation at Lys16 and trimethylation at Lys20 of histone H4 is a common hallmark of human cancer. Nat Genet 37:391–400

53. Bartel DP (2009) MicroRNAs: target recognition and regulatory functions. Cell 136:215–233
54. Carthew RW, Sontheimer EJ (2009) Origins and mechanisms of miRNAs and siRNAs. Cell 136:642–655
55. Guo H, Ingolia NT, Weissman JS, Bartel DP (2010) Mammalian microRNAs predominantly act to decrease target mRNA levels. Nature 466:835–840
56. Garzon R, Calin GA, Croce CM (2009) MicroRNAs in cancer. Annu Rev Med 60:167–179
57. Ventura A, Jacks ST (2009) MicroRNAs and cancer: short RNAs go a long way. Cell 136:586–591
58. Di Leva G, Croce CM (2010) Roles of small RNAs in tumor formation. Trends Mol Med 16:257–267
59. Pogribny IP (2009) MicroRNA dysregulation during chemical carcinogenesis. Epigenomics 1:281–290
60. Loeb LA, Harris CC (2008) Advances in chemical carcinogenesis: a historical review and prospective. Cancer Res 68:6863–6872
61. Wild CP (2009) Environmental exposure measurement in cancer epidemiology. Mutagenesis 24:117–125
62. Clapp RW, Jacobs MM, Loechler EL (2008) Environmental and occupational causes of cancer: new evidence 2005–2007. Rev Environ Health 23:1–37
63. Miller JA (1970) Carcinogenesis by chemicals: and overview—G.H.A. Clowes memorial lecture. Cancer Res 30:559–576
64. Herceg Z (2007) Epigenetics and cancer: towards an evaluation of the impact of environmental and dietary factors. Mutagenesis 22:91–103
65. Pogribny IP, Rusyn I, Beland FA (2008) Epigenetics aspects of genotoxic and non-genotoxic hepatocarcinogenesis: studies in rodents. Environ Mol Mutagen 49:9–15
66. Bollati V, Baccareli A (2010) Environmental epigenetics. Heredity 105:105–112
67. Nakajima T, Enomoto S, Ushijima T (2008) DNA methylation: a marker for carcinogen exposure and cancer risk. Environ Health Prev Med 13:8–15
68. Ziech D, Franco R, Pappa A, Malamou-Mitsi V, Georgakila S, Georgakitas AG, Panayiotidis MI (2010) The role of epigenetics in environmental and occupational carcinogenesis. Chem Biol Interact 188:340–349
69. Hughes MF, Beck BD, Chen Y, Lewis AS, Thomas DJ (2011) Arsenic exposure and toxicology: a historical perspective. Toxicol Sci 123:305–332
70. Rossman TG (2003) Mechanism of arsenic carcinogenesis: an integrated approach. Mutat Res 533:37–65
71. IARC Monographs on the Evaluations of Carcinogenic Risks to Humans (2004) Some drinking-water disinfectants and contaminants, including arsenic, vol 84. IARC, Lyon
72. Liu J, Waalkes MP (2008) Liver is a target of arsenic carcinogenesis. Toxicol Sci 105:24–32
73. Cui X, Wakai T, Shirai Y, Hatakeyama K, Hirano S (2006) Chronic oral exposure to inorganic arsenate interferes with methylation status of p16INK4A and RASSF1A and induces lung cancer in A/J mice. Toxicol Sci 91:372–381
74. Waalkes MP, Ward JM, Liu J, Diwan BA (2003) Transplacental carcinogenicity of inorganic arsenic in the drinking water: induction of hepatic, ovarian, pulmonary, and adrenal tumors in mice. Toxicol Appl Pharmacol 186:7–17
75. Tokar EJ, Diwan BA, Ward JM, Delker DA, Waalkes MP (2011) Carcinogenic effects of "whole-life" exposure to inorganic arsenic in CD1 mice. Toxicol Sci 119:73–83
76. Reichard JF, Puga A (2010) Effects of arsenic exposure on DNA methylation and epigenetic gene regulation. Epigenomics 2:87–104
77. Zhao CQ, Young MR, Diwan BA, Coogan TP, Waalkes MP (1997) Association of arsenic-induced malignant transformation with DNA hypomethylation and aberrant gene expression. Proc Natl Acad Sci USA 94:10907–10912
78. Chen H, Li S, Liu J, Diwan BA, Barrett JC, Waalkes MP (2004) Chronic inorganic arsenic exposure induces hepatic global and individual gene hypomethylation: implications for arsenic hepatocarcinogenesis. Carcinogenesis 25(9):1779–1786

79. Jensen TJ, Novak P, Elbin KE, Gandolfi AJ, Futscher BW (2008) Epigenetic remodeling during-arsenical-induced malignant transformation. Carcinogenesis 29:1500–1508
80. Ren X, McHale CM, Skibola CF, Smith AH, Smith MT, Zhang L (2011) An emerging role for epigenetic dysregulation in arsenic toxicity and carcinogenesis. Environ Health Perspect 119:11–19
81. Coppin JF, Qu W, Waalkes MP (2008) Interplay between cellular methyl metabolism and adaptive efflux during oncogenic transformation from chronic arsenic exposure in human cells. J Biol Chem 283:19342–19350
82. Kitchin KT, Ahmad S (2003) Oxidative stress as a possible mode of action for arsenic carcinogenesis. Toxicol Lett 137:3–13
83. Kojima C, Ramirez DC, Tokar EJ, Himeno S, Drobná Z, Stýblo M, Mason RP, Waalkes MP (2009) Requirement of arsenic biomethylation for oxidative DNA damage. J Natl Cancer Inst 101:1670–1681
84. Valinluck V, Sowers LC (2007) Endogenous cytosine damage products alter the site selectivity of human DNA maintenance methyltransferase DNMT1. Cancer Res 67:946–950
85. Lee DH, Jacobs DR Jr, Porta M (2009) Hypothesis: a unifying mechanism for nutrition and chemicals as lifelong modulators of DNA hypomethylation. Environ Health Perspect 117:1799–1802
86. Mass MJ, Wang L (1997) Arsenic alters cytosine methylation patterns of the promoter of the tumor suppressor gene p53 in human lung cells: a model for a mechanism of carcinogenesis. Mutat Res 386:263–277
87. Huang YC, Huang WC, Chen WT, Yu HS, Chai CY (2009) Sodium arsenite-induced DAPK hypermethylation and autophagy via ERK1/2 phosphorylation in human uroepithelial cells. Chem Biol Interact 181:254–262
88. Zhou X, Sun H, Ellen TP, Chen H, Costa M (2008) Arsenite alters global histone H3 methylation. Carcinogenesis 29:1831–1836
89. Beezhold K, Liu J, Kan H, Meighan T, Castranova V, Shi X, Chen F (2011) miR-190-mediated downregulation of PHLP contributes to arsenic-induced Akt activation and carcinogenesis. Toxicol Sci 123(2):411–420
90. Salnikow K, Zhitkovich A (2008) Genetic and epigenetic mechanisms in metals carcinogenesis and cocarcinogenesis: nickel, arsenic, and chromium. Chem Res Toxicol 21:28–44
91. Martinez-Zamudio R, Ha HC (2011) Environmental epigenetics in metal exposure. Epigenetics 6(7):820–827
92. IARC Monographs on the Evaluations of Carcinogenic Risks to Humans (2008) 1,3-Butadiene, ethylene oxide and vinyl halides (vinyl fluoride, vinyl chloride, and vinyl bromide), vol 97. IARC, Lyon
93. Melnick RL, Sills RC (2001) Comparative carcinogenicity of 1,3-butadiene, isoprene and chloroprene in rats and mice. Chem Biol Interact 135–136:27–42
94. Walker VE, Walker DM, Meng Q, McDonald JD, Scott BR, Selikop SK, Claffey DJ, Upton PB, Powley MW, Swenberg JA, Henderson RF, Committee HR (2009) Genotoxicity of 1,3-butadiene and its epoxy intermediate. Res Rep Health Eff Inst 144:3–79
95. Koturbash I, Scherhag A, Sorrentino J, Sexton K, Bodnar W, Tryndyak V, Latendresse JR, Swenberg JA, Beland FA, Pogribny IP, Rusyn I (2011) Epigenetic alterations in liver of C57BL/6J mice after short-term inhalational exposure to 1,3-butadiene. Environ Health Perspect 119:635–640
96. Schotta G, Lachner M, Sarma K, Ebert A, Sengupta R, Reuter G, Reinberg D, Jenuwein T (2004) A silencing pathway to induce H3-K9 and H4-K20 trimethylation at constitutive heterochromatin. Genes Dev 18:1251–1262
97. Martens JH, O'Sullivan RJ, Braunschweig U, Opravil S, Radolf M, Steinlein P, Jenuwein T (2005) The profile of repeat-associated histone lysine methylation states in the mouse epigenome. EMBO J 24:800–812
98. Jenuwein Y (2006) The epigenetic magic of histone lysine methylation. FEBS J 273:3121–3135
99. Koturbash I, Scherhag A, Sorrentino J, Sexton K, Bodnar W, Swenberg JA, Beland FA, Pardo-Manuel Devillena F, Rusyn I, Pogribny IP (2011) Epigenetic mechanisms of mouse

interstrain variability in genotoxicity of the environmental toxicant 1,3-butadiene. Toxicol Sci 122:448–456
100. Rusyn I, Gatti DM, Wilshire T, Kleeberger SR, Threadgill DW (2010) Toxicogenomics: population-based testing of drug and chemical safety in mouse models. Pharmacogenomics 11:1127–1136
101. IARC Monographs on the Evaluations of Carcinogenic Risks to Humans (2011) A review of human carcinogens, vol 100, Part A: Pharmaceuticals. IARC, Lyon
102. Li S, Washburn KA, Moore R, Uno T, Teng C, Newbold RR, McLachlan JA, Negishi M (1997) Developmental exposure to diethylstilbestrol elicits demethylation of estrogen-responsive lactoferrin gene in mouse uterus. Cancer Res 57:4356–4359
103. Li S, Hansman R, Newbold R, Davis B, McLachlan JA, Barrett JC (2003) Neonatal diethylstilbestrol exposure induces persistent elevation of c-fos expression and hypomethylation in its exon-4 in mouse uterus. Mol Carcinog 38:78–84
104. Tang WY, Newbold R, Mardilovich K, Jefferson W, Cheng RY, Medvedovic M, Ho SM (2008) Persistent hypomethylation in the promoter of nucleosomal binding protein 1 (Nsbp1) with overexpression of Nsbp1 in mouse uteri neonatally exposed to diethylstilbestrol or genistein. Endocrinology 149:5922–5931
105. Sato K, Fukata H, Kogo Y, Ohgane J, Shiota K, Mori C (2009) Neonatal exposure to diethylstilbestrol alters expression of DNA methyltransferases and methylation of genomic DNA in the mouse uterus. Endocr J 56:131–139
106. Hsu PY, Detherage DE, Rodriguez BA, Liyanarachchi S, Weng YI, Zuo T, Liu J, Cheng AS, Huang TH (2009) Xenoestrogen-induced epigenetic repression of microRNA-9-3 in breast epithelial cells. Cancer Res 69:5936–5945
107. Lehmann U, Hasemeier B, Christgen M, Müller M, Römermann D, Länger F, Kreipe H (2008) Epigenetic inactivation of microRNA gene has-mir-9-1 in human breast cancer. J Pathol 214:17–24
108. Jordan VC (2006) Tamoxifen (ICI146,474) as a target therapy to treat and prevent breast cancer. Br J Pharmacol 147:S269–S276
109. Shang Y (2006) Molecular mechanisms of oestrogen and SERMs in endometrial carcinogenesis. Nat Rev Cancer 6:360–368
110. Wu H, Chen Y, Liang J, Shi B, Wu G, Zhang Y, Wang D, Li R, Yi X, Zhang H, Sun L, Shang Y (2005) Hypomethylation-linked activation of PAX2 mediates tamoxifen-stimulated endometrial carcinogenesis. Nature 438:981–987
111. Wogan GN (1997) Review of the toxicology of tamoxifen. Semin Oncol 24:S87–S97
112. Phillips DH (2001) Understanding the genotoxicity of tamoxifen? Carcinogenesis 22:839–849
113. Gamboa da Costa G, McDaniel-Hamilton LP, Heflich RH, Margues MM, Beland FA (2001) DNA adduct formation and mutant induction in Sprague–Dawley rats treated with tamoxifen and its derivatives. Carcinogenesis 22:130701315
114. Tryndyak VP, Muskhelishvili L, Kovalchuk O, Rodriguez-Juarez R, Churchwell MI, Ross SA, Beland FA, Pogribny IP (2006) Effect of long-term tamoxifen exposure on genotoxic and epigenetic changes in rat liver: implications for tamoxifen-induced hepatocarcinogenesis. Carcinogenesis 27:1713–1720
115. Tryndyak VP, Kovalchuk O, Muskhelishvili L, Montgomery B, Rodriguez-Juarez R, Melnyk S, Ross SA, Beland FA, Pogribny IP (2007) Epigenetic reprogramming of liver cells in tamoxifen-induced rat hepatocarcinogenesis. Mol Carcinog 46:187–197
116. Pogribny IP, Tryndyak VP, Boyko A, Rodriguez-Juarez R, Beland FA, Kovalchuk O (2007) Induction of microRNAome deregulation in rat liver by long-term tamoxifen exposure. Mutat Res 619:30–37
117. Bachman AN, Phillips JM, Goodman JI (2006) Phenobarbital induces progressive patterns of GC-rich and gene-specific altered DNA methylation in the liver of tumor-prone B6C3F1 mice. Toxicol Sci 91:393–405
118. Phillips JM, Goodman JI (2009) Multiple genes exhibit Phenobarbital-induced constitutive active/androstane receptor-mediated DNA methylation changes during liver tumorigenesis and in liver tumors. Toxicol Sci 108:273–289

119. Lempiäinen H, Müller A, Brasa S, Teo SS, Roloff TC, Morawiec L, Zamurovic N, Vicart A, Funhoff E, Couttet P, Schübeler D, Grenet O, Marlowe J, Moggs J, Terranova R (2011) Phenobarbital mediates an epigenetic switch at the constitutive androstane receptor (CAR) target gene Cyp2b10 in the liver of B6C3F1 mice. PLoS One 6:e18216
120. Bucher JR, Shackelford CC, Haseman JK, Johnson JD, Kurtz PJ, Persing RL (1994) Carcinogenicity studies of oxazepam in mice. Fundam Appl Toxicol 23:280–297
121. Iida M, Anna CH, Hartis J, Bruno M, Wetmore B, Dubin JR, Sieber S, Bennett L, Cunningham ML, Paules RS, Tomer KB, Houle CD, Merrick AB, Sills RC, Devereux TR (2003) Changes in global gene and protein expression during early mouse liver carcinogenesis induced by non-genotoxic model carcinogens oxazepam and Wyeth-14,643. Carcinogenesis 24:757–770
122. Lahousse SA, Hoenerhoff M, Collins J, Ton TV, Massinde T, Olson D, Rebolosso Y, Koujitani T, Tomer KB, Hong HH, Bucher J, Sills RC (2011) Gene expression and mutation assessment provide clues of genetic and epigenetic mechanisms in liver tumors of oxazepam-exposed mice. Vet Pathol 48:678–699
123. Ferguson LR, Philpott M (2008) Nutrition and mutagenesis. Annu Rev Nutr 28:313–329
124. IARC Monographs on the Evaluations of Carcinogenic Risks to Humans (2002) Some traditional herbal medicines, some mycotoxins, naphthalene and styrene, vol 82. IARC, Lyon
125. Wild CP, Gong YY (2010) Mycotoxins and human disease: a largely ignored global health issue. Carcinogenesis 31:71–82
126. Knasmüller S, Bresgen N, Kassie F, Mersch-Sundermann V, Gelderblom W, Zöhrer E, Eckl PM (1997) Genotoxic effects of three Fusarium mycotoxins, fumonisin B1, moniliformin and vomitoxin in bacteria and primary cultures of rat hepatocytes. Mutat Res 391:39–48
127. Wang JS, Groopman JD (1999) DNA damage by mycotoxins. Mutat Res 424:167–181
128. Zhang YJ, Ahsan H, Chen Y, Lunn RM, Wang LY, Chen SY, Lee PH, Chen CJ, Santella RM (2002) High frequency of promoter hypermethylation of RASSF1A and p16 and its relationship to aflatoxin B1-DNA adduct levels in human hepatocellular carcinoma. Mol Carcinog 35:85–92
129. Zhang YJ, Chen Y, Ahsan H, Lunn RM, Chen SY, Santella RM (2003) Inactivation of the DNA repair gene O6-methylguanine-DNA methyltransferase by promoter hypermethylation and its relationship to aflatoxin B1-DNA adducts and the p53 mutations in hepatocellular carcinoma. Int J Cancer 103:440–444
130. Su H, Zhao J, Xiong Y, Xu T, Zhou F, Yuan Y, Zhang Y, Zhuang SM (2008) Large-scale analysis of the genetic and epigenetic alterations in hepatocellular carcinoma from Southeast China. Mutat Res 641:27–35
131. Zhao W, Liu H, Liu W, Wu Y, Chen W, Jiang B, Zhou Y, Xue R, Luo C, Wang L, Jiang JD, Liu J (2006) Abnormal activation of the synuclein-gamma gene in hepatocellular carcinomas by epigenetic alteration. Int J Oncol 28:1081–1088
132. Kouadio JH, Dano SD, Moukha S, Mobio TA, Creppy EE (2007) Effects of combinations of Fusarium mycotoxins on the inhibition of macromolecular synthesis, malondialdehyde levels, DNA methylation and fragmentation, and viability in Caco-2 cells. Toxicon 49:306–317
133. Marin-Kuan M, Cavin C, Delatour T, Schilter B (2008) Ochratoxin A carcinogenicity involves a complex network of epigenetic mechanisms. Toxicon 52:195–202
134. Hu W, Feng Z, Tang M (2003) Preferential carcinogen-DNA adduct formation at codons 12 and 14 in the human K-ras gene and their possible mechanisms. Biochemistry 42: 10012–10023
135. IARC Monographs on the Evaluations of Carcinogenic Risks to Humans (2011) A review of human carcinogens, vol 100, Part B: Biological agents. IARC, Lyon
136. Maekita T, Nakazawa K, Mihara M, Nakajima T, Yanaoka K, Iguchi M, Arii K, Kaneda A, Tsukamoto T, Tatematsu M, Tamura G, Saito D, Sugimura T, Ichinose M, Ushijima T (2006) High levels of aberrant DNA methylation in Helicobacter pylori-infected gastric mucosae and its possible association with gastric cancer risk. Clin Cancer Res 12:989–995
137. Nakajima T, Yamashita S, Maekita T, Niwa T, Nakazawa K, Ushijima T (2009) The presence of methylation fingerprint of Helicobacter pylori infection in human gastric mucosae. Int J Cancer 124:905–910

138. Niwa T, Tsukamoto T, Toyoda T, Mori A, Tanaka H, Maekita T, Ichinose M, Tatematsu M, Ushijima T (2010) Inflammatory processes triggered by Helicobacter pylori infection cause aberrant DNA methylation in gastric epithelial cells. Cancer Res 70:1430–1440
139. Shin CM, Kim N, Jung Y, Park JH, Kang GH, Kim JS, Jung HC, Song IS (2010) Role of Helicobacter pylori infection in aberrant DNA methylation along multistep gastric carcinogenesis. Cancer Sci 101:1337–1346
140. Nakajima T, Enomoto S, Yamashita S, Ando T, Nakanishi Y, Nakazawa K, Oda I, Gotoba T, Ushijima T (2010) Persistence of a component of DNA methylation in gastric mucosa after Helicobacter pylori eradication. J Gastroenterol 45:37–44
141. LeBaron MJ, Rasoulpour RJ, Klapacz J, Ellis-Hutchings RG, Hollnagel HM, Gollapudi BB (2010) Epigenetics and chemical safety assessment. Mutat Res 705:83–95
142. Huang YW, Kuo CT, Stoner K, Huang TH, Wang LS (2011) An overview of epigenetics and chemoprevention. FEBS Lett 585:2129–2136
143. Goodman JI, Augustine KA, Cunningham ML, Dixon D, Dragan YP, Falls JG, Rasoulpour RJ, Sills RC, Storer RD, Wolf DC, Pettit SD (2010) What do we need to know prior to thinking about incorporating an epigenetic evaluation into safety assessments? Toxicol Sci 116:375–381
144. Rasoulpour RJ, LeBaron MJ, Ellis-Hutchings RG, Klapacz J, Gpllapudi BB (2011) Epigenetic screening in product safety assessment: are we there yet? Toxicol Mech Methods 21:298–311
145. Kalari S, Pfeifer GP (2010) Identification of driver and passenger DNA methylation in cancer by epigenomic analysis. Adv Genet 70:277–308

Chapter 12
Blood-Derived DNA Methylation Markers of Cancer Risk

Carmen Marsit and Brock Christensen

Abstract The importance of somatic epigenetic alterations in tissues targeted for carcinogenesis is now well recognized and considered a key molecular step in the development of a tumor. Particularly, alteration of gene-specific and genomic DNA methylation has been extensively characterized in tumors, and has become an attractive biomarker of risk due to its specificity and stability in human samples. It also is clear that tumors do not develop as isolated phenomenon in their target tissue, but instead result from altered processes affecting not only the surrounding cells and tissues, but other organ systems, including the immune system. Thus, alterations to DNA methylation profiles detectable in peripheral blood may be useful not only in understanding the carcinogenic process and response to environmental insults, but can also provide critical insights in a systems biological view of tumorigenesis. Research to date has generally focused on how environmental exposures alter genomic DNA methylation content in peripheral blood. More recent work has begun to translate these findings to clinically useful endpoints, by defining the relationship between DNA methylation alterations and cancer risk. This chapter highlights the existing research linking the environment, blood-derived DNA methylation alterations, and cancer risk, and points out how these epigenetic alterations may be contributing fundamentally to carcinogenesis.

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12.1 Introduction

Epigenetic alterations within cells that give rise to tumors are believed to be causal contributors to the development of malignancy [27, 38]. The most widely studied epigenetic mechanism in cancer is DNA methylation and it is well recognized that cancer cells concomitantly exhibit both gene-specific increases in DNA methylation and genome-wide hypomethylation compared to their normal tissue counterparts. Because DNA methylation is tissue-specific, perhaps it is no surprise that a multitude of studies seeking to detect tumor-specific DNA methylation for early detection/diagnosis have used cell-free fractions (serum, plasma) of peripheral blood. Studies measuring DNA methylation in serum and plasma aim to reduce the potential noise contributed by leukocyte methylation patterns in whole blood and to specifically detect tumor-derived DNA methylation. However, interindividual variability in leukocyte methylation patterns may be—akin to genetic variation—related to an individual's cancer risk while acquired alterations to leukocyte methylation may represent both a cause and consequence of carcinogenesis in solid tissues. As new measurement technologies and analytic strategies are being developed, and there is an improved understanding of the contribution of the immune system to solid tumor development, there may be great utility in peripheral blood methylation analysis for predicting cancer risk.

This chapter will cover evidence from human studies that peripheral blood DNA methylation states can inform cancer risk. First, investigations of repetitive element and global DNA methylation will be presented. Then, epimutation and gene-specific methylation markers of cancer risk will be discussed, followed by more recent and larger-scale investigations of blood methylation and cancer risk. Notably, as mentioned above, cancer epigenetics includes a large body of research on cell-free (plasma, serum) DNA methylation for diagnostic and prognostic purposes that is not within this chapter's scope. To end, potential mechanisms underlying the basis for blood-based methylation markers of cancer risk, and future directions for this avenue of research will be covered.

12.2 Repetitive Elements, Global Methylation, and Cancer Risk

12.2.1 Introduction to Global Methylation and Repetitive Elements

While the classic example of altered DNA methylation in cancer would likely describe promoter hypermethylation-induced gene silencing of a tumor suppressor gene, before this phenomenon was understood it was recognized that tumors are heavily hypomethylated relative to their normal tissue counterparts. In contrast to tumor suppressor gene promoters, moderately to highly repetitive,

non-coding sequences of the genome are normally methylated [26, 63]. Indeed, generally non-specific methylation of repeat and non-coding elements is considered an important part of normal development, cellular differentiation, and X-chromosome inactivation. Hence, changes in this methylation can lead to specific human disease states including cancer. In fact, genomic or global hypomethylation is now thought to occur early in tumorigenesis, including in pre-cancerous lesions [60, 66, 67], and may promote cancer development by contributing to genomic instability.

A few studies have directly assessed the relationship between total genomic methyl-cytosine in blood and cancer risk. Pufulete and colleagues measured genome-wide reduction in 5-methylcytosine content with a (relatively insensitive) ^{3}H-thymidine incorporation assay in peripheral blood lymphocytes and found that hypomethylation significantly increased risk for colon adenoma and indicated a trend in risk of colon cancer [60]. In an investigation of colorectal adenoma among women, Lim et al. measured total genomic leukocyte methylation utilizing DNA digestion followed by liquid chromatography and mass spectrometry for quantitation in 230 cases and controls. When setting the referent group as the women in the *lowest tertile of methylation*, women in the second tertile had a reduced risk of colorectal adenoma (odds ratio (OR): 0.72, 95% CI: 0.34–1.52), and women in highest methylation tertile had a significantly decreased risk of colorectal adenoma (OR: 0.17, 95% CI: 0.06–0.49) [47]. Around the same time, a hospital-based case–control study in Spain found that reduced total percent methyl-cytosine content (using high-performance capillary electrophoresis, *HpaII* digestion, and densitometry) was significantly associated with bladder cancer risk [54]: compared to the quartile of subjects with the highest percent 5-methyl-cytosine, the adjusted OR for subjects in the lowest quartile of methylation was 2.67 (95% CI: 1.8–4.0). Further, when stratifying by smoking status, global hypomethylation was a strong risk factor for bladder cancer in never smokers (OR: 6.4, 95% CI: 2.4–17.2).

Early links between genomic hypomethylation and pathogenesis generated great interest in developing additional methods to determine global DNA methylation. Total genomic methyl-cytosine content can be directly measured, though large amounts of substrate and highly specialized equipment are required. In the mid 1990s, founded on the basis of chemical modification of DNA with sodium bisulfite, a PCR-based method for measuring DNA methylation was developed: methylation-specific PCR (MSP) [30]. Later, a quantitative version of MSP known as MethyLight was developed in Peter Laird's lab [21]. Using MethyLight to measure *LINE-1*, *Alu*, and satellite element repeats, Weisenberger et al. showed that methylation of repetitive elements were reasonably well correlated with total methyl-cytosine content [74]. Around the same time, the first report of bisulfite sequencing for *LINE-1* and *Alu* elements was published and was claimed as a simple method to estimate global DNA methylation [80]. Alone, *LINE-1* and *Alu* elements comprise about 30% of the human genome, making them an attractive target for a surrogate measure of global methylation [80]. With these more accessible methods to measure "global methylation", many groups began evaluating global methylation. As a result, the term "global methylation" lost its specific meaning

and started being used to describe any of these assays even though their measures are potentially non-comparable.

Because repetitive elements such as *LINE-1* and *Alu* are used to signify global methyl-cytosine content, it is important to clarify what these elements are and to point out potential drawbacks of using these as surrogate measures of global methylation. Long interspersed nuclear elements (*LINE-1*) and short interspersed nuclear elements (SINEs, which include *Alu* elements and mammalian interspersed repeats (MIR)), and long terminal repeats (LTRs) are retrotransposons. Collectively, with tandem repeats such as satellite elements (SAT), LINE, SINE, and LTR retrotransposons comprise approximately half of the human genome. The majority of these elements are evolutionary remnants that are truncated or mutated and even if transcribed would have no phenotype. For instance, there are approximately 500,000 *LINE-1* elements in the genome; very few of these are full-length (6 kb) complete with an internal RNA polymerase II promoter in the 5′ UTR, two open reading frames that encode an RNA-binding protein and elements for retrotransposon activity, and a 3′ UTR with a polyadenylation signal [17]. Unlike *LINE-1*, *Alu* elements use an internal RNA polymerase III promoter and lack any coding sequence. For retrotransposition, *Alu* elements require the retrotransposon machinery encoded by *LINE-1* elements [19]. LTRs are considered endogenous retroviruses, and with over 400,000 copies, these repeat elements account for 8% of the human genome [43]. Lastly, satellite repeated sequences (SAT) are small DNA transposons that are the oldest type of transposable element, having arisen as a result of simple repeat amplification [39, 43].

Because repeat elements can have transposition activity, largely outnumber coding genes and make up a large fraction of the genome, it is critical that they are appropriately regulated. Hence, in normal cells repeat elements are maintained as silenced with relatively high levels of DNA methylation in their promoter regions. However, if methylation is lost at repeat elements they may be re-expressed and insert into various regions of the genome, possibly leading to the inactivation of tumor suppressor genes, or activation of oncogenes, thereby contributing to cancer as well as other human diseases [18, 41].

12.2.2 Satellite Elements and Long Terminal Repeats

Although satellite elements and long terminal repeats are numerous and make up a considerable portion of the human genome, their potential role in carcinogenesis remains understudied. Nonetheless, initial investigations into LTR repeats in tumors have indicated that inappropriate activation of LTR repeats is linked to cancers. The methylation status of one type of LTR, the endogenous retrovirus type K (HERV-K) was hypomethylated in bladder tumor tissue compared to normal bladder [23]. Similarly, in a small number of ovarian tumors, HERV-W was hypomethylated compared to non-tumor tissue [53]. More recently, an examination of satellite repeat expression in pancreatic ductal adenocarcinomas revealed that HSATII

transcripts were highly cancer-specific, alpha satellite transcripts were abundantly expressed, and that increased satellite expression in these cancers was likely due to loss of methylation [71]. Unfortunately, to our knowledge there have not yet been any studies examining methylation of satellite or LTRs in blood to test for association with risk of cancer. However, as large-scale sequencing efforts continue, non-coding elements are becoming better annotated and may allow for better-informed approaches to investigate the potential role of satellite and LTR repeat methylation in blood as it relates to cancer risk [1].

12.2.3 Long Interspersed Nuclear Elements and Alu elements

Using bisulfite pyrosequencing assays, a number of studies on *LINE-1* methylation in human peripheral blood have now been conducted. First, it is interesting to note that there are several studies investigating the association of *LINE-1* methylation in blood DNA with exposures that are etiologically relevant to human cancers. Examples of exposures that are associated with *LINE-1* hypomethylation include benzene [10], particulate matter including traffic particles [4, 68], polycyclic aromatic hydrocarbons [58], and persistent organic pollutants [62].

One of the first case–control studies of cancer to measure *LINE-1* methylation in blood was conducted in head and neck squamous cell carcinoma (HNSCC) [36]. Hsiung et al. measured *LINE-1* methylation with a modified version of combined bisulfite restriction analysis in over 800 HNSCC cases and controls. The between-subject variability in *LINE-1* methylation ranged from 54 to 87%, with a significant ($P<0.002$) increase in the *LINE-1* methylation in males compared to females, and significant increases in *LINE-1* methylation associated with positive HPV16 antibody serology and for subjects of non-Caucasian race compared to Caucasians ($P<0.02$ and $P<0.03$, respectively). In cases, controlling for age, gender, race, lifetime average drinks per week, and HPV16 serology, dietary folate in the lowest tertile, compared to the upper two tertiles, had a borderline significant reduction in *LINE-1* methylation. Similarly, subjects with the *MTHFR* 677 variant had a significant ($P<0.04$) reduction in *LINE-1* methylation; whereas, among cases, smoking was significantly associated ($P<0.04$) with increased *LINE-1* methylation. With respect to risk of HNSCC, patients in the lowest tertile of *LINE-1* methylation had a significant relative risk of HNSCC (OR: 1.6, 95% CI: 1.1–2.4), while those in the mid tertile showed an elevated OR of 1.3 (95% CI: 0.9–2.0) when controlling for age, gender, race, smoking, drinking, and HPV16 serology. Across tertiles there was a significant trend ($P<0.03$) for increased HNSCC risk with lower *LINE-1* methylation, and suggested that epigenetic variation, in this case extent of repetitive region methylation, is associated with disease risk [36].

In a study of breast cancer risk, Choi et al. measured *both* total methyl-cytosine content and *LINE-1* methylation in blood DNA from cases and controls [15]. With 176 cases and 173 controls, the authors first measured methyl-cytosine content and *LINE-1* methylation in a pilot subset of 19 cases and 18 controls, and found that

cases had significantly reduced methyl-cytosine content ($P=0.001$) compared to controls, whereas *LINE-1* methylation was not associated with case status or correlated ($r=-0.2$, $P=0.23$). Based on the results from the pilot cases and controls, the remaining cases and controls were evaluated for total methyl-cytosine only. Among several demographic factors examined (including age, race, BMI, smoking, parity, and menopausal status), high alcohol intake (>median) was the only factor significantly associated with reduced methyl-cytosine, and this was true in each of the case ($P<0.04$) and control groups ($P<0.04$). Further, among all cases and controls total methyl-cytosine content in blood DNA was significantly lower in cases than controls: when compared to women in the highest tertile of methylation, women in the lowest tertile of methylation had a significantly increased risk of breast cancer (OR: 2.9, 95% CI: 1.7–4.9). Despite the association between methyl-cytosine levels and alcohol intake, alcohol consumption did not affect the association between methyl-cytosine content and breast cancer risk. However, when stratifying on demographic and lifestyle factors, the authors found that risk was further increased by lower methyl-cytosine content in women with a family history of disease, as well as among women who were never smokers.

Studying the risk of gastric cancer in relation to repeat element methylation, Hou et al. used pyrosequencing and measured both *LINE-1* and *Alu* methylation in blood DNA from 302 gastric cancer cases and 421 age- and gender-matched controls [35]. This population-based case–control study enrolled participants from Warsaw, Poland. Methylation data were stratified into tertiles and in an analysis adjusted for age, sex, education level, smoking, and alcohol there were borderline significant associations between reduced methylation and gastric cancer risk for *LINE-1* (OR: 1.4, 95% CI: 0.9–2.0) and *Alu* (OR: 1.3, 95% CI: 0.9–1.9). Yet, in stratified analyses the association between *LINE-1* hypomethylation and gastric cancer risk was stronger for individuals with a family history of disease (OR: 3.1, 95% CI: 1.4–7.0), current drinkers of alcohol (OR: 1.9, 95% CI: 1.0–3.6), current smokers (OR: 2.3, 95% CI: 1.1–4.6), subjects who rarely or never consumed fruit, as well as carriers of either of two polymorphisms in 5-methyltetrahydrofolate-homocysteine methyltransferase reductase (*MTRR*). However, associations between *LINE-1* methylation and cancer risk were not modified by sex, infection with *Helicobacter pylori*, or intake of protein, vitamin B6, or folate.

An investigation of *LINE-1* blood DNA methylation and bladder cancer risk in a population-based case–control study in New Hampshire also indicated that reduced *LINE-1* methylation is associated with cancer risk [76]. Among 285 cases and 465 controls, *LINE-1* methylation values from bisulfite pyrosequencing ranged from 58 to 92%. Comparing subjects in the lowest methylation decile to all other subjects, controlling for age, gender, and smoking status indicated a significantly increased risk of bladder cancer for the lowest decile subjects (OR: 1.8, 95% CI: 1.1–2.9). In addition, these authors stratified their analysis by invasive and non-invasive disease and found that the lowest decile of *LINE-1* methlyation was associated with a significantly increased risk of non-invasive disease, but not invasive disease. Similar to the results from Hsiung et al., which showed that males had significantly higher *LINE-1* methylation levels, Wilhelm et al. stratified their analysis by gender and

found the association between *LINE-1* hypomethylation and bladder cancer to be stronger in women than in men (OR_{women}: 2.5, 95% CI: 1.2–5.2; OR_{men}: 1.5, 95% CI: 0.8–2.7). Finally, recalling the studies of *LINE-1* methylation and environmental exposures with etiologic relevance to cancer, these authors showed a significant association between high exposure to arsenic and reduced *LINE-1* methylation in control subjects.

Along with the studies of global and repeat element hypomethylation and bladder cancer risk in Europeans from Moore et al., and in American Caucasians from Wilhelm et al., a third study in Chinese subjects has also been published [13]. Among 510 cases and 528 controls from a case–control study based in Shanghai China, *LINE-1* methylation values from bisulfite pyrosequencing ranged from 73 to 93%. Notably, the low-end of *LINE-1* methylation in these subjects was higher than studies of Caucasians, 73% compared to 58% from [36], and 53% from [76]. Nonetheless, similar to previous research, men had significantly higher *LINE-1* methylation than women ($P = 0.004$), and perhaps some of the disparity in low-range methylation among studies may be attributable to a higher prevalence of men in this study (77%) compared to the studies from Hsiung et al. (69%) and Wilhelm et al. (69%). Among all cases and controls in adjusted models comparing tertiles of *LINE-1* methylation, the lowest methylation tertile compared to the highest revealed an elevated risk of bladder cancer that did not reach statistical significance (OR: 1.3, 95% CI: 0.95–1.7). However, when stratifying by smoking status, never smokers in the lowest tertile of *LINE-1* methylation had a significantly increased risk of bladder cancer (OR: 1.9, 95% CI: 1.2–3.1). Further, lifelong non-smokers with *GSTM1* and/or *GSTT1* null genotypes had an even higher risk of bladder cancer (OR: 2.4, 95% CI: 1.3–4.1).

12.2.4 Challenges and Caveats

Despite recent advances in measuring repetitive element methylation with bisulfite pyrosequencing, a full understanding of the biology of these elements is still lacking, and there are technical limitations that should be carefully considered. Although reported in numerous studies, the relatively greater extent of methylation of *LINE-1* elements in men compared to women is not understood, and may represent fundamental differences that need to be further explored. Though the CpGs targeted for methylation measurement in pyrosequencing assays are generally 3–6 CpG sites in the 5′ UTR, because it is unclear how many of the 500,000 *LINE-1* elements are full length (6 kb) it is not known *how many* copies of *LINE-1* elements are actually being measured in any given individual. From an evolutionary standpoint the newer *LINE-1* sequences are more likely to be fully intact, though the 5′ end of the repeat can be deleted and it is not yet known what the prevalence of these deletions are. In addition, the total number of newer *LINE-1* sequence elements is polymorphic in the population. Together, these issues make it difficult if not impossible to know how many *LINE* elements are being measured and whether the number is similar across samples

or individuals. So, although a number of studies are now identifying and confirming associations between epigenetic alterations to these elements and cancer, the biological mechanism towards carcinogenesis that these observations represent is not understood. A more in-depth treatment of the challenges associated with repeat element and global methylation measures is available in Nelson et al. [56].

12.3 Gene-Specific Methylation and Epimutation

12.3.1 Epimutation

There is growing recognition that gene-specific soma-wide and/or germline DNA methylation, often called epimutation, can predispose individuals to cancer [20, 31]. Initial work in the area of epimutation identified changes to gene imprinting status that was phenotypically equivalent to disease attributable to genetic alterations. For example, Wilm's tumor can derive from inherited mutation in the *IGF2* gene leading to a change in the imprinting status and therefore the biallelic expression of this gene. A change in the DNA methylation status of the maternally imprinted allele without change to the underlying sequence can lead to loss of imprinting (LOI) at the *H19/IGF2* locus, which similarly results in biallelic expression and risk for Wilm's tumor [52, 65]. A number of other imprinting disorders have been identified and have been linked not only with genetic etiology but also epimutations, including Beckwith–Wiedeman, Silver–Russel, Prader–Willi, and Angelman syndromes [28, 57]. Epimutations resulting in LOI are relatively rare due to the scarcity of imprinted genes in the genome.

Epimutations also have been shown to occur in the context of biallelic expression and such epimutations have been linked to cancer. For example, 37% of individuals presenting with Cowden's syndrome or with Cowden-like features, but without genetic alteration to the *PTEN* gene, harbor germline allele-specific DNA methylation upstream of *PTEN*. This leads to reduced expression of the *KILLIN* gene and a greatly increased risk of breast and renal cancer [7]. Similarly, a subset of familial breast and ovarian cancer without *BRCA1* or *BRCA2* mutation is linked to mosaic epimutation of *BRCA1* [78], and hereditary non-polyposis colorectal cancer (HNPCC) without germline mutation is observed with allele-specific mosaic methylation of *MLH1* or *MSH2* [14, 32–34]. In some cases, multiple generations of individuals within these HNPCC families could be identified [33, 55]. In other cases, the aberrant methylation present in the affected individuals germline (i.e., spermatozoa), could not be identified in family members, suggesting a potential de novo germline or early embryonic event [32, 66, 67]. This lack of a consistent direct inheritance of the epimutation itself, but the potential for familial transmission suggests that it may, in fact, be a predisposition to epimutation in general that is truly being inherited.

These are examples of highly penetrant but rare epimutation in genes known to contribute to specific disease. Such findings are analogous to decades of work in

genetic susceptibility to cancer which originated with studies of highly penetrant, rare mutations leading to rare genetic disease and provided the profound understanding of the key genes involved in tumorigenesis. As genetic susceptibility studies later evolved into the investigation of more common, polymorphic variation associated with sporadic cancer, so has the study of epimutation begun to move beyond these rare variants to studies of common epigenetic variability association with common disease.

12.3.2 Gene-specific methylation

A number of investigations of peripheral blood DNA methylation have focused on the examination of candidate tumor suppressor gene methylation, taking cues from the alterations detected in targeted solid tissues to define candidates. Often these studies are based upon the assumption that the alterations driving carcinogenesis in a target tissue will be identified in blood and potentially other non-target tissues, although the somatic nature of methylation would argue against such assumption. Nevertheless, there is a large and growing literature utilizing candidate approaches to examine in populations single and multi-gene panels of candidate tumor suppressor genes in peripheral blood as markers of solid tumor risk.

Wong and colleagues [77] measured *CDKN2B* methylation in buffy coat DNA from 15 hepatocellular carcinoma (HCC) patients, 15 patients without cancer but with chronic hepatitis/cirrhosis, and 20 healthy controls with MSP. Among the 15 HCC patients, eight had *CDKN2B* methylation buffy coat DNA, whereas none of the healthy controls or individuals with hepatitis/cirrhosis had methylated *CDKN2B*. Further, the eight HCC patients with *CDKN2B* methylation in blood DNA also had *CDKN2B* methylation in their tumor tissue.

In colon cancer, Ally and colleagues measured methylation in blood DNA from 27 cases, 30 individuals with adenoma, 16 with hyperplastic polyps, and 57 disease-free controls [2]. Using bisulfite pyrosequencing the authors examined seven CpG sites in the promoter region of the estrogen receptor alpha gene (*ESR1*) and across all subjects the methylation of *ESR1* ranged from 0 to 13% (median, 4.3%). Across disease groups there was not a difference in *ESR1* methylation ($P > 0.05$). However, *ESR1* methylation was 60% lower in peripheral blood samples than in normal colonic tissues. Further, the authors observed a correlation between colonic tissue methylation and blood methylation of *ESR1* that was independent of age, gender, disease status, and body mass index (BMI).

Another interesting example comes from studies of *BRCA1* methylation in peripheral blood DNA of cases with breast cancer. Germline *BRCA1* mutations are related to the development of hereditary breast cancers, which account for ~5–10% of cases, and generally present at a younger age and with a more aggressive phenotype. Although mRNA levels of *BRCA1* have been shown to be reduced in a subset of sporadic breast cancer cases [70], mutations of *BRCA1* in sporadic breast cancer are rarely (if ever) present [25, 42]. As *BRCA1* is known to contain a CpG island in

its promoter region, it was hypothesized that DNA methylation-induced silencing may be present in a subset of sporadic breast tumors. It has been shown that up to 44% of sporadic breast tumors are methylated at *BRCA1*, and tumors with methylated *BRCA1* share pathologic features of tumors with mutated *BRCA1* [9, 12]. In 2008, Snell and colleagues measured *BRCA1* methylation in blood DNA from seven familial breast cancer cases that did not have detectable *BRCA1* or *BRCA2* mutations and seven age-matched controls. These authors used several techniques to measure *BRCA* methylation including MethyLight, methylation-sensitive high-resolution melting, and a digital version of the latter. Three of the seven patients studied had >0% methylation of *BRCA1* in peripheral blood DNA and the corresponding tumors were found to be heavily methylated. Among the control subjects, six of seven had no detectable *BRCA1* methylation, only one subject had low-level *BRCA1* (0.1%) methylation [64].

Al-Moghrabi et al. measured *BRCA1* methylation with MSP in 47 breast tumor tissues, and in peripheral blood from seven breast cancer cases and 73 disease-free controls [3]. Among tumor tissues, 13 (27%) had *BRCA1* methylation. Similarly, two (29%) of the seven blood samples from breast cancer cases were methylated at *BRCA1*. Further, there was a significant association between a younger age at diagnosis (≤40 years) and *BRCA1* methylation ($P < 0.004$). However, 8 of the 73 (11%) disease-free controls also had *BRCA1* methylation in blood, which was not a significantly lower prevalence of *BRCA1* methylation than in cases. Nonetheless, with only seven breast cancer cases providing blood DNA, this study may have been underpowered to detect a significant association between *BRCA1* methylation in blood and risk of breast cancer. In addition, it is possible that the disease-free women with *BRCA1* methylation in blood are still at an increased risk of developing breast cancer.

Iwamoto and colleagues presented a similar study of *BRCA1* methylation in peripheral blood DNA from 200 cases and 200 controls [37]. In peripheral blood samples from cases and controls, *BRCA1* methylation was measured with quantitative MSP and found to be associated with a significantly increased risk of breast cancer (OR: 1.7, 95% CI: 1.01–2.96), controlling for age, family history, age at menarche, parity, menopausal status, and BMI. In addition, these authors also measured *BRCA1* methylation in 162 breast tumors where 31 (19%) were *BRCA1* methylation-positive and these tumors were more likely to be estrogen receptor and progesterone receptor-negative. When stratifying by presence of *BRCA1* methylation in tumors (and controlling for covariates above), peripheral blood methylation of *BRCA1* was highly associated with risk of developing *BRCA1* methylation-positive breast cancer (OR: 17.8, 95% CI: 6.7–47.1).

Blood DNA methylation of *BRCA1* in relation to the risk of ovarian cancer has also been reported. Bosivel and colleagues [11] measured blood DNA methylation of both *BRCA1* and *BRCA2* promoter regions in 51 ovarian cancer cases (without *BRCA* mutation) and 349 controls using quantitative analysis of methylated alleles. Although they did not observe an association between *BRCA2* methylation level and case status, these authors reported significantly *reduced BRCA1* methylation in ovarian cancer cases compared to controls. However, the implications of a significantly

hypomethylated *BRCA1* promoter region in association with ovarian cancer are somewhat counterintuitive and warrant further investigation.

In a case–control study of lung cancer, Li et al. measured methylation of the putative tumor suppressor gene *FHIT* in peripheral blood DNA samples from Han Chinese subjects with MSP [46]. Among 123 lung cancer cases, 42 (34%) had *FHIT* promoter methylation, whereas none of the 105 control subjects' blood DNAs were methylated, indicating a significantly increased risk of lung cancer associated with peripheral blood methylation of *FHIT* (OR: 2.3, 95% CI: 2.0–2.7). Additionally, these authors reported that blood methylation of *FHIT* was significantly associated with cases who had early stage (I) disease ($P < 0.05$), and not cases with high-stage (IV) disease.

12.3.3 Panels of candidate genes

Some groups have reported blood methylation data for panels of candidate genes. The heterogeneity in molecular alterations of specific tumor types could be motivation for studies that examine multiple gene-loci, and the results from Iwamoto et al. are apropos: peripheral blood methylation of *BRCA1* was highly associated with risk of developing *BRCA1* methylation-positive breast cancer [37]. Of course, within a particular tumor type, not every tumor will have the same repertoire of molecular alterations. Hence, a more comprehensive approach to study blood-based methylation markers of cancer risk would measure methylation of several genes known (or suspected) to be methylated in a moderate to high proportion of tumors.

One such study from Liu et al. used an approach directed at six genes on chromosome 3p because a previous report from these authors had demonstrated a CpG island methylator phenotype (CIMP) associated with genes on 3p in lung tumors [48, 49]. Here, the authors used peripheral blood DNA from 80 cases of non-small cell lung cancer (NSCLC) and 80 matched controls and measured methylation of six genes (*OGG1*, *RARB*, *SEMA3B*, *RASSF1A*, *BLU*, *FHIT*) on chromosome 3p with MSP. If at least three of these genes were methylated the sample was considered 3pCIMP+. The prevalence of methylation in blood DNA from cases was higher than controls for all genes except *FHIT* where the same number of cases and controls were methylated. Further, almost all case blood samples (78/80, 98%) had at least one methylated gene, whereas 78% (62/80) of control blood samples had at least one methylated gene. When comparing 3pCIMP status in cases and controls, 44% of NSCLC cases were 3pCIMP + and only 6% of control blood DNA samples were 3pCIMP + ($P < 0.001$). In a model adjusting for age, sex, and smoking status, subjects with 3pCIMP + blood DNA were at a significantly increased risk of NSCLC (OR: 12.8, 95% CU: 4.4–37.4) [49].

Another gene-panel approach to investigate the role of blood-based DNA methylation markers of lung cancer risk was recently published by Vineis and colleagues using nested cases and controls from the European Prospective Investigation into Cancer and Nutrition (EPIC) [72]. This group measured methylation of multiple

CpGs in five genes: *CDKN2A*, *RASSF1A*, *GSTP1*, *MTHFR*, and *MGMT* with a bisulfite pyrosequencing approach in 93 lung cancer cases and 99 controls. Stratifying pyrosequencing methylation data for each gene on the median, adjusted models revealed that increased *RASSF1A* methylation was associated with a significantly increased risk of lung cancer (OR: 1.9, 95% CI: 1.0–3.5), though none of the other genes, or combination thereof were associated with disease. The authors also reported that serum levels of B vitamins and one-carbon metabolites were associated with methylation; increased folate was associated with increased *RASSF1A* and *MTHFR* methylation, whereas increased methionine was associated with decreased *RASSF1A* methylation [72].

Prior to these works, a group in France published a comparison of blood DNA methylation of ten genes in a study of prostate cancer [61]. Using prostate cancer cases with disease relapse ($n=20$), patients without relapse ($n=22$), as well as control subjects ($n=22$), the authors measured methylation of ten genes; *RASSF1A*, *CDH1*, *APC*, *DAPK*, *MGMT*, *CDKN2A* (*p16* and *p14*), *GSTP1*, *RARB*, and *TIMP3* using quantitative MSP. Compared to all cases, methylation levels of all ten genes were lower in control subject blood DNA, and five were significantly lower; *DAPK* ($P=0.04$) *RASSF1A*, *GSTP1*, *APC*, and *RARB* (all $P<0.0001$).

An interesting final example of small gene-panel studies comes from Flanagan and colleagues who developed a tiling microarray with a methylation-sensitive enzyme-based approach to study 17 breast cancer susceptibility genes [22]. With the tiling array the authors took an unbiased approach to examining the promoter and gene-coding regions for the 17 candidate genes. In the pilot phase, 14 cases with bilateral breast cancer and 14 control subjects had their blood DNA methylation measured. Notably, the authors described 181 regions in the 17 genes analyzed that had significantly variable methylation ($P<0.001$) across all 28 individuals, and the majority of these regions were significantly closer (within 200 bp) to repetitive elements than would be expected ($P=7.4e-07$). As a follow up, the authors validate two regions of variable methylation 4 kb downstream of the *ATM* gene in 190 cases and 190 controls and observed significantly increased methylation of *ATM* variable region 2 in cases compared to controls ($P=0.002$). In an inter-quartile analysis of the methylation data from this same region, subjects in the highest quartile of methylation were at a significantly increased risk of breast cancer (OR: 3.2, 95% CI: 1.8–5.9) compared to subjects in the lowest quartile [22]. One of the key facets of this particular study is that unlike most other investigations, these authors did not restrict their methylation measurements to promoter regions and argues that future studies should consider the distribution of regions measured for methylation.

12.4 Larger Gene-Panels and Commercial Methylation Arrays

A separate class of studies that has undertaken larger-scale approaches (25 genes to genome-wide) to investigate blood-based markers of DNA methylation and cancer risk will be covered here. One such study from Widschwendter et al. used a

three-step approach to investigate blood DNA methylation and the risk of breast cancer [75]. First, these authors chose 49 estrogen receptor target (ERT) and polycomb group target (PCGT) genes and second, used MethyLight to measure methylation in 83 healthy post-menopausal women. Thirdly, based on the distribution of methylation in these individuals 25 of the 49 genes were selected for measurement in 353 cases and 730 controls. After controlling for age and family history of breast cancer, methylation of 5 of the 25 genes (*ZNF217*, *NEUROD1*, *SFRP1*, *TITF1* (officially *NKX2-1* as of 8/14/11), *NUP155*) was associated with a significantly increased risk of breast cancer (ORs range: 1.40–1.49, median OR: 1.48) [75]. This study provides further proof of principle for the utility of blood-based methylation markers of cancer risk. However, because methylation of five separate genes were independently associated with breast cancer, it would have been interesting to know whether an analytic approach that combined the methylation markers would have increased the effect estimate.

A similar study of small cell lung cancer (SCLC) risk from Wang et al. also used a multi-step approach to curate a group of genes measured for methylation in a small pilot group of cases and controls before expanding into additional cases and controls [73]. This study took advantage of recent technologic advances that allow for the simultaneous resolution of hundreds to hundreds of thousands of methylation events, providing an epigenotyping platform for rapid epigenetic profiling [8]. First, bisulfite-modified blood DNA from 44 cases and 44 controls was applied to the Illumina GoldenGate methylation array which measures 1,505 CpG sites associated with >800 cancer-related genes. Testing 1,332 CpGs (those with methylation states not associated with cancer treatment) the authors observed 62 CpG sites associated with 52 genes to be significantly associated with cases status (FDR $P<0.05$). To follow up, the authors chose nine of these 62 CpGs for validation by bisulfite pyrosequencing in 138 cases and 138 controls. Controlling for age, sex, and smoking history, the methylation status of the nine CpG sites collectively were able to correctly classify 86% of cases as being at a higher risk of SCLC. Further, when considering specific CpGs, for the risk of SCLC increased ~4-fold for each 5% decrease in *ERCC1* methylation (OR: 3.9, 95% CI: 2.0–6.1) and ~1.5-fold for each 5% decrease in *CSF3R* methylation (OR: 1.5, 95% CI: 1.1–2.0) [73].

A group from the Mayo Clinic in Minnesota also used a two-phase study and the GoldenGate array to study blood methylation and risk of cancer, though they focused on pancreatic cancer [59]. First, these authors measured blood DNA methylation with the array in 132 cases and 60 controls and reported 110 CpGs with significantly differential methylation between cases and controls (FDR $P<0.05$). Then, using analogous technology in a custom platform from Illumina (VeraCode), the top 96 CpGs associated with case control status were subjected to validation in a further 240 cases and 240 matched controls. Leveraging the potential of combining methylation measures a prediction model was built and included five CpG sites associated with five genes: *IL10*, *LCN2*, *ZAP70*, *AIM2*, and *TAL1*. Collectively, these five CpGs demonstrated good discrimination between pancreatic cancer cases and controls (c-statistic phase I = 0.85, phase II = 0.72) [59].

Teschendorff et al. published an investigation of blood methylation profiles to predict ovarian cancer using a more comprehensive array platform, the Illumina Infinium 27K array [69]. Following exclusions for batch effects and quality control, methylation array data from 148 controls, 113 pre-treatment, and 122 post-treatment cases from the UK Ovarian Cancer Population Study were included in the analysis. Comparing methylation among controls to pre-treatment cases, the authors identified 2,714 CpG sites that were significantly (FDR $P<0.05$) associated with ovarian cancer. Notably, among the top 50 CpGs, 87% were hypomethylated in cases compared to controls ($P=9e-09$). To construct a DNA methylation signature associated with ovarian cancer, these authors used a supervised approach to the data with 100 iterations of training and testing sets (each with 90 controls and 70 pre-*t* cases) and multivariate logistic regression. With these iterations and a cross-validation step, the top 100 CpG sites were determined to be an optimal number of CpG sites for their classifier. The performance of these 100 CpGs as a classifier for ovarian caner in a blinded test set was very good (AUC: 0.8, 95% CI: 0.74–0.87), and was validated in the post-treatment cases (AUC: 0.76, 95% CI: 0.72–0.81) [69].

In a New Hampshire population-based bladder cancer case–control study, Marsit et al. examined peripheral blood DNA methylation profiles using the Infinium 27K array. Using a novel, semi-supervised recursively partitioned mixture modeling (SS-RPMM) strategy [40] involving classifier training in a series of subjects consisting of 118 controls and 112 cases, and validation in an independent series of 119 controls and 111 cases, Marsit et al. identified a panel of 9 CpG loci whose profile of DNA methylation was significantly associated ($P<0.0001$) with bladder cancer [50]. Membership in any of the three classes of DNA methylation associated with risk demonstrated a 5.2-fold increased risk of bladder cancer (95% CI 2.8, 9.7), when controlled for subject age, gender, smoking status, and family history of bladder cancer. Notably, the methylation classes whose membership was predominantly bladder cancer cases had higher levels of mean methylation across the 9 CpG loci. Gene-set enrichment analysis of the loci most associated with bladder cancer demonstrated that transcription-factor binding sites related to immune modulation and forkhead family transcription were over-represented among regions whose methylation differed in bladder cases compared to controls. The key role of immune modulation in both aging and carcinogenesis, and particularly bladder carcinogenesis, lends mechanistic significance to these findings.

Using the same array platform and SS-RPMM analytical approach, the association between peripheral blood methylation profiles and HNSCC was assessed by Langevin et al. [44] in 96 HNSCC cases and 96 cancer-free control subjects. In this study, cases and controls were best differentiated by a methylation profile of six CpG loci (associated with *FGD4*, *SERPINF1*, *WDR39*, *IL27*, *HYAL2,* and *PLEKHA6*), and after adjustment for subject age, gender, smoking, alcohol consumption, and HPV16 serostatus, the AUC was 0.85 (95% CI: 0.76–0.92). Notably, the methylation classes whose membership was predominantly head and neck cancer cases had lower mean methylation across the 6 CpG loci. Although this is not yet adequate for use in clinical settings, these results further demonstrate the poten-

tial of DNA methylation measured in blood for development of non-invasive applications for detection of head and neck cancer and the utility of the proposed methods for the analysis of the array-based methylation data.

12.5 Mechanisms

Just as normal genetic variation is now understood to be associated with a predisposition to a vast array of human diseases [51], it is important to consider interindividual variation in tissue-specific DNA methylation to better understand the ability of this variation to inform disease risk. Epigenetic variation has been hypothesized to cause underlying differences in disease susceptibility among monozygotic twins, and young twin-pairs have been shown to be more epigenetically similar than older monozygotic twins [24]. The aging process and differences in environment have been hypothesized to influence clinically significant changes in methylation profiles as individuals accumulate varying exposures with age.

Marks of DNA methylation are entirely reprogrammed during in-utero development. This reprogramming, during the pre-implantation period, necessitates a rapid de-methylation of the genome, thought to be accomplished through an active process [29, 45], followed by appropriate, cell and tissue-specific methylation of the genome. The mechanisms through which these processes of de-methylation and reprogramming of the DNA methylation marks and particularly, the appropriate targeting of enzymes responsible for establishing those marks remains unclear. Importantly, epigenetic reprogramming during in-utero development constitutes a critical period during which environmental stimuli and insults can alter the establishment of cell-type-specific DNA methylation profiles and may constitute one point at which variation in methylation profiles is established. Therefore, alteration to epigenetic profiles has been posited as the molecular basis of the developmental origins of health and disease phenomenon, which links the environment (taken broadly) in-utero, with outcomes throughout the life course of the individual [5, 6].

Beyond the variation in DNA methylation profile which is established in-utero, additional variation may arise resulting from exposures and the environment encountered throughout life, or from the process of aging itself. Work from Christensen et al. [16] demonstrated that features of the patterns of age-associated methylation were conserved irrespective of tissue-type, suggesting a common mechanism or dysregulation to explain these alterations. Potential mechanisms include reduced fidelity of maintenance methyltransferases with aging leading to hypomethyation events. Although age-related methylation alterations may not functionally result in a pathologic process, drifts of normal epigenomes may nonetheless confer significantly increased risk of conversion to a pathologic phenotype by enhancing both the likelihood and frequency of subsequent methylation events that ultimately result in aberrant expression or altered genomic stability.

Particularly when considering profiles of methylation in a heterogeneous tissue sample such as blood, it should be recognized that the quantitative measure of

methylation truly represents the fraction of cells within the sampled population exhibiting a methyl-group at any CpG site. Therefore, differences in DNA methylation profiles could and likely do indicate aging or exposure-related changes to the underlying populations of cells comprising that mixture. In the case of blood these shifts may indicate changes to the profile of immune cells and thus alterations to the immune system permissive to or resulting from carcinogenesis. In fact, comparing *LINE1*, Sat2, and Alu methylation levels in whole blood, granulocytes, mononuclear cells, and lymphoblastoid lines with multiple methylation assays (MethyLight, luminometric methylation assay, and a methyl acceptance assay) Wu et al. have demonstrated differences in methylation dependent upon substrate and assay used [79]. As additional studies are conducted to identify differentially methylated regions among various leukocyte subtypes, it may soon be possible to identify proportional shifts in specific leukocyte subtypes that may contribute to cancer, or indicate immune response to an existing tumor.

12.6 Conclusions

The extent of variability of the cellular epigenome in non-pathologic tissues, particularly at gene promoter regions, remains a critical question; the amount of variation in genomic methylation across the population is not currently known. It is clear that epigenetic variability detectable in human blood is influenced, in part, by aging and exposures, and in turn, specific profiles of methylation in blood are associated with cancer risk (Fig. 12.1). The ease of collection of blood samples and the rapidly advancing technologies to assess DNA methylation in genomic DNA from this tissue make this an ideal focus of study for novel biomarkers of disease risk and of disease prognosis. Additionally, as we better understand functional consequences of altered methylation profiles, there will be an improved understanding at the systems level of the contribution of non-target tissues and systems on carcinogenesis, likely yielding novel approaches not only of diagnosis but treatment as well.

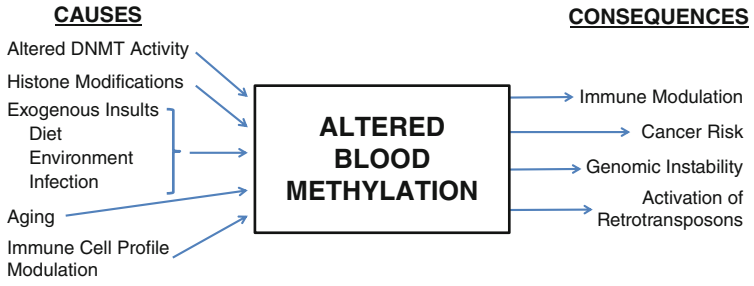

Fig. 12.1 Causes and consequences of altered blood DNA methylation

References

1. Alexander RP, Fang G et al (2010) Annotating non-coding regions of the genome. Nat Rev Genet 11(8):559–571
2. Ally MS, Al-Ghnaniem R et al (2009) The relationship between gene-specific DNA methylation in leukocytes and normal colorectal mucosa in subjects with and without colorectal tumors. Cancer Epidemiol Biomarkers Prev 18(3):922–928
3. Al-Moghrabi N, Al-Qasem AJ et al (2011) Methylation-related mutations in the BRCA1 promoter in peripheral blood cells from cancer-free women. Int J Oncol 39(1):129–135
4. Baccarelli A, Wright RO et al (2009) Rapid DNA methylation changes after exposure to traffic particles. Am J Respir Crit Care Med 179(7):572–578
5. Barker DJ (2004) Developmental origins of adult health and disease. J Epidemiol Community Health 58(2):114–115
6. Barker DJ (2004) The developmental origins of well-being. Philos Trans R Soc Lond B Biol Sci 359(1449):1359–1366
7. Bennett KL, Mester J et al (2010) Germline epigenetic regulation of KILLIN in Cowden and Cowden-like syndrome. JAMA 304(24):2724–2731
8. Bibikova M, Lin Z et al (2006) High-throughput DNA methylation profiling using universal bead arrays. Genome Res 16(3):383–393
9. Birgisdottir V, Stefansson OA et al (2006) Epigenetic silencing and deletion of the BRCA1 gene in sporadic breast cancer. Breast cancer research: BCR 8(4):R38
10. Bollati V, Baccarelli A et al (2007) Changes in DNA methylation patterns in subjects exposed to low-dose benzene. Cancer Res 67(3):876–880
11. Bosviel R, Michard E et al (2011) Peripheral blood DNA methylation detected in the BRCA1 or BRCA2 promoter for sporadic ovarian cancer patients and controls. Clin Chim Acta 412(15–16):1472–1475
12. Butcher DT, Rodenhiser DI (2007) Epigenetic inactivation of BRCA1 is associated with aberrant expression of CTCF and DNA methyltransferase (DNMT3B) in some sporadic breast tumours. Eur J Cancer 43(1):210–219
13. Cash HL, Tao L et al (2011) LINE-1 hypomethylation is associated with bladder cancer risk among nonsmoking Chinese. Int J Cancer 130(5):1151–1159
14. Chan TL, Yuen ST et al (2006) Heritable germline epimutation of MSH2 in a family with hereditary nonpolyposis colorectal cancer. Nat Genet 38(10):1178–1183
15. Choi JY, James SR et al (2009) Association between global DNA hypomethylation in leukocytes and risk of breast cancer. Carcinogenesis 30(11):1889–1897
16. Christensen BC, Houseman EA et al (2009) Aging and environmental exposures alter tissue-specific DNA methylation dependent upon CpG island context. PLoS Genet 5(8):e1000602
17. Cordaux R, Batzer MA (2009) The impact of retrotransposons on human genome evolution. Nat Rev Genet 10(10):691–703
18. Deininger PL, Batzer MA (1999) Alu repeats and human disease. Mol Genet Metab 67(3): 183–193
19. Dewannieux M, Esnault C et al (2003) LINE-mediated retrotransposition of marked Alu sequences. Nat Genet 35(1):41–48
20. Dobrovic A, Kristensen LS (2009) DNA methylation, epimutations and cancer predisposition. Int J Biochem Cell Biol 41(1):34–39
21. Eads CA, Danenberg KD et al (2000) MethyLight: a high-throughput assay to measure DNA methylation. Nucleic Acids Res 28(8):E32
22. Flanagan JM, Munoz-Alegre M et al (2009) Gene-body hypermethylation of ATM in peripheral blood DNA of bilateral breast cancer patients. Hum Mol Genet 18(7):1332–1342
23. Florl AR, Lower R et al (1999) DNA methylation and expression of LINE-1 and HERV-K provirus sequences in urothelial and renal cell carcinomas. Br J Cancer 80(9):1312–1321
24. Fraga MF, Ballestar E et al (2005) Epigenetic differences arise during the lifetime of monozygotic twins. Proc Natl Acad Sci USA 102(30):10604–10609

25. Futreal PA, Liu Q et al (1994) BRCA1 mutations in primary breast and ovarian carcinomas. Science 266(5182):120–122
26. Gama-Sosa MA, Wang RY et al (1983) The 5-methylcytosine content of highly repeated sequences in human DNA. Nucleic Acids Res 11(10):3087–3095
27. Gaudet F, Hodgson JG et al (2003) Induction of tumors in mice by genomic hypomethylation. Science 300(5618):489–492
28. Gicquel C, Rossignol S et al (2005) Epimutation of the telomeric imprinting center region on chromosome 11p15 in Silver-Russell syndrome. Nat Genet 37(9):1003–1007
29. Hajkova P, Erhardt S et al (2002) Epigenetic reprogramming in mouse primordial germ cells. Mech Dev 117(1–2):15–23
30. Herman JG, Graff JR et al (1996) Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci USA 93(18):9821–9826
31. Hitchins MP (2010) Inheritance of epigenetic aberrations (constitutional epimutations) in cancer susceptibility. Adv Genet 70:201–243
32. Hitchins M, Williams R et al (2005) MLH1 germline epimutations as a factor in hereditary nonpolyposis colorectal cancer. Gastroenterology 129(5):1392–1399
33. Hitchins MP, Wong JJ et al (2007) Inheritance of a cancer-associated MLH1 germ-line epimutation. N Engl J Med 356(7):697–705
34. Hitchins M, Owens S et al (2011) Identification of new cases of early-onset colorectal cancer with an MLH1 epimutation in an ethnically diverse South African cohort(dagger). Clin Genet 80(5):428–434
35. Hou L, Wang H et al (2010) Blood leukocyte DNA hypomethylation and gastric cancer risk in a high-risk Polish population. Int J Cancer 127(8):1866–1874
36. Hsiung DT, Marsit CJ et al (2007) Global DNA methylation level in whole blood as a biomarker in head and neck squamous cell carcinoma. Cancer Epidemiol Biomarkers Prev 16(1):108–114
37. Iwamoto T, Yamamoto N et al (2011) BRCA1 promoter methylation in peripheral blood cells is associated with increased risk of breast cancer with BRCA1 promoter methylation. Breast Cancer Res Treat 129(1):69–77
38. Jones PA, Laird PW (1999) Cancer epigenetics comes of age. Nat Genet 21(2):163–167
39. Jordan IK, Rogozin IB et al (2003) Origin of a substantial fraction of human regulatory sequences from transposable elements. Trends Genet 19(2):68–72
40. Koestler DC, Marsit CJ et al (2010) Semi-supervised recursively partitioned mixture models for identifying cancer subtypes. Bioinformatics 26(20):2578–2585
41. Kolomietz E, Meyn MS et al (2002) The role of Alu repeat clusters as mediators of recurrent chromosomal aberrations in tumors. Genes Chromosomes Cancer 35(2):97–112
42. Lancaster JM, Wooster R et al (1996) BRCA2 mutations in primary breast and ovarian cancers. Nat Genet 13(2):238–240
43. Lander ES, Linton LM et al (2001) Initial sequencing and analysis of the human genome. Nature 409(6822):860–921
44. Langevin SM, Koestler DC et al (2012) Peripheral blood DNA methylation profiles are predictive of head and neck squamous cell carcinoma: an epigenome-wide association study. Epigenetics 7(3):291–299
45. Lee J, Inoue K et al (2002) Erasing genomic imprinting memory in mouse clone embryos produced from day 11.5 primordial germ cells. Development 129(8):1807–1817
46. Li W, Deng J et al (2010) Association of 5′-CpG island hypermethylation of the FHIT gene with lung cancer in southern-central Chinese population. Cancer Biol Ther 10(10):997–1000
47. Lim U, Flood A et al (2008) Genomic methylation of leukocyte DNA in relation to colorectal adenoma among asymptomatic women. Gastroenterology 134(1):47–55
48. Liu Z, Zhao J et al (2008) CpG island methylator phenotype involving tumor suppressor genes located on chromosome 3p in non-small cell lung cancer. Lung Cancer 62(1):15–22
49. Liu Z, Li W et al (2010) CpG island methylator phenotype involving chromosome 3p confers an increased risk of non-small cell lung cancer. J Thorac Oncol 5(6):790–797

50. Marsit CJ, Koestler DC et al (2011) DNA methylation array analysis identifies profiles of blood-derived DNA methylation associated with bladder cancer. J Clin Oncol 29(9): 1133–1139
51. McCarthy MI, Hirschhorn JN (2008) Genome-wide association studies: potential next steps on a genetic journey. Hum Mol Genet 17(R2):R156–R165
52. McKay JD, Truong T et al (2011) A genome-wide association study of upper aerodigestive tract cancers conducted within the INHANCE consortium. PLoS Genet 7(3):e1001333
53. Menendez L, Benigno BB et al (2004) L1 and HERV-W retrotransposons are hypomethylated in human ovarian carcinomas. Mol Cancer 3:12
54. Moore LE, Pfeiffer RM et al (2008) Genomic DNA hypomethylation as a biomarker for bladder cancer susceptibility in the Spanish Bladder Cancer Study: a case–control study. Lancet Oncol 9(4):359–366
55. Morak M, Schackert HK et al (2008) Further evidence for heritability of an epimutation in one of 12 cases with MLH1 promoter methylation in blood cells clinically displaying HNPCC. Eur J Hum Genet 16(7):804–811
56. Nelson HH, Marsit CJ et al (2011) "Global methylation" in exposure biology and translational medical science. Environ Health Perspect 119(11):1528–1533
57. Netchine I, Rossignol S et al (2007) 11p15 imprinting center region 1 loss of methylation is a common and specific cause of typical Russell-Silver syndrome: clinical scoring system and epigenetic-phenotypic correlations. J Clin Endocrinol Metab 92(8):3148–3154
58. Pavanello S, Bollati V et al (2009) Global and gene-specific promoter methylation changes are related to anti-B[a]PDE-DNA adduct levels and influence micronuclei levels in polycyclic aromatic hydrocarbon-exposed individuals. Int J Cancer 125(7):1692–1697
59. Pedersen KS, Bamlet WR et al (2011) Leukocyte DNA methylation signature differentiates pancreatic cancer patients from healthy controls. PLoS One 6(3):e18223
60. Pufulete M, Al-Ghnaniem R et al (2003) Folate status, genomic DNA hypomethylation, and risk of colorectal adenoma and cancer: a case control study. Gastroenterology 124(5): 1240–1248
61. Roupret M, Hupertan V et al (2008) Promoter hypermethylation in circulating blood cells identifies prostate cancer progression. Int J Cancer 122(4):952–956
62. Rusiecki JA, Baccarelli A et al (2008) Global DNA hypomethylation is associated with high serum-persistent organic pollutants in Greenlandic Inuit. Environ Health Perspect 116(11): 1547–1552
63. Sano H, Imokawa M et al (1988) Detection of heavy methylation in human repetitive DNA subsets by a monoclonal antibody against 5-methylcytosine. Biochim Biophys Acta 951(1): 157–165
64. Snell C, Krypuy M et al (2008) BRCA1 promoter methylation in peripheral blood DNA of mutation negative familial breast cancer patients with a BRCA1 tumour phenotype. Breast Cancer Res 10(1):R12
65. Steenman MJ, Rainier S et al (1994) Loss of imprinting of IGF2 is linked to reduced expression and abnormal methylation of H19 in Wilms' tumour. Nat Genet 7(3):433–439
66. Suter CM, Martin DI et al (2004) Germline epimutation of MLH1 in individuals with multiple cancers. Nat Genet 36(5):497–501
67. Suter CM, Martin DI et al (2004) Hypomethylation of L1 retrotransposons in colorectal cancer and adjacent normal tissue. Int J Colorectal Dis 19(2):95–101
68. Tarantini L, Bonzini M et al (2009) Effects of particulate matter on genomic DNA methylation content and iNOS promoter methylation. Environ Health Perspect 117(2):217–222
69. Teschendorff AE, Menon U et al (2009) An epigenetic signature in peripheral blood predicts active ovarian cancer. PLoS One 4(12):e8274
70. Thompson ME, Jensen RA et al (1995) Decreased expression of BRCA1 accelerates growth and is often present during sporadic breast cancer progression. Nat Genet 9(4):444–450
71. Ting DT, Lipson D et al (2011) Aberrant overexpression of satellite repeats in pancreatic and other epithelial cancers. Science 331(6017):593–596

72. Vineis P, Chuang SC et al (2011) DNA methylation changes associated with cancer risk factors and blood levels of vitamin metabolites in a prospective study. Epigenetics 6(2):195–201
73. Wang L, Aakre JA et al (2010) Methylation markers for small cell lung cancer in peripheral blood leukocyte DNA. J Thorac Oncol 5(6):778–785
74. Weisenberger DJ, Campan M et al (2005) Analysis of repetitive element DNA methylation by MethyLight. Nucleic Acids Res 33(21):6823–6836
75. Widschwendter M, Apostolidou S et al (2008) Epigenotyping in peripheral blood cell DNA and breast cancer risk: a proof of principle study. PLoS One 3(7):e2656
76. Wilhelm CS, Kelsey KT et al (2010) Implications of LINE1 methylation for bladder cancer risk in women. Clin Cancer Res 16(5):1682–1689
77. Wong IH, Lo YM et al (2000) Frequent p15 promoter methylation in tumor and peripheral blood from hepatocellular carcinoma patients. Clin Cancer Res 6(9):3516–3521
78. Wong EM, Southey MC et al (2011) Constitutional methylation of the BRCA1 promoter is specifically associated with BRCA1 mutation-associated pathology in early-onset breast cancer. Cancer Prev Res (Phila) 4(1):23–33
79. Wu HC, Delgado-Cruzata L et al (2011) Global methylation profiles in DNA from different blood cell types. Epigenetics 6(1):76–85
80. Yang AS, Estecio MR et al (2004) A simple method for estimating global DNA methylation using bisulfite PCR of repetitive DNA elements. Nucleic Acids Res 32(3):e38

Chapter 13
Epigenetic Therapies in MDS and AML

Elizabeth A. Griffiths and Steven D. Gore

Abstract The use of low dose hypomethylating agents for patients with myelodysplastic syndrome (MDS) and secondary acute myeloid leukemia (AML) has had made a significant impact. In the past, therapies for these diseases were limited and patients who elected to receive treatment were subject to highly toxic, inpatient chemotherapeutics, which were often ineffective. In the era of hypomethylating agents (azacitidine and decitabine), a patient with high grade MDS or AML with multilineage dysplasia can be offered the alternative of outpatient, relatively low-toxicity therapy. Despite the fact that CR (CR) rates to such agents remain relatively low at 15–20%, a much larger percentage of patients will have clinically significant improvements in hemoglobin, platelet, and neutrophil counts while maintaining good outpatient quality of life. As our clinical experience with azanucleotides expands, questions regarding patient selection, optimal dosing strategy, latency to best response and optimal duration of therapy following disease progression remain, but there is no question that for some patients these agents offer, for a time, an almost miraculous clinical benefit. Ongoing clinical trials in combination and in sequence with conventional therapeutics, with other epigenetically active agents, or in conjunction with bone marrow transplantation continue to provide promise for optimization of these agents for patients with myeloid disease. Although the mechanism(s) responsible for the proven efficacy of these agents remain a matter of some controversy, activity is thought to stem from induction of DNA hypomethylation, direct DNA damage, or possibly even immune modulation; there is no question that they have become a permanent part of the armamentarium against myeloid neoplasms.

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13.1 Introduction

Myelodysplastic syndromes (MDS) are a heterogenous group of malignant myeloid disorders characterized by peripheral blood cytopenias in association with bone marrow hypercellularity and dysplasia [1]. Patients with high grade MDS (int-2 or high by IPSS criteria, Fig. 13.1) have a high rate of transformation to acute myeloid leukemia (AML) and poor long-term survival with a life expectancy in the absence of treatment between 0.4 and 1.8 years [2]. The International Prognostic Scoring System (IPSS) was developed as a tool for stratifying patient outcomes based upon readily available clinical characteristics. Figure 13.1 details the components necessary for the generation of an IPSS score and the expected survival for each designation [2]. "Secondary" AMLs such as those arising in patients with an antecedent MDS diagnosis are generally resistant to traditional chemotherapeutics and the overall survival (OS) in this group of patients is universally poor [3–5]. Both MDS and AML are diseases of the elderly with a majority of patients diagnosed when they are older than 60 years [5]. Although a small minority of patients with MDS will present with mild cytopenias and low grade disease, a majority do not [2]. Patients with MDS associated with multilineage cytopenias (anemia, thrombocytopenia, and neutropenia), high bone marrow blast percentages, or characteristic adverse chromosomal features often progress rapidly to AML and in the absence of bone marrow transplantation, ultimately die of their disease [2].

For these patients, and for a large number of older people who present with putatively de novo myeloid leukemias, but with unrecognized low grade cytopenias and

Prognostic Variable	Score value				
	0	0.5	1.0	1.5	2.0
Bone Marrow Blast %	<5	5-10		11-20	21-30
Karyotype	Good= normal,-y, del(5q), del(20q)	Intermediate= all others	Poor= ≥3 or any 7 abnormality		
Cytopenias	0/1	2/3			

IPSS risk group	Score	Median Survival
Low	0	5.7 years
Int-1	0.5-1	3.5 years
Int-2	1.5-2.0	1.2 years;
High	≥2.5	0.4 year

Fig. 13.1 Clinical criteria for and IPSS risk group classification of patients with myelodysplasia, from ref. [2]

bone marrow dysplasia, conventional induction chemotherapeutics (IC, with daunorubicin and cytarabine) have been in large measure disappointing [6]. Furthermore many such patients are unfit for intensive treatment and are offered instead low dose cytarabine, clinical trials or supportive care [7]. In this group the OS rates at 2 and 5 years remain only 10% and 2% respectively [3, 4]. Patients who are fit to receive traditional IC require long periods of time (often 4–6 weeks) in the hospital, and this treatment offers a complete remission rate of only 20–30%, with median survivals ranging between 5 and 13 months [6, 8, 9]. In addition to induction failure and early relapse, even in those who achieve remission, prolonged hospitalization can have the side effect of physical deconditioning and the 3 or more weeks of neutropenia resulting from this treatment can result in resistant bacterial and fungal infections [6]. These burdens create patients who are unable to return to good quality of life and who become ineligible for salvage therapy or clinical trials upon relapse due to poor performance status, organ dysfunction or infection. Even in those who retain an excellent performance status following induction, primary refractory AML remains a significant quality of life problem, requiring frequent blood transfusions, extensive prophylactic antibiotic regimens, and regular hospital visits [9].

Until recently, toxic traditional IC was the only real option for fit patients with high grade MDS or AML with MDS related changes [1]. Recently however, the epigenetically active drugs azacitidine (Aza, Vidaza, Celgene, Concord OH) and decitabine (Dac, Dacogen, Esai Inc., Mars, PA) have been approved both in the United States and Europe for the treatment of MDS and low blast count (<30%) AML [7, 10]. These drugs, both of which are incorporated into DNA resulting in the depletion of the intracellular methyltransferases (DNMTs) when given at low dose, were the first epigenetically active therapy to be approved for cancer. They have resulted in a significant change in the approach to patients with MDS and required the development of the International Working Group (IWG) response criteria in MDS in order to measure meaningful improvements in cytopenias that did not fit into the traditional response assessment which designated only complete (CR) or partial (PR) responses as meaningful [11, 12]. A summary of the IWG response criteria in MDS are provided in Table 13.1. In particular, Aza has been shown to improve OS, delay the transformation to AML in high-grade MDS patients, and produce significant responses in patients with low blast count AML [7]. Although a statistically significant survival benefit has not been demonstrated following treatment with Dac, this drug has been shown to produce both CRs and hematological improvements in both MDS and AML patients who receive it [10, 13]. Taken together these drugs offer an effective alternative to induction chemotherapy and have become the standard of care for patients with MDS as well as selected patients with AML.

As with conventional chemotherapeutic strategies for these patients, responses are usually limited to a year or two, but therapy is largely outpatient, with minimal end organ toxicity and few side effects [14]. Despite notable limitations, these drugs have made a significant impact upon quality of life for a large number of patients with high grade MDS and AML. Ongoing work to understand the mechanism responsible for the efficacy of these drugs and the ultimate loss of response observed

Table 13.1 Selected clinical trials with azacitidine (aza) or decitabine (dac) in MDS

Trial	CALGB 9221	D-0007	ICD03-180	AZA-001	US Oncology	ADOPT	EORTC 06011
Author (publication year)	Silverman (2002) [27]	Kantarjian (2006) [10]	Kantarjian (2007) [55]	Fenaux (2009) [7]	Lyons (2009) [36]	Steensma (2009) [56]	Lubbert (2011) [13]
Number enrolled	191	170	95	358	151	99	233
Number treated with study drug	150 (99 upfront, 51) crossovers	89	95	179	151	99	119
Phase	III	III	II	III	II	II	III
Study regimen	Aza SQ 75 mg/m^2×7 days	Dac IV 15 mg/m^2 q8h×3 days	Dac IV 10 mg/m^2×10 days IV 20 mg/m^2×5 days SQ 20 mg/m^2×5 days	Aza SQ 75 mg/m^2×7 days	Aza SQ 75 mg/m^2 ×5days–2 days off-2days ×5 days–2days off-5 days ×5 days	Dac IV 20 mg/m^2×5 days	Dac IV 15 mg/m^2 q8h×3days
Int-2 or high IPSS (%)	46	70	66	87	Not reported	46	93
Median cycles administered	4	3	7	9	6	5	4
CR % (by IWG 2000)	9	9	37	17	Not reported	15	13
CR+PR+HI% (by IWG 2000)	48	30	73	49	48	43	34

clinically is ongoing. Furthermore, the development of novel dosing strategies, combinations, and the appropriate use of allogenic transplantation provide hope for improving response duration and possibly even providing an opportunity for long-term remission to these unfortunate patients.

13.2 Single Agent "Hypomethylating" Therapy for MDS and AML

13.2.1 Azacitidine

Aza is a nucleoside analog of cytidine in which the carbon 5 position of the pyrimidine ring has been substituted with nitrogen (Fig. 13.2a) [15]. It is imported into cells by the action of nucleotide transporters, where it is activated by uridine–cytidine kinase and incorporated into RNA (Fig. 13.3) [15]. Sixty to 80% of the Aza dose given is incorporated into RNA and this has impacts upon protein synthesis and RNA metabolism [15]. Twenty to 40% of the dose is converted into the deoxyribonucleoside Dac by the action of ribonucleotide reductase [15]. This deoxyribonucleoside base is then phosphorylated and incorporated into DNA where it acts as a suicide substrate for DNMTs and induces DNA hypomethylation during cellular replication as well as DNA damage due to adduct formation [15]. Aza was first synthesized and tested in 1960s and 1970s [16, 17]. In early clinical trials as a traditional chemotherapeutic, it was demonstrated to be effective in myeloid malignancy, however its efficacy was limited by significant gastrointestinal toxicity and prolonged cytopenias [16–18]. Cytarabine or AraC was developed at about the same time. This drug, another nucleoside analog of cytidine whose activity is thought to result in chain termination, is among the most active drugs used for myeloid malignancy. Ultimately the toxicity of 5-Aza limited its further

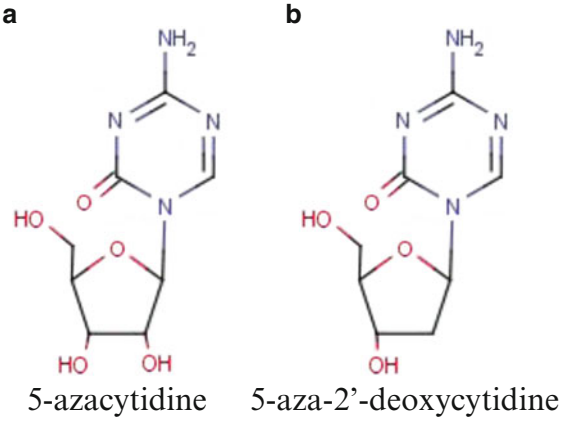

Fig. 13.2 Molecular structure of Aza (**a**) and Dac (**b**)

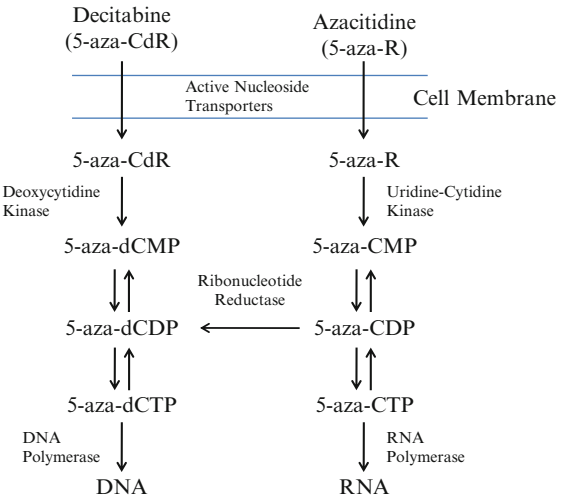

Fig. 13.3 Uptake and serial steps for the incorporation of Aza and Dac into RNA and DNA

clinical development, and cytarabine became the nucleoside analog of choice in myeloid malignancy [17, 18].

In 1978, Peter Jones and colleagues demonstrated that treatment of mouse embryo cells in vitro with Aza and its deoxy analog 5-aza-2′-deoxycytitine (Dac) could induce differentiation into functional myotubes [19]. Jones and Taylor went on to show that this differentiation resulted from changes in DNA methylation elicited by treatment with azanucleosides [20, 21]. Further work, by Dr. Jones and others, identified methylation as a common event in many malignancies, including the pre-leukemic condition known as MDS, a disease for which no treatment was available [22]. Although initially used as a laboratory tool to test gene and chromosome specific methylation changes, the identification of methylation as a potentially reversible cancer specific event spurred interest in the possibility that cancers treated with these drugs might be induced to differentiate and potentially to apoptose and die.

Ultimately in the 1990s, insights into methylation events common to MDS, specifically identification of recurrent methylation of tumor suppressor genes such as p15INK4B, resulted in the development of a number of phase I and II clinical trials of azanucleotides in this disease [23, 24]. Table 13.2 reviews the key published trials with single agent azanucleotides in MDS.

Among the first published trials with Aza for the treatment of MDS delivered the drug at 75 mg/m^2 as a continuous intravenous infusion for 7 days every 4 weeks [25]. This trial enrolled high grade MDS patients with symptomatic disease characterized by red cell and platelet transfusion dependence and poor life expectancy (refractory anemia with excess blasts (10–20%), or refractory anemia with excess blasts in transformation (20–30%). Forty three patients were evaluable and responses were seen in 21 (49%) of these patients [25]. Five patients (12%) achieved a CR, 11 (25%) achieved a partial response (PR), and 5 “improved” (a response characterized in the study as a ≥50% reduction in transfusion requirements, or improvement in platelets,

Table 13.2 Selected clinical trials with azacitidine (aza) or decitabine (dac) in MDS

Trial	CALGB 9221	D-0007	ICD03-180	AZA-001	US Oncology	ADOPT	EORTC 06011
Author (publication year)	Silverman (2002) [27]	Kantarjian (2006) [10]	Kantarjian (2007) [55]	Fenaux (2009) [7]	Lyons (2009) [36]	Steensma (2009) [56]	Lubbert (2011) [13]
Number enrolled	191	170	95	358	151	99	233
Number treated with study drug	150 (99 upfront, 51) crossovers	89	95	179	151	99	119
Phase	III	III	II	III	II	II	III
Study regimen	Aza SQ 75 mg/m^2×7 days	Dac IV 15 mg/m^2 q8h×3 days	Dac IV 10 mg/m^2×10 days IV 20 mg/m^2×5 days SQ 20 mg/m^2×5 days	Aza SQ 75 mg/m^2×7 days	Aza SQ 75 mg/m^2 ×5d–2 days off-2 days ×5 days–2 days off-5 days ×5 days	Dac IV 20 mg/m^2/days×5 days	Dac IV 15 mg/m^2 q8h×3 days
Int-2 or high IPSS (%)	46	70	66	87	Not reported	46	93
Median cycles administered	4	3	7	9	6	5	4
CR % (by IWG 2000)	9	9	37	17	Not reported	15	13
CR+PR+HI% (by IWG 2000)	48	30	73	49	48	43	34

hemoglobin or neutrophils) [25]. OS in these high risk patients was 13.3 months and for those achieving CR or PR was 14.7 months, and the chief toxicities were mild to moderate nausea [25]. A number of other clinical trials using this drug were published suggesting that Aza had significant activity in MDS and these results were sufficient to prompt two larger, randomized trials of Aza in MDS [26].

In 2004, the FDA approved Aza for the treatment of MDS based upon results from a single phase III clinical trial (described in detail below) [27]. A second trial demonstrating survival was required by European regulators, and this was published formally in 2009 [7]. These trials established Aza as the standard of care approach to patients with int-2 and high risk MDS by demonstrating a prolongation in the time to progression to AML, decreased transfusion requirements, improvements in neutropenia, and ultimately, improvements in OS.

13.2.1.1 CALGB 9221

The first phase III trial of Aza in patients with MDS was published by investigators from the CALGB [27]. CALGB 9221 enrolled 191 patients of median age 68 with French American British-defined MDS (reference for FAB classification), to receive either supportive care or Aza at a dose of 75 mg/m^2/day subcutaneously for 7 of 28 days. Patients were maintained on their randomized arm for 4 months, after which patients who were deemed to have progressed on the supportive care arm could crossover to the Aza arm. Patient characteristics were distributed evenly across both arms with 59% of the patients overall having RAEB or RAEB-T by FAB criteria (46% Int-2 or high by IPSS) [27]. Sixty five percent of the enrolled patients were red blood cell transfusion dependent (69% Aza arm, 61% supportive care arm) [27].

Responses were evaluated in both arms. Among patients randomized to receive supportive care, 5% met criteria for improvement; no patients on this arm achieved a CR or PR. Of the 99 patients randomized to receive Aza, 60% ($n=60$) achieved a response ($p<0.0001$) [28]. Responses were classified as CR in 7% ($n=7$), PR in 16% ($n=16$), and improvement in 37% ($n=37$). Of those patients demonstrating "improvement," 35% had increases in three cell lines inadequate to qualify as a PR, 30% had improvement in two cell lines, and 35% had improvement in only one cell line. Responses did not depend upon MDS sub-classification. Forty nine patients crossed over to receive Aza, of these 47% ($n=23$) responded and 10% ($n=5$) achieved a CR [27]. Patients treated with Aza had a median time to progression to AML or death of 21 months vs. 12 months in those patients treated with supportive care alone, and this was statistically significant ($p=0.007$), median OS in an intention to treat analysis was 20 months in the Aza treated patients vs. 14 months for those randomized to supportive care, although this difference was not statistically significant ($p=0.10$) [27].

Due to the design of this study, the survival analysis was confounded by the 49 patients who crossed over to receive Aza. In order to eliminate this bias, a landmark analysis at the 6 month date was performed. Three subgroups were identified, the

first included patients randomized to supportive care who did not crossover, or who crossed over after the six 6 month time point, the second were patients who were randomized to Aza, and the third were patients who crossed over after 4 months, but before 6 months [27]. This analysis excluded 36 patients who died before the landmark date. The median survival in these three groups was 11 (supportive care only), 14 (early crossover), and 18 (randomized to Aza) months respectively. A statistically significant difference in survival was observed between the Aza treated and supportive care groups ($p=0.03$), but not between supportive care and early crossovers [27].

Transfusion requirements were tracked in both groups. In the Aza treated group transfusion needs increased during the first cycle, and thereafter declined, whereas in the supportive care arm transfusion requirements remained stable or increased. Of the 99 patients initially randomized to receive Aza, 51% had an improvement in hemoglobin, 45% (29) became RBC transfusion independent, and 6 (9%) had a reduction in transfusion dependence by at least 50%. Improved platelet counts were observed in 47%, and increased white cell counts were seen in 40% of the Aza treated patients [27].

In addition to objective improvements in transfusion requirements, white cell counts, survival and prolonged time to AML transformation, patients treated with Aza on this trial experienced significant improvements in quality of life. These were reported as improvements in fatigue, physical functioning, dyspnea, psychological distress, and positive effect, all of which demonstrated statistical significance when compared to patients treated with supportive care alone with a p value ≤ 0.01 [27]. Similar results were observed in the patients who crossed over to Aza. Toxicities among the Aza treated patients were most frequently related to myelosuppression and were difficult to distinguish from the underlying disease. It was notable that treatment with Aza did not appear to increase the infection or bleeding rates above background, and furthermore only one treatment related death was reported on the study, emphasizing the safety of this therapy, even for older patients [27].

13.2.1.2 AZA-001

Although the data from CALGB 9221 was compelling, this study did not, in the final analysis, demonstrate a difference in OS between the patients randomized to receive Aza and those randomized to supportive care, likely as a result of the crossover trial design. The AZA-001 study was designed to address the question of whether Aza provided an OS benefit for high grade MDS patients [7]. This cleverly conceived, international, randomized trial definitively demonstrated that Aza 75 mg/m^2 given subcutaneously for 7 days of a 28 day schedule prolonged OS when compared with conventional care regimens (CCRs) as selected by the patients physician. The investigators aimed to provide at least six cycles of Aza to those patients randomized to the experimental arm. Conventional care was assigned by the patient's physician prior to randomization depending upon the patient's age, performance status co-morbidities and patient preference. CCR consisted of the three most

common treatments for patients with int-2 or high risk MDS: IC including cytarabine 100–200 mg/m^2/day × 7 days plus, daunorubicin 45–60 mg/m^2 × 3 days or idarubicin 9–12 mg/m^2/day × 3 days or mitoxantrone 8–12 mg/m^2/day, low dose cytarabine (LDAC) at a dose of 20 mg/m^2 for 14 days every 28 days, or best supportive care (BSC). All patients randomized received CCR as selected by their physician or Aza on trial. A total 358 patients were randomized. In this way a pre-specified subgroup analysis based upon physician assignment was possible and helped to eliminate differences in outcome based upon issues of performance status and patient fitness.

The primary OS endpoint of this study was met after a median follow-up of 21.1 months [7]. At this analysis the OS in the Aza treated patients was 24.5 months vs. 15 months for patients assigned to CCR and this result was found to be statistically significant ($p \leq 0.0001$). Two year OS also favored Aza, at 51% vs. 25% for CCRs ($p \leq 0.0001$) [7]. Predefined subgroup analysis was also done in order to compare Aza responses with each of the CCRs selected and within specific cytogenetic and IPSS risk groups. There were significant differences between Aza and BSC with an OS benefit for azacytidine treatment of 9.6 months (HR 0.58, $p = 0.0045$), as well as between Aza and LDAC with an OS benefit of 9.2 months (HR 0.36, $p = 0.0006$) [7]. No statistically significant differences in OS were seen when Aza was compared with IC; OS was prolonged by 9.4 months with a hazard ratio of 0.76, but the p value was not significant at 0.51 [7]. This apparent discrepancy was likely due to the low numbers in this subgroup ($n = 42$); 17 patients in this group were randomized to Aza and 25 to intensive chemotherapy.

No differences in response to Aza were seen across the IPSS risk groups enrolled (although most patients were int-2 or high risk $n = 313$ (87%)), nor within the cytogenetic risk groups identified by the IPSS (good, intermediate, poor). Patients with del-7 or del(7q), a group recognized to have particularly poor prognosis, had an OS of 13.1 months vs. 4.6 months in the CCR group [7, 29].

Responses on this trial were similar to those observed in CALGB 9221. Overall, 29% of those assigned to Aza achieved either CR (17%) or PR (12%) compared with 12% (8% CR, 4% PR) assigned to CCR ($p = 0.0001$) [7]. Any hematological improvement (HI) was observed in 49% of those treated with Aza vs. 29% of those treated with CCR ($p = 0.0001$) [7]. In addition, for those treated with Aza, major erythroid responses were seen in 40% of patients, major platelet responses in 33% and major neutrophil responses in 19%. By contrast, for those receiving CCR major erythroid responses were seen in 11% ($p < 0.0001$), major platelet responses were seen in 14% ($p < 0.0003$) and major neutrophil responses were seen in 18% ($p = 0.58$, not statistically significant) [7]. Patients treated with Aza experienced a statistically significant reduction in the need for intravenous antibiotics (33% relative risk reduction vs. CCR; RR 0.66 95% CI:0.49–0.87 $p = 0.0032$). Furthermore of the 111 patients with red cell transfusion dependence at the time of study enrollment, 50 (45%) became transfusion independent vs. 13 (11.4%) of the 114 patients randomized to receive CCR (p value significant at 0.0032) [7].

Secondary endpoints in this trial included time to AML transformation and hematological response according to the IWG 2000 criteria for MDS [11]. Treatment with Aza in the entire group was associated with delayed leukemic transformation;

the median time to transformation was 17.8 months in the Aza treated group vs. 11.5 months in the CCR group ($p < 0.0001$) [7].

Among the most notable findings on this trial was that achievement of CR or PR was not necessary in order to achieve an improvement in OS; any patient who achieved a hematological response showed a survival benefit.

13.2.1.3 AZA in AML

Changes in the diagnostic criteria for AML based upon the WHO guidelines published in 2008 resulted in the reclassification of patients enrolled on both the CALGB and AZA-001 from the previous FAB classification of Refractory Anemia with Excess Blasts in Transformation (RAEB-T; 20–30% bone marrow blasts) to a new diagnosis of AML [1, 30, 31]. The WHO now defines any patients with ≥20% blasts as having AML [30].

A pooled analysis of previously published CALGB studies including 9221, 8921, and 8421, in which enrolled patients treated with Aza would now be re-assigned as AML was published in 2006 [28]. This reported the response to Aza given either intravenously or subcutaneously at a dose of 75 mg/m^2/day for 7 days of a 28 day cycle in 103 patients who would now be classified as having AML, 91 of whom received Aza [28]. Of these patients 33 (36%) developed a response (8 CRs, 2 PRs, 23 HIs), with a median duration of response of 7.3 months (range 2.2–25.9 months) [28]. Formal comparison with supportive care alone across the three studies was not possible, but 27 patients enrolled in 9221 were randomized to upfront Aza and a further 13 crossed over to receive Aza before the 6 month analysis. Of these, 7% in the Aza group achieved CR or PR compared with 0% in the observation-only group [28]. Median survival time for the 27 patients assigned upfront to Aza was 19.3 months compared with 12.9 months for the 25 AML patients randomly assigned to observation. Of 13 patients with WHO AML at the time of study entry who crossed over to receive Aza, one achieved a PR, and one HI.

Of the 358 patients originally enrolled on AZA-001, a third would now be identified as having AML. A second analysis of these patients was undertaken in order to assess outcome in this group of older adults treated with either Aza or CCR [7, 32]. Of the 113 patients now designated as AML, 63 were assigned to BSC, 34 to LDAC and 16 to IC [32]. The median age in all groups was 70 years with a range of 58–80. Patients were evenly distributed with respect to age, cytogenetic risk group, and ECOG scores. Bone marrow blast percentages were similar in both groups at 23% with a range of 20–34%. In all, 55 patients were randomized to the Aza arm and 53 to CCR. After a median follow-up of 20.1 months, OS was significantly ($p = 0.005$) longer in those patients treated with Aza (24.5 months) than in those receiving CCR (16 months). The 2 year survival was also superior in the Aza group at 50% compared with16% in the CCR group ($p = 0.001$) [32]. Adverse events in this group of patients were primarily grade 3 and 4 cytopenias, which remain difficult to distinguish from the underlying disease. Four patients in the Aza group and three patients in the CCR group discontinued treatment as a result of adverse events.

Several prospective studies of Aza given on the conventional schedule of 75 mg/m^2/day for 7 days in patients identified as AML at diagnosis have been reported. One such study enrolled 82 patients with AML (27 (33%) with secondary disease) and a median age of 72 years (range, 29–87 years) [33]. Thirty-five patients (43%) received Aza as their first treatment, and 47 patients (57%) had previously received 1 or more lines of chemotherapy. The overall response rate in this group was 32% (26/82 patients) with 16 patients (20%) achieving a CR or a CR with incomplete count recovery, and 10 patients (12%) achieving a PR [33]. Untreated patients responded more often than those previously treated with 31% of untreated patients achieving either a CR or a CR with incomplete count recovery compared with only 9 (19%) such responses in the previously treated group ($p=0.006$). The response duration in untreated patients who achieved a response was 13 months with 1 and 2 year survivals of 58 and 24% respectively [33]. Another study from Germany evaluated medically unfit ($n=20$) or relapsed/refractory ($n=20$) patients with AML and a median bone marrow blasts count of 42% [34]. This study showed similar statistically significant differences in response between untreated patients, who demonstrated overall responses (CR + PR + HI) of 50%, and patients with relapsed or refractory disease, who had an overall response rate of only 10% ($p=0.008$) [34]. These response rates are striking and compare favorably with responses seen with induction therapy although additional data are necessary in order to determine whether Aza or Dac will end up the therapeutic agent of choice in this context [6, 35].

Results from the CALGB trials were sufficient in the United States and the AZA-001 trial satisfied the European regulators for the approval of Aza as standard therapy for patients with MDS and low blast count AML. In the United States, approval was granted for all IPSS defined MDS subtypes, while in Europe approval is confined to patients with Int-2 and high risk IPSS scores not eligible for bone marrow transplantation, those with CMML-2 and those with WHO defined AML with 20–30% blasts or multilineage dysplasia.

Both large phase III trials demonstrated this drugs activity in MDS and AML, and further showed that unlike previous therapies, DNMTi require prolonged exposure to elicit a clinical benefit. In the CALGB trials most responses were seen by cycle 4 (75%), with a median number of cycles to any response (CR, PR, HI) of three cycles [27]. The range for this response was 1–17 cycles, however and although 90% of patients achieved a response by cycle 6, some patients got their response as late as cycle 17 [27]. In the AZA-001 trial where the goal was to provide at least six cycles of therapy and there was no predefined stopping point, the investigators demonstrated that continuing the Aza dosing as long as possible can result in improvements in the observed responses, and these results were re-iterated by additional analysis of the studies conducted by the CALGB [28, 32]. The secondary analysis of CALGB studies demonstrated a response in 91 of 179 patients, and responders received a median of 14 cycles of therapy (range 2–30) [28]. The median time to first response in this study was slightly shorter than that seen in 9221, at 2 cycles (but with a range of 1–16) and although most responses (91%) were achieved by the sixth cycle, continuation of Aza was able to improve the quality of the first response in 48% of those treated, and this best response was seen in most patients (92%) by

the 12th cycle [28]. Overall 30 patients achieved a best response of CR 3.5 cycles beyond the first response (with a 95% CI of 3.0–6.0 cycles), and in 21 patients whose best response was PR, this was seen as a median of 3.0 cycles after the first response (95% CI was 1.0–3.0) [28].

13.2.1.4 Other Considerations of Dose and Schedule

Additional questions which remain about the use of single agent Aza therapy are related to administration schedule (to weekend or not to weekend, are 7 days enough) and optimal drug delivery (subcutaneous vs. intravenous vs. oral).

In community practice there is often difficulty in giving this drug on the FDA approved schedule due to inadequate availability of personnel to administer the drug on weekends. This practical consideration resulted in a trial of several schedules of Aza administered in a community setting during weekdays only [36]. In this trial, 151 patients, for the most part with lower risk MDS (low, int-1 in 63% of patients), were randomized to receive Aza on one of the three schedules: 75 mg/m^2 daily for 5 days, off 2 days and then on 2 days (5-2-2), 50 mg/m^2 daily for 5 days, off 2 days and then on for 5 further days (5-2-5), and lastly 75 mg/m^2 daily for 5 days alone (5-0-0) [36]. These schedules seemed to result in similar hematological improvement rates (44%, 45%, 56%, respectively), but this study was not designed to produce statistically significant results, nor have these schedules been directly compared with the approved 7 day schedule. Thus it is difficult to condone alteration of the schedule at this time, based upon the lack of survival data in these schedules and the demonstrated survival benefit with administration of these drugs on the approved schedule. One additional schedule question has been raised by the preliminary data from the Eastern Cooperative Oncology Group trial 1905, which was a randomized phase II trial comparing Aza 50 mg/m^2/day subcutaneously for 10 days to the same Aza schedule given in combination with the Histone deacetylase (HDAC) inhibitor entinostat (4 mg/m^2/day PO days 3 and day 10) [37]. This abstract reported only on patients with baseline cytogenetic abnormalities ($n = 40$ evaluable) but demonstrated complete cytogenetic responses of 13% and a partial cytogenetic responses of 23% for an overall response in this subgroup of 51% (21/40) [37]. No differences in response were seen between the two treatment groups. Notably the responses observed were significantly higher than those reported with conventional Aza dosing raising the question of whether a lower dose, longer administration schedule may be of some benefit. At present these data are insufficient to change practice, however as additional groups publish the results of ongoing clinical trials of different dosing schedules, practice changes may be in order.

With respect to optimal drug delivery there is only a single study which directly compares the pharmacokinetics of intravenous to subcutaneous dosing within individual patients. In this study the pharmacokinetic profile of intravenous administration was almost identical to that seen with subcutaneous dosing, although the peak drug concentration was higher in patients receiving intravenous drug [38]. Despite these

data, published clinical trials using 20 min IV infusion schedules are limited to two studies, one which gave Aza for 5 days and the other for 7 [39, 40]. Both of these studies demonstrated response rates which were similar to those seen with subcutaneous dosing (27% in the 5 day and 56% for the 7 day schedule), but neither of them was powered to detect a survival benefit [39, 40]. Despite the dearth of published response data, it seems reasonable to switch to intravenous administration in patients who suffer significant injection site reactions with subcutaneous dosing, and the FDA approved a New Drug Application for intravenous Aza in January 2007, supporting this practice [41].

Initial studies with oral Aza were limited by rapid catabolism of the compound in aqueous environments but the development of a film-coated formulation improved stability [42, 43]. Since that time the first phase I study of oral Aza has been published, demonstrating activity for the oral drug in patients with both MDS and CMML, with promising response rates [44]. Six of 17 (35%) previously treated patients had a response (CR + PR + HI) and 11 of 15 (73%) untreated patients responded (CR + PR + HI). This study demonstrated no overall response in the 8 patients with AML, however two patients had stable disease for 14 and 15 cycles [44]. Overall these results suggest that oral Aza may be a real possibility for the future and clinical trials of this drug are ongoing.

13.2.2 Dac

5-Aza-2′-deoxycytidine (Dac) is a deoxynucleoside analog of cytidine in which the carbon 5 position of the pyrimidine ring has been substituted with nitrogen (Fig. 13.2b) [15]. It is imported into cells by the action of nucleotide transporters, where it is activated by deoxycytidine kinase and then phosphorylated (Fig. 13.3) [15]. After its phosphorylation to the triphosphate form, 100% of the drug is incorporated into DNA, where it interrupts the action of DNA methyltransferases as described above for Aza. Similar to Aza, Dac has been demonstrated to cause both DNA hypomethylation and DNA damage, albeit at lower concentrations [45]. The identification of DNA hypomethylation as a functional consequence of exposure to both Aza and Dac, in conjunction with the recognition of DNA methylation changes as a frequent abnormality in cancer, spurred significant clinical interest in the development of these drugs for clinical use [20, 45].

Although effects upon DNA methylation were recognized and noted early in its development, initial clinical trials focused on conventional dosing strategies aimed at developing a maximum tolerated dose schedules [46–48]. These studies demonstrated considerable activity but with toxicity not significantly superior to cytarabine, with several studies performed investigating combinations with other chemotherapeutics in the salvage setting [49, 50].

Several early studies showed promising results with “low dose” Dac regimens, however these studies provided the drug at doses of 40–50 mg/m^2/day, and toxicity remained a serious problem [51–53]. The first study to investigate the “optimal”

lower dose Dac schedule for maximal demethylation was published in Blood in 2002 by Jean-Pierre Issa and colleagues [54]. This trial enrolled 48 patients at doses ranging from 5 to 20 mg/m^2/day for 10–20 days of a 6 week schedule depending upon count recovery. Most interestingly in this study, responses appeared to be superior for the lower dose schedules studied, prompting the authors to suggest further investigations of the drug be undertaken at truly lower dose schedules [54].

Based upon extensive phase I/II data at moderate to higher doses, the first large scale trial of Dac enrolled 170 patients with MDS between 2001 and 2004 and randomized them to either Dac (89 patients), given at 15 mg/m^2 iv every 8 h (45 mg/m^2/day) for 5 days, or BSC (81 patients) [10]. Patients were removed from the study for disease progression, transformation to AML, failure to achieve a PR after six cycles of therapy, or failure to achieve a CR after eight cycles of therapy. Additionally, patients who did achieve a CR were removed from therapy after two cycles of sustained CR. The groups were well matched for all important variables with a median age of 70 years (range, 30–85 years). A majority of the patients (71%) had int-2 or high risk disease by IPSS criteria. The primary study endpoints were overall response rate and time to AML transformation or death. Overall 30% ($n=27$) of patients experienced improvement on the study (CR + PR + HI) compared with 7% ($n=6$) patients randomized to BSC, and this difference was statistically significant $p=0.001$ [10]. In a retrospective central review of pathology nine patients enrolled on Dac and three patients on the supportive care arm were designated as having AML (by FAB criteria, >30% bone marrow blasts). Response rates in these nine patients were 56% (5/9), while none of the patients enrolled on the supportive care arm developed a response [10]. It is important to note that in this randomized controlled non-crossover trial there was no survival benefit for the use of Dac, although one might argue that the dose used (45 mg/m^2/day × 5 days) was not low enough to maximize hypomethylation over cytotoxicity and the median number of cycles administered was low (3).

Following the results of this trial (which were disappointing from a survival perspective, but represented the first active agent for patients with high grade myelodysplasia), in 2006 the FDA approved Dac for all MDS subtypes. Based upon the results of earlier studies suggesting that lower dose Dac dosing might be superior, two pivotal phase II studies were performed aimed at identifying the "optimal" hypomethylating dose for Dac [55, 56]. The first of these was published in 2007 and enrolled 95 patients, again with a majority (66%) of patients having int-2 or high risk disease [55]. All patients were randomized to receive one of the three different Dac schedules, 10 mg/m^2 intravenously over 1 h daily for 10 days, 20 mg/m^2 intravenously over 1 h daily for 5 days, or 20 mg/m^2 subcutaneously daily for 5 days. Patients received a median of seven cycles of treatment and the CR rate overall was significantly better than anticipated at 37%, and an overall improvement (including CR + PR + HI) was observed in a staggering 73% of patients [55]. The 5 day schedule was deemed superior with 25/64 patients on this arm achieving CR and this schedule was selected for further investigation in subsequent trials [55]. The second analogous trial published in 2009 by Steensma and colleagues enrolled 99 patients in a single arm trial of Dac 20 mg/m^2 over 1 h daily for 5 days [56]. A lower percentage of patients on this trial were high grade (46%), and the median number of administered courses were slightly

lower (5) than in the prior investigation. These authors observed a 15% CR rate and an overall response rate of 43% (CR + PR + HI) [56]. Both trials demonstrated that the lower dose schedule of Dac 20 mg/m^2/day for 5 days had at least equivalent efficacy when compared with the FDA approved schedule, and furthermore that maintaining 4 week dosing intervals and repeated cycles of therapy were important in order to maximize response.

One additional phase III study of Dac has been published [13]. It is important to note that this study did not employ the 5 day, 20 mg/m^2/day schedule described above. This trial was designed to demonstrate a survival benefit for the use of Dac in patients with MDS, comparable to that observed with Aza. Two-hundred and thirty-three patients with a median age of 70 years (range 60–90) were enrolled; 53% had poor-risk cytogenetics and 33% fulfilled WHO AML diagnostic criteria (≥20% blasts) [13]. The primary end point for this trial was OS. Patients were stratified by IPSS risk group, cytogenetics and enrollment site, and were randomly assigned to receive either Dac or BSC. This study design specifically prohibited patient crossover to the experimental arm in an effort to eliminate crossover bias. The Dac was given intravenously at a dose of 15 mg/m^2 every 8 h for 3 days. Cycles were scheduled to repeat every 6 weeks, but the interval could be extended up to 10 weeks for failure of count recovery, eight cycles of treatment were planned. In total 119 patients were randomized to receive decitabine and 114 patients were randomized to the control arm; only 21% of patients received the planned eight cycles of treatment. At the planned analysis point of 2 years, OS in the Dac treatment cohort was 10.1 months vs. 8.5 months in the supportive care arm, this difference was not statistically significant ($p = 0.38$, HR, 0.88; 95% CI, 0.66–1.17) [13]. Sixteen patients on the Dac arm (13%) achieved a CR and 25 patients (21%) improved (PR + HI), for an overall response rate of 34%. The median time to best response was 3.8 months (range, 1.4–11.8 months) for all responders, with a median of 5.8, 2.9, and 3.8 months to reach CR, PR, and HI, respectively. Two patients (2%) in the supportive care arm had a HI, there were no CRs or PRs in this group. Dac did not have a statistically significant impact upon time to AML transformation; patients on Dac transformed to AML after 8.8 months vs. 6.1 months in the supportive care arm (HR 0.85; 95% CI 0.64–1.12; $p = 0.24$) [13].

Disappointing results, in terms of survival benefit, from two large phase III trials of Dac in MDS have resulted in a significant shift in terms of practice away from Dac in this population [10, 13]. Despite these results, some clinicians continue to use Dac in the first line treatment of MDS patients, and it is certainly notable that none of the three phase III studies of Dac used the most common low dose schedule of Dac at 20 mg/m^2/day for 5 days, a dose schedule which is pharmacologically more consistent with the 75 mg/m^2 Aza dose demonstrated to prolong survival. Additionally, the European phase III trial delayed subsequent Dac cycles based upon cytopenias, a strategy which is increasingly recognized as inferior. As a result of these caveats it is likely that Dac has similar efficacy to Aza, although at present the data have not definitively demonstrated this equivalence.

13.2.2.1 DAC in AML

Despite disappointing results in patients with MDS, many clinicians favor Dac in patients presenting with AML, particularly in those with very proliferative disease, as a result of its relative cytotoxicity when compared with Aza. A dosing strategy employing 20 mg/m^2 for 10 days has been studied by investigators at the Ohio State James Cancer Center [35, 57]. This dose schedule was initially developed in a phase I trial designed to assess combination therapy with valproic acid, however a single agent response of 73% in a group of very elderly (median age 70) patients with high risk AML prompted phase II investigation (see below) [57]. The Phase II trial enrolled 53 patients of median age 74 years (range 60–85) with AML (16 complex karyotype, 19 with an antecedent hematological diagnosis) and produced a response rate of 64% (34/53) composed of 25 CRs and 9 CRs without count recovery [35]. Patients enrolled on study had a median survival of more than a year, suggesting that this strategy is similarly effective to conventional chemotherapeutics in this patient population [6, 8, 9]. These very promising results have produced an ongoing cooperative trial using this dose schedule in older patients with AML and may yet demonstrate statistically significant improvements in survival for this particular subgroup of elderly AML patients.

13.3 Azanucleotides and CMML

Dac remains the most studied drug in patients with CMML, a distinct entity within the WHO diagnostic criteria form MDS. Several studies have examined the activity of Dac both prospectively and retrospectively in this group. One recently published phase II study enrolled 39 patients of median age of 71 years with advanced CMML to receive Dac on the 20 mg/m^2/day intravenous schedule for 5 days of a 28 day cycle [58]. Enrolled patients received a median of ten cycles of drug (range, 1–24) and the overall response rate was 38%, composed of 4 (10%) CRs, 8 (21%) marrow responses, and 3(8%) His [58]. With a median on trial follow-up of 23 months the OS was 48%. Another study examined the response to Dac in 31 patients diagnosed with CMML who were treated on two phase II and one phase III clinical trials [59]. Patients included in the analysis had similar demographics and disease characteristics across the three studies. The median age was 70 and patients were predominantly male (71%). The overall response rate in this group was 36% (14% CR + 11% PR + 11% HI) [59]. Although Aza has also been shown to have activity in this disease, the number of published reports in this group are limited, and thus most experts would likely favor the use of Dac for patients with CMML outside the context of a clinical trial [60]. An ongoing clinical trial designed to prospectively enroll patients with CMML is ongoing in order to address the efficacy of Aza in this disease.

13.4 Outcomes Following Azanucleotide Failure

As we develop our experience with azanucleotides it has become clear that patients who lose their response to azanucleotides have a dismal prognosis [14]. As a result of these poor outcomes, current standard practice is to maintain patients on therapy with hypomethylating drugs on a monthly schedule indefinitely and to stop only in the context of overt progression. Unfortunately, analysis of patients enrolled on early studies of Aza who develop disease progression have now been published, showing that in patients who fail azanucleotides, survival is remarkably short with a median life expectancy of 5.6 months and a 2-year survival probability of 15% [61]. Similar results have been reported in patients who fail Dac [14, 62]. Outcomes in these reports suggest that enrollment on clinical trials and bone marrow transplantation may result in superior outcome in these patients, however in the absence of successful bone marrow transplantation the OS reported at 1 year remains a mere 28% [14, 61, 62].

13.5 Histone Deacetylase Inhibitors

Histone deacetylase inhibitors (HDACis) are a novel class of drugs whose putative mechanism of action depends upon the ability to alter gene expression. Intracellularly, DNA is stored in the form of "beads on a string" in which the DNA duplex winds around a nucleosome composed of eight histones (two each of H2A, H2B, H3 and H4) [63]. The DNA/histone unit (the nucleosome) is condensed to form higher order chromatin structures such as heterochromatin, which has densely packed nucleosomes and euchromatin, which has loosely packed nucleosomes [63]. Modifications, including ubiquitination, methylation, phosphorylation, poly(ADP) ribosylation, and acetylation, of specific amino acid residues within each histone make up the "histone code" which determines the state of the regional chromatin at specific genes and thus their transcriptional activity [63]. DNA methylation events are thought to induce changes within the local "histone code" which promote gene silencing, although whether methylation events or histone marks are primary remains a matter of some controversy. Perhaps the most studied histone modification is acetylation of lysine N-terminal tails which are common to most histones. Acetylation of lysine results in an open chromatin conformation and promotes gene transcription while deacetylation of lysine residues promotes gene silencing [63].

HDACs are enzymes that remove acetyl groups from a variety of different protein targets including histones. Increased HDAC activity has been described in cancer cells, and aberrant HDAC activity is characteristic of a number of well recognized recurrent genetic anomalies characteristic of leukemia including the core binding factor gene fusions (t(8;21)(q22;22) and inv(16)), and the sine qua non of acute promyelocytic leukemia t(15;17)(q24;21) [64–66]. The gene products of such fusions result in aberrant recruitment of HDACs to genes important for myeloid

differentiation. Recognition of HDACi as a potential novel therapy in myeloid malignancy resulted from the observation that drugs known to induce differentiation in vitro induced histone hyperacetylation, potentially leading to re-expression of epigenetically silenced genes [67]. Many different diverse chemical compounds can inhibit HDACs, including short chain fatty acids (e.g., phenylbutyrate), hydroxamic acid derivatives (e.g., vorinostat), non-hydroxamate small molecules (e.g., entinostat), and cyclic peptides (e.g., romidepsin) [68].

Most of the published clinical trials of HDACi in MDS and AML are phase I. As single agents the response rates observed have been relatively low, usually between 10 and 20% [68]. Toxicities with these agents demonstrate a common pattern and include fatigue, nausea, vomiting, and diarrhea. Although most of these studies evaluated the correlative endpoint of histone acetylation, no associations between hyperacetylation of histones and response to therapy have been demonstrated. For a more complete review of HDACi in cancer please see Chap. 3, Sect. 3.5 of this book.

13.6 Azanucleotides and HDACis

There has been significant enthusiasm for a combination strategy which includes azanucleotides in conjunction with HDACis. This stems from the observation in vitro that sequential exposure to Dac or Aza followed by HDACi result in synergistic re-expression of DNA methylation silenced genes [69]. Several studies evaluating such combinations have been published to date and the results remain mixed. Although some studies suggest a higher response rate than for single agent azanucleotides, most data are in the phase I or II setting, at a single center, and employ alternative dosing strategies for the azanucleotide making it difficult to distinguish whether these responses are truly superior. In those studies where a single agent arm was also enrolled response rates do not appear to be consistently superior [37, 57]. Although early correlative endpoints did demonstrate evidence to support a connection between reversal of methylation events and response to therapy, subsequent studies (even at the same institution by the same investigators) have failed to substantiate a correlation between gene specific reversal of methylation and response [70, 71].

The first two studies published reports on a combination of Aza at doses between 25 and 75 mg/m^2/day subcutaneously for 5–10 days [70, 72]. These studies enrolled a total of 42 patients with MDS (16) and AML (26), of median age 66. These studies reported that the combination was well tolerated and resulted in response rates of 34 (11/32, 5 CRs) and 50% (5/10, no CRs) respectively (CR + PR + stable disease) [70, 72]. The second study reported correlative epigenetic data in three responders and three non-responders, with those patients who developed a response showing robust demethylation of the tumor suppressor gene *p15^{INK4B}* while those who did not retained methylation at this locus, suggesting that changes in methylation were indeed a marker for responsiveness [70].

Two phase I/II studies have evaluated the combination of Dac with valproic acid. The first employed Dac 15 mg/m^2/day for 10 days with a dose escalation of valproic acid from 20 to 50 mg/kg/day for 10 days in patients with high grade MDS or AML [73]. Fifty four patients of median age 60 (range 5–80 years) were enrolled, 48 patients had AML and 6 had MDS, 11 patients were previously untreated. Twelve patients responded to therapy; 10 developed a CR and 2 a CR with incomplete platelet recovery. Median responses were seen after 2 months (range 29–130 days) and responders survived a median of 15.3 (range 4.6–20.2+) months vs. 4.9 (0.6–17.8+) months in non-responders [73]. Responders were more likely to have been randomized to a higher dose of valproic acid. Although changes in methylation (both gene specific events, including $p15^{INK4B}$, and genome wide methylation, by LINE-1 pyrosequencing) and gene expression were analyzed in the patients on this study no correlations with response were observed [73]. All patients experienced a decrease in genome wide methylation which correlated with Dac exposure. In a second study, this one employing Dac 20 mg/m^2/day for 10 days intravenously, responses were also encouraging with an overall response rate of 44% in 11 of 25 enrolled patients [57]. This trial enrolled 25 AML patients, in whom the median age was 70 years; 12 patients were untreated and 13 had relapsed disease. In this group of slightly older patients, encephalopathy was the principal toxicity and this was dose limiting at 20–25 mg/kg/day. In an intent-to-treat analysis, the response rate was 52% (13). CR was observed in 8 patients and PR in 4. Responses appeared similar for patients who received Dac alone and for those who received valproic acid in addition. In this study, re-expression of estrogen receptor was statistically significantly associated with clinical response ($p=0.05$), however although the investigators also demonstrated ER promoter demethylation, global DNA hypomethylation, depletion of DNA methyltransferase enzyme, and histone hyperacetylation, these markers did not correlate with response [57].

The combination of Aza with vorinostat (SAHA) has also been explored. In one phase I trial in patients with MDS and AML this combination produced an impressive overall response rate of 64%[74]. A second phase II trial of this combination in patients with MDS and AML has also been reported [75]. This trial enrolled 17 untreated patients and demonstrated an overall response rate of 41% ($n=7$) [75]. Similar outcomes (overall response of 37%) were observed in patients receiving a combination of Aza with the compound MGCD0103, an oral isotype-selective HDACi [76]. Although these responses appear to be encouraging, a majority of these combination studies have been published to date only in abstract form and larger studies are necessary in order to verify their superiority.

Data from one of the first randomized phase II studies to enroll patients either on single or double agent therapy was presented at the 2010 ASH meeting and reviewed in detail earlier in this manuscript (see Aza section under Sect. 5.2.1.4), this study, at least, suggests that combination therapy may not be superior [37]. In this trial patients with either MDS or AML with MDS related changes were randomized to receive either Aza at 50 mg/m^2 for 10 days subcutaneously alone or Aza in combination

with entinostat 4 mg/m^2 orally on days 3 and 10. Although the final results of this trial have not yet been published, it is important to note that the response rates for patients enrolled to receive Aza alone were indistinguishable from those who got the combination.

These results and others with a variety of HDACis may underestimate the value of combined therapy. It is important to note that among the many mechanisms postulated to be responsible for the efficacy of HDACis are induction of apoptosis and cell cycle arrest [77]. Since azanucleotides require DNA replication in order to produce DNA demethylation, it may be that administration of HDACi simultaneously or even in advance of the azanucleotide may result in diminished incorporation and limit responsiveness. Presently, a multi-institution phase II sequence study designed to address this question is open for enrollment [78].

13.7 Azanucleotides and Conventional Chemotherapy

One study has been published which explores the possible role of azanucleotide in "priming" leukemia cells for death [79]. This open label, phase I study was designed to address the safety and feasibility of Dac at a dose of 20 mg/m^2 either as a continuous infusion or a short infusion for 3, 5, or 7 days followed by standard dose 7 + 3 IC (cytarabine 100 mg/m^2/day continuous intravenous infusion for 7 days + daunorubicin 60 mg/m^2/day for 3 days). The study enrolled 30 patients of median age 55 (range 23–60) with newly diagnosed AML and a less than favorable karyotype (inv(16), t(8;21) and APL patients were excluded). Thirteen patients had complex, 11q23 or chromosome 7 abnormality associated leukemias and 8 had an antecedent hematological diagnosis. Toxicity was not dissimilar to that seen with 7 + 3 alone, although there appeared to be slightly more gastrointestinal toxicity in the group treated with 7 days of Dac priming, and there were no deaths. All subjects received consolidation, 20 patients went on to receive allogeneic bone marrow transplantation. Overall 27 (90%) of patients responded to one course of induction therapy, 17 patients achieved a CR and 10 a PR, patients scored as a PR all achieved hematological remission, but went on to receive a second course of induction resulting in a CR in 8/10 patients [79]. The overall CR rate following 1 or 2 cycles of induction therapy was therefore 83%. With a median follow-up of 32 months, 53% of patients (16/30) remained alive and in CR, 14 subjects died, 3 of complications related to allogeneic bone marrow transplant and the remainder died of relapsed or refractory AML [79]. The correlative DNA methylation analysis of this study revealed universal demethylation at both gene specific and genome wide loci with all schedules of Dac. The most potent hypomethylation was observed in patients treated with bolus, rather than continuous infusion schedules of Dac.

Although preliminary, this phase I trial demonstrated a remarkably good CR rate and a randomized phase II study designed to assess the two most potent demethylation schedules of Dac priming identified by this study should begin accrual in 2012.

13.8 Azanucleotides and Bone Marrow Transplantation

Allogeneic bone marrow transplant (allo-transplant) is the only curative strategy currently available for patients with MDS and high risk AML. Presently the role of hypomethylating agents both prior to and following transplant is under investigation.

Several small retrospective studies of azanucleotide induction prior to allo-transplant have been reported, two using Dac and two using Aza. The first of these reported outcomes in 17 patients with MDS of median age 55.5 (range 36–66) years undergoing allo-transplant (12 sibling donor, 5 unrelated donor) after prior therapy with Dac (various dosing regimens) [80]. These patients received predominantly reduced intensity conditioning and peripheral blood stem cells (13/17). With median follow-up of 12 (range 3–35) months, 8 patients remained in CR [80]. A second prospective study performed in Europe reported similar results in 15 patients of median age 69 (range 60–75) years with either MDS ($n=10$) or AML ($n=5$) [81]. All patients were treated with upfront Dac followed by reduced intensity allo-transplant (4 sibling donor, 11 unrelated donor). Fourteen patients achieved a CR (93%), with a median duration of 5 (range 1–51) months [81]. The relapse rate in this group was similar (4/15) to that reported retrospectively. The third study examined outcomes in 54 patients with MDS or CMML who either received (30) or did not receive (24) prior therapy with Aza [82]. Patients treated with Aza received a median of 4 (range 1–7) courses prior to transplant. The overall, relapse free and cumulative relapse 1 year following transplant were 47, 41, and 20%, for those patients treated with Aza and 60, 51, and 32% for untreated patients and these results were not statistically significantly different [82]. The final trial using Aza was a retrospective review of 68 patients undergoing allo-transplant for MDS or AML arising from MDS [83]. Thirty five patients received Aza followed by either myeloablative (40%) or reduced intensity (60%) conditioning. Thirty three patients received IC followed by allo-transplant. In these two, albeit somewhat different groups, the OS at 1 year was 57% in those treated with Aza and 36% in the IC group [83]. Overall these data suggest that Dac and Aza are a reasonable pre-transplantation strategy that does not adversely affect outcome when compared with high dose induction or supportive care. A phase II clinical trial of Dac prior to allo-transplant is ongoing in Singapore using the currently favored schedule of 20 mg/m^2/day for 5 days intravenously.

Post-transplant relapse remains a significant problem in MDS and high risk AML patients. Traditionally relapses in this population have been managed with donor lymphocyte infusions (DLI) (in those who do not demonstrate graft vs. host disease) or re-induction with traditional chemotherapeutic agents. Although limited prospective data exist on the use of azanucleotides for salvage of patients relapsing following allogeneic transplant, or as a preventive strategy following transplant, several small studies have been published, suggesting that these agents may have a significant role to play.

The first of these examined the efficacy of Aza at a flat dose of 100 mg subcutaneously days 1–3 followed by planned DLI on day 10 [84]. Cycles were repeated

every 22 days for a median of 2 (range 1–10) courses to 26 patients with relapsed AML ($n=24$) or CMML ($n=2$) following allo-transplant. Toxicity with this combination was as expected and consisted of infections and GVHD. Four patients (15%) were salvaged with a complete and lasting CR following this combination [84].

A second study, this one retrospective, described the results of salvage with Aza 100 mg/m^2 for 5 days in 22 patients of median age of 50 (range 28–69) years, with either AML (17) or MDS (5) relapsed following allo-transplant [85]. A majority (20/23) of these patients had received a myeloablative conditioning regimen and half (10/23) had a sibling donor. On average two cycles of Aza were administered (range 1–8). Most patients also received DLI (18/23). In this group, 5 patients (23%) achieved a CR lasting a median of 433 days (range 114–769) with a 2-year survival rate of 23%[85].

A third single institution study, retrospectively reviewed Aza 75 mg/m^2 for 5 or 7 days as salvage in 10 patients with MDS (9) or AML (1) of median age 55 (range 25–67) years [86]. Seven patients achieved CR or stable disease with this regimen, 3 of whom progressed after a median of 6 cycles. The median OS (OS) for the group was 422.5 days (range 127–1,411).

Taken together these results are encouraging and a variety of studies are ongoing to determine prospectively the role of azanucleotides both before and after allo-transplant [87].

13.9 Molecular Determinants of DNMTi Response in MDS and AML

Early on in the development of azanucleosides for the treatment of myeloid disease there was considerable enthusiasm for the identification of molecular markers of disease response. Initially several authors examined gene specific methylation reversal, including *p15^{INK4B}* and ER as discussed earlier in this manuscript [10, 55, 57, 70, 71]. Disappointingly, although reversal of methylation at many loci has been documented following azanucleotide exposure, it has not been demonstrate to correlate with or predict response to treatment, but rather seems to reflect duration of exposure to hypomethylating agents [88]. Another marker of response which has been studied is p53-inducible-ribonucleotide-reductase (p53R2), a gene identified in cell line screens to be induced following decitabine exposure [89, 90]. Link and colleagues demonstrated a statistically significant concordance between response to therapy and induction of p53R2 both at the mRNA and protein levels [90]. Although these results are thought provoking, they require sampling after many cycles of therapy and it is difficult to determine how useful a biomarker of response this would be clinically.

The identification of mutations in the genes encoding *TET2* (ten–eleven translocation2) and *DNMT3A* in patients with MDS and AML have raised questions about whether response to therapy may depend upon genetic characteristics of the underlying myeloid neoplasm. Recently a number of authors have demonstrated that up

to 26% of patients with MDS demonstrate mutations in TET2, and further that MDS patients with *TET2* mutations appear to have a superior prognosis (although this is not as clear in patients with AML) [91, 92]. Since *TET2* encodes a dioxygenase which functions to convert 5-methylcytosine to 5-hydroxymethylcytosine resulting in DNA demethylation at selective loci, defects in TET2 function would be expected to result in hypermethylation. One recent study suggests that patients bearing *TET2* mutations have a superior response (CR + PR + HI) to Aza treatment 82% vs. 45% ($p = 0.007$), although OS was not different in the two groups and these results have yet to be validated [93]. By contrast with mutations involving *TET2,* mutations in *DMNT3A* have been demonstrated to predict adverse outcome in both MDS and AML, although as yet no evaluation has been made of the impact of such mutations on response to epigenetic therapies [94–96].

13.10 Conclusions

Azanucleotides have changed the landscape of treatment for patients with MDS and AML with MDS related changes. Ongoing work with these agents in patients with a variety of myeloid diseases is likely to result in advances over the next few years. Despite the considerable efficacy of these drugs, patients with underlying myelodysplasia continue to have a remarkably poor outcome and novel strategies in these diseases remain essential. As we continue to develop insight into the mechanism(s) which underlie the activity of these drugs, perhaps we will be able to understand why they work so well for some patients and what strategies will maximize the longevity of these responses. Certainly it has become clear that single agent azanucleotides given on a conventional schedule are not a panacea. Whether responses can be optimized with continuous dosing strategies, combination with other drugs, or allogeneic bone marrow transplantation remains a question yet to be answered by well designed clinical trials.

References

1. Vardiman JW, Harris NL, Brunning RD (2002) The World Health Organization (WHO) classification of the myeloid neoplasms. Blood 100:2292–2302
2. Greenberg PL (1998) Risk factors and their relationship to prognosis in myelodysplastic syndromes. Leuk Res 22(Suppl 1):S3–S6
3. Menzin J, Lang K, Earle CC, Kerney D, Mallick R (2002) The outcomes and costs of acute myeloid leukemia among the elderly. Arch Intern Med 162:1597–1603
4. Lowenberg B, Downing JR, Burnett A (1999) Acute myeloid leukemia. N Engl J Med 341:1051–1062
5. Howlader N, Noone AM, Krapcho M, Neyman N, Aminou R, Waldron W, Altekruse SF, Kosary CL, Ruhl J, Tatalovich Z, Cho H, Mariotto A, Eisner MP, Lewis DR, Chen HS, Feuer EJ, Cronin KA, Edwards BK (2011) SEER Cancer Statistics Review, 1975-2008, National Cancer Institute. Bethesda, MD, based on November 2010 SEER data submission, posted to the SEER web site

6. Kantarjian H, O'brien S, Cortes J, Giles F, Faderl S, Jabbour E, Garcia-Manero G, Wierda W, Pierce S, Shan J, Estey E (2006) Results of intensive chemotherapy in 998 patients age 65 years or older with acute myeloid leukemia or high-risk myelodysplastic syndrome: predictive prognostic models for outcome. Cancer 106:1090–1098
7. Fenaux P, Mufti GJ, Hellstrom-Lindberg E, Santini V, Finelli C, Giagounidis A, Schoch R, Gattermann N, Sanz G, List A, Gore SD, Seymour JF, Bennett JM, Byrd J, Backstrom J, Zimmerman L, McKenzie D, Beach C, Silverman LR (2009) International Vidaza High-Risk MDS Survival Study Group: Efficacy of azacitidine compared with that of conventional care regimens in the treatment of higher-risk myelodysplastic syndromes: a randomised, open-label, phase III study. Lancet Oncol 10:223–232
8. Tilly H, Castaigne S, Bordessoule D, Casassus P, Le Prise PY, Tertian G, Desablens B, Henry-Amar M, Degos L (1990) Low-dose cytarabine versus intensive chemotherapy in the treatment of acute nonlymphocytic leukemia in the elderly. J Clin Oncol 8:272–279
9. Gardin C, Turlure P, Fagot T, Thomas X, Terre C, Contentin N, Raffoux E, de Botton S, Pautas C, Reman O, Bourhis JH, Fenaux P, Castaigne S, Michallet M, Preudhomme C, de Revel T, Bordessoule D, Dombret H (2007) Postremission treatment of elderly patients with acute myeloid leukemia in first complete remission after intensive induction chemotherapy: results of the multicenter randomized Acute Leukemia French Association (ALFA) 9803 trial. Blood 109:5129–5135
10. Kantarjian H, Issa JP, Rosenfeld CS, Bennett JM, Albitar M, DiPersio J, Klimek V, Slack J, de Castro C, Ravandi F, Helmer R III, Shen L, Nimer SD, Leavitt R, Raza A, Saba H (2006) Decitabine improves patient outcomes in myelodysplastic syndromes: results of a phase III randomized study. Cancer 106:1794–1803
11. Cheson BD, Bennett JM, Kantarjian H, Pinto A, Schiffer CA, Nimer SD, Lowenberg B, Beran M, de Witte TM, Stone RM, Mittelman M, Sanz GF, Wijermans PW, Gore S, Greenberg PL (2000) World Health Organization(WHO) international working group: Report of an international working group to standardize response criteria for myelodysplastic syndromes. Blood 96:3671–3674
12. Cheson BD, Cassileth PA, Head DR, Schiffer CA, Bennett JM, Bloomfield CD, Brunning R, Gale RP, Grever MR, Keating MJ (1990) Report of the National Cancer Institute-sponsored workshop on definitions of diagnosis and response in acute myeloid leukemia. J Clin Oncol 8:813–819
13. Lubbert M, Suciu S, Baila L, Ruter BH, Platzbecker U, Giagounidis A, Selleslag D, Labar B, Germing U, Salih HR, Beeldens F, Muus P, Pfluger KH, Coens C, Hagemeijer A, Eckart Schaefer H, Ganser A, Aul C, de Witte T, Wijermans PW (2011) Low-dose decitabine versus best supportive care in elderly patients with intermediate- or high-risk myelodysplastic syndrome (MDS) ineligible for intensive chemotherapy: final results of the randomized phase III study of the European Organisation for Research and Treatment of Cancer Leukemia Group and the German MDS Study Group. J Clin Oncol 29:1987–1996
14. Kadia TM, Jabbour E, Kantarjian H (2011) Failure of hypomethylating agent-based therapy in myelodysplastic syndromes. Semin Oncol 38:682–692
15. Yang X, Lay F, Han H, Jones PA (2010) Targeting DNA methylation for epigenetic therapy. Trends Pharmacol Sci 31:536–546
16. Weiss AJ, Stambaugh JE, Mastrangelo MJ, Laucius JF, Bellet RE (1972) Phase I study of 5-azacytidine (NSC-102816). Cancer Chemother Rep 56:413–419
17. Karon M, Sieger L, Leimbrock S, Finklestein JZ, Nesbit ME, Swaney JJ (1973) 5-Azacytidine: a new active agent for the treatment of acute leukemia. Blood 42:359–365
18. McCredie KB, Bodey GP, Burgess MA, Gutterman JU, Rodriguez V, Sullivan MP, Freireich EJ (1973) Treatment of acute leukemia with 5-azacytidine (NSC-102816). Cancer Chemother Rep 57:319–323
19. Constantinides PG, Taylor SM, Jones PA (1978) Phenotypic conversion of cultured mouse embryo cells by aza pyrimidine nucleosides. Dev Biol 66:57–71
20. Jones PA, Taylor SM (1980) Cellular differentiation, cytidine analogs and DNA methylation. Cell 20:85–93

21. Jones PA, Taylor SM (1981) Hemimethylated duplex DNAs prepared from 5-azacytidine-treated cells. Nucleic Acids Res 9:2933–2947
22. Quesnel B, Guillerm G, Vereecque R, Wattel E, Preudhomme C, Bauters F, Vanrumbeke M, Fenaux P (1998) Methylation of the p15(INK4b) gene in myelodysplastic syndromes is frequent and acquired during disease progression. Blood 91:2985–2990
23. Esteller M (2003) Profiling aberrant DNA methylation in hematologic neoplasms: a view from the tip of the iceberg. Clin Immunol 109:80–88
24. Voso MT, Scardocci A, Guidi F, Zini G, Di Mario A, Pagano L, Hohaus S, Leone G (2004) Aberrant methylation of DAP-kinase in therapy-related acute myeloid leukemia and myelodysplastic syndromes. Blood 103:698–700
25. Silverman LR, Holland JF, Weinberg RS, Alter BP, Davis RB, Ellison RR, Demakos EP, Cornell CJ Jr (1993) Carey RW, Schiffer C: Effects of treatment with 5-azacytidine on the in vivo and in vitro hematopoiesis in patients with myelodysplastic syndromes. Leukemia 7(Suppl 1):21–29
26. Chitambar CR, Libnoch JA, Matthaeus WG, Ash RC, Ritch PS, Anderson T (1991) Evaluation of continuous infusion low-dose 5-azacytidine in the treatment of myelodysplastic syndromes. Am J Hematol 37:100–104
27. Silverman LR, Demakos EP, Peterson BL, Kornblith AB, Holland JC, Odchimar-Reissig R, Stone RM, Nelson D, Powell BL, DeCastro CM, Ellerton J, Larson RA, Schiffer CA, Holland JF (2002) Randomized controlled trial of azacitidine in patients with the myelodysplastic syndrome: a study of the cancer and leukemia group B. J Clin Oncol 20:2429–2440
28. Silverman LR, McKenzie DR, Peterson BL, Holland JF, Backstrom JT, Beach CL, Larson RA (2006) Cancer and Leukemia Group B: Further analysis of trials with azacitidine in patients with myelodysplastic syndrome: studies 8421, 8921, and 9221 by the Cancer and Leukemia Group B. J Clin Oncol 24:3895–3903
29. Heim S (1992) Cytogenetic findings in primary and secondary MDS. Leuk Res 16:43–46
30. Arber DA, Brunning RD, Orazi A et al (2008) Acute myeloid leukaemaia with myelodysplastic-related changes. In: Swerdlow SH, Campo E, Harris NL et al (eds) WHO classification of tumors of haematopoietic and lympohoid tissues (4th edn). Lyon, International Agency for Research on Cancer
31. Harris NL, Jaffe ES, Diebold J, Flandrin G, Muller-Hermelink HK, Vardiman J, Lister TA, Bloomfield CD (1999) World Health Organization classification of neoplastic diseases of the hematopoietic and lymphoid tissues: report of the Clinical Advisory Committee meeting-Airlie House, Virginia, November 1997. J Clin Oncol 17:3835–3849
32. Fenaux P, Mufti GJ, Hellstrom-Lindberg E, Santini V, Gattermann N, Germing U, Sanz G, List AF, Gore S, Seymour JF, Dombret H, Backstrom J, Zimmerman L, McKenzie D, Beach CL, Silverman LR (2010) Azacitidine prolongs overall survival compared with conventional care regimens in elderly patients with low bone marrow blast count acute myeloid leukemia. J Clin Oncol 28:562–569
33. Maurillo L, Venditti A, Spagnoli A, Gaidano G, Ferrero D, Oliva E, Lunghi M, D'Arco AM, Levis A, Pastore D, Di Renzo N, Santagostino A, Pavone V, Buccisano F, Musto P (2012) Azacitidine for the treatment of patients with acute myeloid leukemia: Report of 82 patients enrolled in an Italian compassionate program. Cancer 118:1014–1022
34. Al-Ali HK, Jaekel N, Junghanss C, Maschmeyer G, Krahl R, Cross M, Hoppe G, Niederwieser D (2012) Azacitidine in patients with acute myeloid leukemia medically unfit for or resistant to chemotherapy: a multicenter phase I/II study. Leuk Lymphoma 53:110–117
35. Blum W, Garzon R, Klisovic RB, Schwind S, Walker A, Geyer S, Liu S, Havelange V, Becker H, Schaaf L, Mickle J, Devine H, Kefauver C, Devine SM, Chan KK, Heerema NA, Bloomfield CD, Grever MR, Byrd JC, Villalona-Calero M, Croce CM, Marcucci G (2010) Clinical response and miR-29b predictive significance in older AML patients treated with a 10-day schedule of decitabine. Proc Natl Acad Sci USA 107:7473–7478
36. Lyons RM, Cosgriff TM, Modi SS, Gersh RH, Hainsworth JD, Cohn AL, McIntyre HJ, Fernando IJ, Backstrom JT, Beach CL (2009) Hematologic response to three alternative dosing schedules of azacitidine in patients with myelodysplastic syndromes. J Clin Oncol 27:1850–1856

37. Prebet T, Sun Z, Ketterling RP, Hicks G, Beach CL, Greenberg PL, Paietta EM, Czader M, Gabrilove J, Erba H, Tallman MS, Gore SD (2010) A 10 day schedule of azacitidine induces more complete cytogenetic remissions than the standard schedule in myelodysplasia and acute myeloid leukemia with myelodysplasia-related changes: results of the E1905 US Leukemia Intergroup Study. Blood 116(21):Abst. 4013
38. Marcucci G, Silverman L, Eller M, Lintz L, Beach CL (2005) Bioavailability of azacitidine subcutaneous versus intravenous in patients with the myelodysplastic syndromes. J Clin Pharmacol 45:597–602
39. Martin MG, Walgren RA, Procknow E, Uy GL, Stockerl-Goldstein K, Cashen AF, Westervelt P, Abboud CN, Kreisel F, Augustin K, Dipersio JF, Vij R (2009) A phase II study of 5-day intravenous azacitidine in patients with myelodysplastic syndromes. Am J Hematol 84:560–564
40. Uchida T, Ogawa Y, Kobayashi Y, Ishikawa T, Ohashi H, Hata T, Usui N, Taniwaki M, Ohnishi K, Akiyama H, Ozawa K, Ohyashiki K, Okamoto S, Tomita A, Nakao S, Tobinai K, Ogura M, Ando K, Hotta T (2011) Phase I and II study of azacitidine in Japanese patients with myelodysplastic syndromes. Cancer Sci 102:1680–1686
41. Kaminskas E, Farrell AT, Wang YC, Sridhara R, Pazdur R (2005) FDA drug approval summary: azacitidine (5-azacytidine, Vidaza) for injectable suspension. Oncologist 10:176–182
42. Ziemba A, Hayes E, Freeman BB III, Ye T, Pizzorno G (2011) Development of an oral form of azacytidine: 2'3'5'triacetyl-5-azacytidine. Chemother Res Pract 2011:965826
43. Garcia-Manero G, Stoltz ML, Ward MR, Kantarjian H, Sharma S (2008) A pilot pharmacokinetic study of oral azacitidine. Leukemia 22:1680–1684
44. Garcia-Manero G, Gore SD, Cogle C, Ward R, Shi T, Macbeth KJ, Laille E, Giordano H, Sakoian S, Jabbour E, Kantarjian H, Skikne B (2011) Phase I study of oral azacitidine in myelodysplastic syndromes, chronic myelomonocytic leukemia, and acute myeloid leukemia. J Clin Oncol 29:2521–2527
45. Hollenbach PW, Nguyen AN, Brady H, Williams M, Ning Y, Richard N, Krushel L, Aukerman SL, Heise C, MacBeth KJ (2010) A comparison of azacitidine and decitabine activities in acute myeloid leukemia cell lines. PLoS One 5:e9001
46. Pinto A, Attadia V, Fusco A, Ferrara F, Spada OA, Di Fiore PP (1984) 5-Aza-2′-deoxycytidine induces terminal differentiation of leukemic blasts from patients with acute myeloid leukemias. Blood 64:922–929
47. Petti MC, Mandelli F, Zagonel V, De Gregoris C, Merola MC, Latagliata R, Gattei V, Fazi P, Monfardini S, Pinto A (1993) Pilot study of 5-aza-2′-deoxycytidine (Decitabine) in the treatment of poor prognosis acute myelogenous leukemia patients: preliminary results. Leukemia 7(Suppl 1):36–41
48. Zagonel V, Lo Re G, Marotta G, Babare R, Sardeo G, Gattei V, De Angelis V, Monfardini S, Pinto A (1993) 5-Aza-2′-deoxycytidine (Decitabine) induces trilineage response in unfavourable myelodysplastic syndromes. Leukemia 7(Suppl 1):30–35
49. Willemze R, Archimbaud E, Muus P (1993) Preliminary results with 5-aza-2′-deoxycytidine (DAC)-containing chemotherapy in patients with relapsed or refractory acute leukemia. The EORTC Leukemia Cooperative Group. Leukemia 7(Suppl 1):49–50
50. Kantarjian HM, O'Brien SM, Estey E, Giralt S, Beran M, Rios MB, Keating M, de Vos D, Talpaz M (1997) Decitabine studies in chronic and acute myelogenous leukemia. Leukemia 11(Suppl 1):S35–S36
51. Wijermans PW, Krulder JW, Huijgens PC, Neve P (1997) Continuous infusion of low-dose 5-Aza-2′-deoxycytidine in elderly patients with high-risk myelodysplastic syndrome. Leukemia 11(Suppl 1):S19–S23
52. Wijermans P, Lubbert M, Verhoef G, Bosly A, Ravoet C, Andre M, Ferrant A (2000) Low-dose 5-aza-2′-deoxycytidine, a DNA hypomethylating agent, for the treatment of high-risk myelodysplastic syndrome: a multicenter phase II study in elderly patients. J Clin Oncol 18:956–962
53. Lubbert M, Wijermans P, Kunzmann R, Verhoef G, Bosly A, Ravoet C, Andre M, Ferrant A (2001) Cytogenetic responses in high-risk myelodysplastic syndrome following low-dose

treatment with the DNA methylation inhibitor 5-aza-2′-deoxycytidine. Br J Haematol 114:349–357
54. Issa JP, Garcia-Manero G, Giles FJ, Mannari R, Thomas D, Faderl S, Bayar E, Lyons J, Rosenfeld CS, Cortes J, Kantarjian HM (2004) Phase 1 study of low-dose prolonged exposure schedules of the hypomethylating agent 5-aza-2′-deoxycytidine (decitabine) in hematopoietic malignancies. Blood 103:1635–1640
55. Kantarjian H, Oki Y, Garcia-Manero G, Huang X, O'Brien S, Cortes J, Faderl S, Bueso-Ramos C, Ravandi F, Estrov Z, Ferrajoli A, Wierda W, Shan J, Davis J, Giles F, Saba HI, Issa JP (2007) Results of a randomized study of 3 schedules of low-dose decitabine in higher-risk myelodysplastic syndrome and chronic myelomonocytic leukemia. Blood 109:52–57
56. Steensma DP, Baer MR, Slack JL, Buckstein R, Godley LA, Garcia-Manero G, Albitar M, Larsen JS, Arora S, Cullen MT, Kantarjian H (2009) Multicenter study of decitabine administered daily for 5 days every 4 weeks to adults with myelodysplastic syndromes: the alternative dosing for outpatient treatment (ADOPT) trial. J Clin Oncol 27:3842–3848
57. Blum W, Klisovic RB, Hackanson B, Liu Z, Liu S, Devine H, Vukosavljevic T, Huynh L, Lozanski G, Kefauver C, Plass C, Devine SM, Heerema NA, Murgo A, Chan KK, Grever MR, Byrd JC, Marcucci G (2007) Phase I study of decitabine alone or in combination with valproic acid in acute myeloid leukemia. J Clin Oncol 25:3884–3891
58. Braun T, Itzykson R, Renneville A, de Renzis B, Dreyfus F, Laribi K, Bouabdallah K, Vey N, Toma A, Recher C, Royer B, Joly B, Vekhoff A, Lafon I, Sanhes L, Meurice G, Orear C, Preudhomme C, Gardin C, Ades L, Fontenay M, Fenaux P, Droin N, Solary E (2011) Groupe Francophone des Myelodysplasies: Molecular predictors of response to decitabine in advanced chronic myelomonocytic leukemia: a phase 2 trial. Blood 118:3824–3831
59. Wijermans PW, Ruter B, Baer MR, Slack JL, Saba HI, Lubbert M (2008) Efficacy of decitabine in the treatment of patients with chronic myelomonocytic leukemia (CMML). Leuk Res 32:587–591
60. Costa R, Abdulhaq H, Haq B, Shadduck RK, Latsko J, Zenati M, Atem FD, Rossetti JM, Sahovic EA, Lister J (2011) Activity of azacitidine in chronic myelomonocytic leukemia. Cancer 117:2690–2696
61. Prebet T, Gore SD, Esterni B, Gardin C, Itzykson R, Thepot S, Dreyfus F, Rauzy OB, Recher C, Ades L, Quesnel B, Beach CL, Fenaux P, Vey N (2011) Outcome of high-risk myelodysplastic syndrome after azacitidine treatment failure. J Clin Oncol 29:3322–3327
62. Jabbour E, Garcia-Manero G, Batty N, Shan J, O'Brien S, Cortes J, Ravandi F, Issa JP, Kantarjian H (2010) Outcome of patients with myelodysplastic syndrome after failure of decitabine therapy. Cancer 116:3830–3834
63. Talbert PB, Henikoff S (2010) Histone variants–ancient wrap artists of the epigenome. Nat Rev Mol Cell Biol 11:264–275
64. Lin RJ, Nagy L, Inoue S, Shao W, Miller WH Jr (1998) Evans RM: Role of the histone deacetylase complex in acute promyelocytic leukaemia. Nature 391:811–814
65. Altucci L, Gronemeyer H (2001) The promise of retinoids to fight against cancer. Nat Rev Cancer 1:181–193
66. Gelmetti V, Zhang J, Fanelli M, Minucci S, Pelicci PG, Lazar MA (1998) Aberrant recruitment of the nuclear receptor corepressor-histone deacetylase complex by the acute myeloid leukemia fusion partner ETO. Mol Cell Biol 18:7185–7191
67. Richon VM, Emiliani S, Verdin E, Webb Y, Breslow R, Rifkind RA, Marks PA (1998) A class of hybrid polar inducers of transformed cell differentiation inhibits histone deacetylases. 95:3003–3007
68. Quintas-Cardama A, Santos FP, Garcia-Manero G (2011) Histone deacetylase inhibitors for the treatment of myelodysplastic syndrome and acute myeloid leukemia. Leukemia 25:226–235
69. Cameron EE, Bachman KE, Myohanen S, Herman JG, Baylin SB (1999) Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. Nat Genet 21:103–107

70. Gore SD, Baylin S, Sugar E, Carraway H, Miller CB, Carducci M, Grever M, Galm O, Dauses T, Karp JE, Rudek MA, Zhao M, Smith BD, Manning J, Jiemjit A, Dover G, Mays A, Zwiebel J, Murgo A, Weng LJ, Herman JG (2006) Combined DNA methyltransferase and histone deacetylase inhibition in the treatment of myeloid neoplasms. Cancer Res 66:6361–6369
71. Fandy TE, Herman JG, Kerns P, Jiemjit A, Sugar EA, Choi SH, Yang AS, Aucott T, Dauses T, Odchimar-Reissig R, Licht J, McConnell MJ, Nasrallah C, Kim MK, Zhang W, Sun Y, Murgo A, Espinoza-Delgado I, Oteiza K, Owoeye I, Silverman LR, Gore SD, Carraway HE (2009) Early epigenetic changes and DNA damage do not predict clinical response in an overlapping schedule of 5-azacytidine and entinostat in patients with myeloid malignancies. Blood 114:2764–2773
72. Maslak P, Chanel S, Camacho LH, Soignet S, Pandolfi PP, Guernah I, Warrell R, Nimer S (2006) Pilot study of combination transcriptional modulation therapy with sodium phenylbutyrate and 5-azacytidine in patients with acute myeloid leukemia or myelodysplastic syndrome. Leukemia 20:212–217
73. Garcia-Manero G, Kantarjian HM, Sanchez-Gonzalez B, Yang H, Rosner G, Verstovsek S, Rytting M, Wierda WG, Ravandi F, Koller C, Xiao L, Faderl S, Estrov Z, Cortes J, O'brien S, Estey E, Bueso-Ramos C, Fiorentino J, Jabbour E, Issa JP (2006) Phase 1/2 study of the combination of 5-aza-2′-deoxycytidine with valproic acid in patients with leukemia. Blood 108:3271–3279
74. Silverman LR, Verma A, Odchimar-Reissig R, LeBlanc A, Nejfeld V, Gabrilove JL (2008) A phase I trial of the epigenetic modulators vorinostat, in combination with azacitidine (azaC) in patients with the myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML): a study of the New York Cancer Consortium. Blood 112:3656
75. Garcia-Manero G, Estey E, Jabbour E, Kadia TM, Estrov Z, Cortes J (2010) Phase II study of 5-azacitidine and vorinostat in patients (pts) with newly diagnosed myelodysplastic syndrome (MDS) or acute myelogenous leukemia (AML) not eligible for clinicalt trials because poor performance of presence of other comorbidities. Blood 116:Abstr. 604
76. Garcia-Manero G, Yang AS, Giles F, Faderl S, Ravandi F, Cortes J, Newsome WJ, Issa JP, Patterson TA, Dubay M, Li Z, Kantarjian H, Martell RE (2007) Phase I/II study of MGCD0103, an oral isotype-selective histone deacetylase (HDAC) inhibitor, in combination with 5-Azacitidine in higher-risk myelodysplastic syndrome (MDS) and acute myelogenous leukemia (AML). Blood 110
77. Grant S (2009) New agents for AML and MDS. Best Pract Res Clin Haematol 22:501–507
78. Carraway HE, Sidney Kimmel Comprehensive Cancer Center (2000–2012, Feb 11) A trial to evaluate two schedules of MS275 in combination with 5AC in elderly patients with acute myeloid leukemia (AML). In: ClinicalTrials.gov [Internet]. Bethesda (MD): National Library of Medicine (US). Available from: http://www.clinicaltrials.gov/ct2/show/NCT01305499:NCT01305499
79. Scandura JM, Roboz GJ, Moh M, Morawa E, Brenet F, Bose JR, Villegas L, Gergis US, Mayer SA, Ippoliti CM, Curcio TJ, Ritchie EK, Feldman EJ (2011) Phase 1 study of epigenetic priming with decitabine prior to standard induction chemotherapy for patients with AML. Blood 118:1472–1480
80. De Padua SL, de Lima M, Kantarjian H, Faderl S, Kebriaei P, Giralt S, Davisson J, Garcia-Manero G, Champlin R, Issa JP, Ravandi F (2009) Feasibility of allo-SCT after hypomethylating therapy with decitabine for myelodysplastic syndrome. Bone Marrow Transplant 43:839–843
81. Lubbert M, Bertz H, Ruter B, Marks R, Claus R, Wasch R, Finke J (2009) Non-intensive treatment with low-dose 5-aza-2′-deoxycytidine (DAC) prior to allogeneic blood SCT of older MDS/AML patients. Bone Marrow Transplant 44:585–588
82. Field T, Perkins J, Huang Y, Kharfan-Dabaja MA, Alsina M, Ayala E, Fernandez HF, Janssen W, Lancet J, Perez L, Sullivan D, List A, Anasetti C (2010) 5-Azacitidine for myelodysplasia before allogeneic hematopoietic cell transplantation. Bone Marrow Transplant 45:255–260

83. Gerds AT, Gooley TA, Estey EH, Appelbaum FR, Deeg HJ, Scott BL (2012) Pre-transplant therapy with azacitidine vs induction chemotherapy and posttransplant outcome in patients with MDS. Biol Blood Marrow Transplant; in press. [Epub ahead of print]
84. Lubbert M, Bertz H, Wasch R, Marks R, Ruter B, Claus R, Finke J (2010) Efficacy of a 3-day, low-dose treatment with 5-azacytidine followed by donor lymphocyte infusions in older patients with acute myeloid leukemia or chronic myelomonocytic leukemia relapsed after allografting. Bone Marrow Transplant 45:627–632
85. Czibere A, Bruns I, Kroger N, Platzbecker U, Lind J, Zohren F, Fenk R, Germing U, Schroder T, Graf T, Haas R, Kobbe G (2010) 5-Azacytidine for the treatment of patients with acute myeloid leukemia or myelodysplastic syndrome who relapse after allo-SCT: a retrospective analysis. Bone Marrow Transplant 45:872–876
86. Bolanos-Meade J, Smith BD, Gore SD, McDevitt MA, Luznik L, Fuchs EJ, Jones RJ (2011) 5-Azacytidine as Salvage Treatment in Relapsed Myeloid Tumors After Allogeneic Bone Marrow Transplantation. Biol Blood Marrow Transplant 17:754–758
87. Loh Y (2000) Singapore General Hospital: Study of decitabine induction prior to allogeneic hematopoietic cell transplant in newly diagnosed MDS patients [cited 2012, Feb 11]. In: ClinicalTrials.gov [Internet]. Bethesda (MD): National Library of Medicine (US). http://www.clinicaltrials.gov/ct2/show/NCT01333449:NCT01333449
88. Shen L, Kantarjian H, Guo Y, Lin E, Shan J, Huang X, Berry D, Ahmed S, Zhu W, Pierce S, Kondo Y, Oki Y, Jelinek J, Saba H, Estey E, Issa JP (2010) DNA methylation predicts survival and response to therapy in patients with myelodysplastic syndromes. J Clin Oncol 28:605–613
89. Karpf AR, Lasek AW, Ririe TO, Hanks AN, Grossman D, Jones DA (2004) Limited gene activation in tumor and normal epithelial cells treated with the DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine. Mol Pharmacol 65:18–27
90. Link PA, Baer MR, James SR, Jones DA, Karpf AR (2008) p53-inducible ribonucleotide reductase (p53R2/RRM2B) is a DNA hypomethylation-independent decitabine gene target that correlates with clinical response in myelodysplastic syndrome/acute myelogenous leukemia. Cancer Res 68:9358–9366
91. Langemeijer SM, Kuiper RP, Berends M, Knops R, Aslanyan MG, Massop M, Stevens-Linders E, van Hoogen P, van Kessel AG, Raymakers RA, Kamping EJ, Verhoef GE, Verburgh E, Hagemeijer A, Vandenberghe P, de Witte T, van der Reijden BA, Jansen JH (2009) Acquired mutations in TET2 are common in myelodysplastic syndromes. Nat Genet 41:838–842
92. Kosmider O, Gelsi-Boyer V, Cheok M, Grabar S, Della-Valle V, Picard F, Viguié F, Quesnel B, Beyne-Rauzy O, Solary E, Vey N, Hunault-Berger M, Fenaux P, Mansat-De Mas V, Delabesse E, Guardiola P, Lacombe C, Vainchenker W, Preudhomme C, Dreyfus F, Bernard OA, Birnbaum D, Fontenay M, Groupe Francophone des Myélodysplasies (2009) TET2 mutation is an independent favorable prognostic factor in myelodysplastic syndromes (MDSs). Blood 114:3285–3291
93. Itzykson R, Kosmider O, Cluzeau T, Mansat-De Mas V, Dreyfus F, Beyne-Rauzy O, Quesnel B, Vey N, Gelsi-Boyer V, Raynaud S, Preudhomme C, Adès L, Fenaux P, Fontenay M, Groupe Francophone des Myelodysplasies (GFM) (2011) Impact of TET2 mutations on response rate to azacitidine in myelodysplastic syndromes and low blast count acute myeloid leukemias. Leukemia 25:1147–1152
94. Walter MJ, Ding L, Shen D, Shao J, Grillot M, McLellan M, Fulton R, Schmidt H, Kalicki-Veizer J, O'Laughlin M, Kandoth C, Baty J, Westervelt P, DiPersio JF, Mardis ER, Wilson RK, Ley TJ, Graubert TA (2011) Recurrent DNMT3A mutations in patients with myelodysplastic syndromes. Leukemia 25:1153–1158
95. Ley TJ, Ding L, Walter MJ, McLellan MD, Lamprecht T, Larson DE, Kandoth C, Payton JE, Baty J, Welch J, Harris CC, Lichti CF, Townsend RR, Fulton RS, Dooling DJ, Koboldt DC, Schmidt H, Zhang Q, Osborne JR, Lin L, O'Laughlin M, McMichael JF, Delehaunty KD, McGrath SD, Fulton LA, Magrini VJ, Vickery TL, Hundal J, Cook LL, Conyers JJ, Swift GW, Reed JP, Alldredge PA, Wylie T, Walker J, Kalicki J, Watson MA, Heath S, Shannon

WD, Varghese N, Nagarajan R, Westervelt P, Tomasson MH, Link DC, Graubert TA, DiPersio JF, Mardis ER, Wilson RK (2010) DNMT3A mutations in acute myeloid leukemia. N Engl J Med 363:2424–2433

96. Thol F, Damm F, Lüdeking A, Winschel C, Wagner K, Morgan M, Yun H, Göhring G, Schlegelberger B, Hoelzer D, Lübbert M, Kanz L, Fiedler W, Kirchner H, Heil G, Krauter J, Ganser A, Heuser M (2011) Incidence and prognostic influence of DNMT3A mutations in acute myeloid leukemia. J Clin Oncol 29:2889–2896

Chapter 14
Epigenetic Targeting Therapies to Overcome Chemotherapy Resistance

Curt Balch and Kenneth P. Nephew

Abstract It is now well established that epigenetic aberrations occur early in malignant transformation, raising the possibility of identifying chemopreventive compounds or reliable diagnostic screening using epigenetic biomarkers. Combinatorial therapies effective for the reexpression of tumor suppressors, facilitating resensitization to conventional chemotherapies, hold great promise for the future therapy of cancer. This approach may also perturb cancer stem cells and thus represent an effective means for managing a number of solid tumors. We believe that in the near future, anticancer drug regimens will routinely include epigenetic therapies, possibly in conjunction with inhibitors of "stemness" signal pathways, to effectively reduce the devastating occurrence of cancer chemotherapy resistance.

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Chemo-, radio-, and hormonal therapies have proved invaluable for the management of numerous solid and hematologic cancers. Commonly used chemotherapies include topoisomerase inhibitors, microtubule-targeting agents (for mitotic spindle disruption), and DNA-alkylating agents, while hormonal therapies include antiestrogens (such as tamoxifen) and androgen-ablating drugs [1]. Despite the success of these agents (often early during patient therapy), the majority of patients eventually develop resistance to these interventions, and it is believed that >90% of all cancer deaths result from therapy-refractory, metastatic disease [2, 3]. Resistance to therapy is believed to be multifactorial, involving reduced delivery/uptake, membrane efflux, metabolic inactivation, loss of the therapeutic target, and autocrine/paracrine signaling (involving the local tumor microenvironment). Attenuation of cancer cell death pathways, due to hyperactive growth/survival pathways and/or suppression of cell cycle arrest/apoptosis cascades, is considered a major contributor to the loss of therapeutic sensitivity in cancer [4, 5].

While tumor progression is clearly associated with DNA sequence anomalies (e.g., point mutations, DNA gains or losses within specific loci, and/or translocations), *epigenetic* aberrations are now believed to play an equivalent (or even greater) role [6–8]. Epigenetics is classically defined as the study of heritable changes in gene expression that occur without a change in the DNA sequence. Epigenetic modifications include methylation of C5 of cytosines within CG dyads, numerous posttranslational modifications of histone residues, repositioning of whole (histone octomer) nucleosomes, deposition of histone protein variants, and posttranscriptional regulation of protein translation by microRNAs [8–10].

As noted above, cancer progression is characterized by genetic and epigenetic misregulation of signal transduction cascades (often in association with altered microRNA expression) [11, 12], and it has been hypothesized that the cancer cell phenotype resembles a reversion of adult tissue cells to an embryonic-like state (i.e., loss of differentiation), with immortalization replacing age-related apoptosis and senescence [13, 14]. Analogously, one recent, increasingly accepted carcinogenesis paradigm is that a mature, heterogeneous tumor represents a "caricature" of the normal organ from which it derives, due to the abnormal differentiation of "cancer stem cells" (CSCs) [15]. Normal tissue stem cells are relatively long-lived, due to quiescence or relatively slow cell division and expression of various phenotypes that confer resistance to genotoxic or cytotoxic agents, including enhanced DNA repair, metabolic inactivation and/or expulsion of cytotoxins, oxidative stress protection, and enhanced pro-survival (i.e., antiapoptotic) signaling [16]. While not necessarily derived from normal stem cells [16], CSCs have been shown to possess numerous "stemness" phenotypes, including the aforementioned defense mechanisms against environmental insults, thus facilitating resistance to most conventional anticancer agents [15, 16]. In addition to studies of hematologic malignancies, chemoresistant stem-like cells have now been identified and characterized in several solid tumors, including hepatocellular, colon, breast, glioma, pancreatic, and ovarian cancers [16].

To reverse the multi-/pluripotent phenotypes of progenitor tumor cells, numerous well-known differentiation agents are under investigation as potential cancer therapeutics, including vitamin D, retinoids, arsenic trioxide, and phytochemicals

[18, 19]. It is feasible that successful delivery of differentiating agents to CSCs might reduce malignant stem cell populations and improve conventional therapy responses, in addition to hampering tumor regrowth [8]. Similar to normal development, which is governed by epigenetic modifications that allow tissue-specific gene expression [20], abnormal differentiation states of tumor subpopulations are also largely regulated by atypical epigenetic modifications to DNA/chromatin [21]. The existence of "epigenetic plasticity" (associated with extensive chromatin remodeling) [22] was further exemplified by the recent generation of "induced pluripotent," embryonic stem-like cells from terminally differentiated, adult tissue cells [23, 24]. By contrast, it was also demonstrated that even highly aggressive cancer cells (including melanoma and estrogen receptor-negative breast cancer cells) possess a highly "plastic" phenotype capable of reversion to their respective differentiated, normal tissue phenotypes [25, 26].

In this chapter, we discuss agents capable of reversing cancer-associated, repressive epigenetic modifications. The emphasis of this article is on the possible restoration of drug response pathways/targets that could potentially reverse chemoresistance, a destructive and usually fatal complication of numerous malignancies.

14.1 Preclinical Studies of DNA Hypomethylating and Deacetylase-Inhibiting Agents for Overcoming Drug Resistance

As noted above, cancer is often characterized by a loss of differentiated and tissue-specialized phenotypes, which are maintained by epigenetic modifications that drive lineage- and organ-specific development. Over the past 50 years, the L-1210 acute lymphoblastic and Friend erythroleukemia mouse models have been widely used to screen antileukemic compounds, several of which were found to possess differentiating activity [27, 28]. Several of those differentiating agents were later discovered to be inhibitors of repressive epigenetic modifications and more specifically, histone deacetylase and DNA methyltransferase inhibitors (HDACIs and DNMTIs, respectively) [8, 29, 30].

Preclinical cancer studies of DNA methyltransferase inhibitors (DNMTIs). The two best-characterized DNA methyltransferase inhibitors (DNMTIs) are 5-azacytidine (5-aza-C, Vidaza) and its deoxyribose analog, 5-aza-2′-deoxycytidine (5-aza-dC, decitabine), with both compounds possessing the non-methylatable pyrimidine analog azacytosine [31]. Both DNMTIs, first synthesized and shown as antileukemic in the 1960s (Fig. 14.1), are now FDA-approved for therapy of the hematologic malignancy myelodysplastic syndrome (MDS) [10] (see next section). Following cellular uptake, these cytidine analogs are triphosphorylated and incorporated into the newly synthesized DNA strand during S phase (5-aza-C is also integrated into RNA) [32]. However, a C5-to-N5 substitution in the cytosine six-member heterocyclic ring precludes methyl group acceptance, resulting in covalent and irreversible binding of the DNMT enzyme to the fraudulent base, followed by the eventual cel-

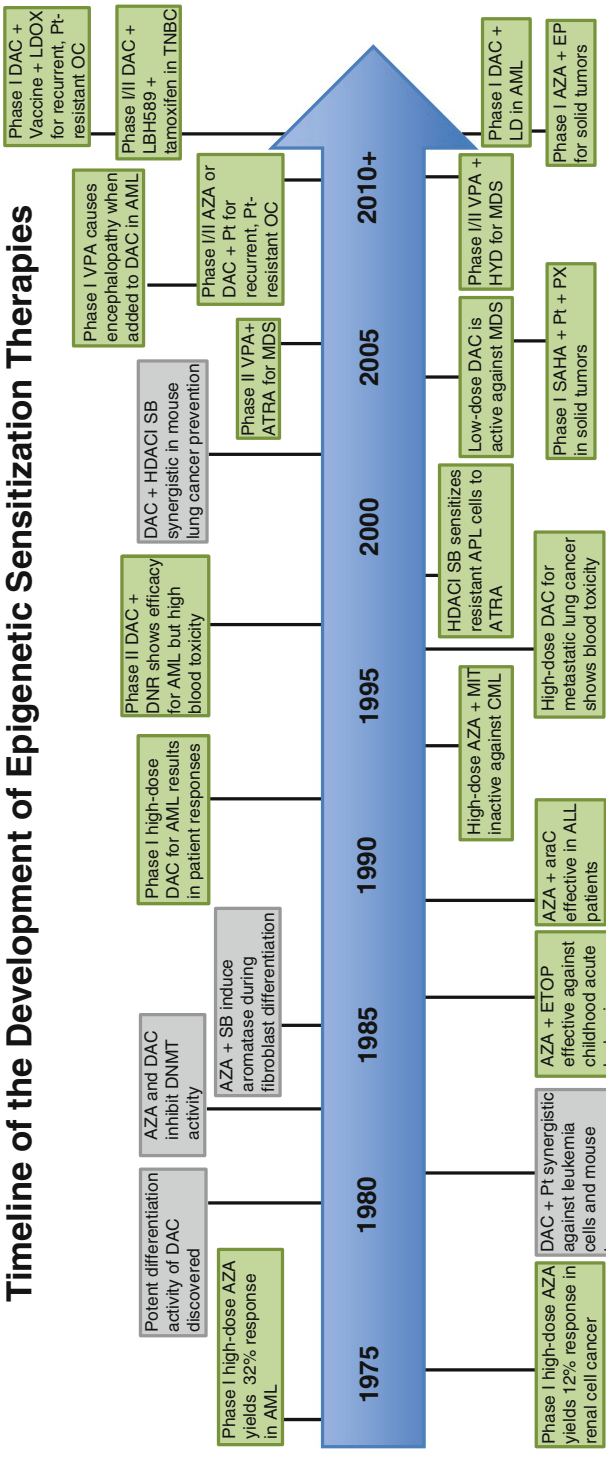

Fig. 14.1 Timeline of notable events supporting epigenetic sensitization to conventional cancer therapies. *Green boxes* indicate clinical studies, and *gray boxes* denote preclinical studies. The timeline chronology begins approximately 10 years after the discovery of DNA methyltransferase inhibitors. The cited studies are discussed in the text. See references [17, 29, 42, 43, 169, 171, 185, 211–222] for details. *AZA* 5-azacytidine or VIDAZA; *DAC* 5-aza-2′-deoxycytidine or decitabine/dacogen; *ALL* acute lymphoblastic leukemia; *AML* acute myeloid leukemia; *ara-C* arabinosylcytosine (cytarabine); *ATRA* all-trans retinoic acid; *CML* chronic myelogenous leukemia; *DNR* daunorubicin; *EP* erlotinib, Tarceva; *ETOP* etoposide; *FDA* Food and Drug Administration; *HDAC* histone deacetylase; *HDACI* histone deacetylase inhibitor; *HYD* hydralazine; *LD* lenalidomide, Revlimid; *LDOX* liposomal doxorubicin; *MDS* myelodyspastic syndrome; *MIT* mitoxatrone; *OC* epithelial ovarian cancer or primary peritoneal carcinoma; *PX* paclitaxel; *Pt* platinum; *SAHA* suberoylanilide hydroxamic acid, Vorinostat, Zolinza; *SB* sodium butyrate; *TNBC* triple negative breast cancer; *Vaccine* NY-ESO-1 protein immunization; *VPA* valproic acid (valproate)

lular depletion of DNMT, via ubiquitin-associated proteasome targeting [8, 10, 33]. Due to their requirement of nascent strand DNA incorporation, the hypomethylating activity of these cytosine analogs is replication-dependent, requiring several cell divisions to complete the demethylation of each DNA strand [34], consistent with successful patient trials typically requiring multiple treatment cycles prior to detectable response ([32, 35] and see following section).

Following their initial syntheses in 1964 [36], 5-aza-C and 5-aza-dC were later found to possess antileukemic activity in mouse disease models, elicit cancer cell differentiation, and enhance response to the chemotherapeutics etoposide and cisplatin [29, 37] (Fig. 14.1). These nucleoside analogs potently hypomethylate a number of tumor suppressor genes (TSGs), resulting in their transcriptional upregulation [6, 38, 39]. Decitabine-mediated DNA demethylation has also been reported to associate with reduced methylation at lysines 9 and 27 of histone H3 (H3K9 and H3K27, respectively), two other repressive chromatin "marks" [40, 41], in addition to enhanced acetylation at H3K9 and H3K14 (two activating chromatin marks). Such "crosstalk" between repressive chromatin modifications is believed to result from coordinated activity of histone and DNA methyltransferase enzymes associated with large, multimeric epigenetic repressive protein complexes.

Since its inception, the cytidine analog 5-aza-C has been extensively studied in cell and animal model systems. Early studies demonstrated potent antileukemic activity in the L1210 mouse model, followed by reports of 5-aza-C efficacy against solid tumors, using various preclinical cancer models (Fig. 14.1) [42, 43]. In medulloblastoma cells, 5-aza-C was also shown to inhibit proliferation, coincident with promoter demethylation and upregulation of a TSG, *KLF4* [44]. More recently, it was shown that intratracheal administration of 5-aza-C, in an orthotopic mouse lung cancer model, exhibited fivefold reduced myelosuppression and threefold enhanced survival, as compared to i.v. administration [45]. While subsequent studies further established 5-aza-C as a differentiating agent, particularly in effecting myogenesis [46–48], other work firmly established its ability to induce TSGs and initiate apoptosis in cancer cells, including those of the liver, colon, and ovary [49–51].

In contrast to 5-aza-C, its deoxyribose analog 5-aza-dC is not incorporated into RNA and is thus more stable and potent (active at submicromolar concentrations), although its activity is similarly attenuated by cytosine deaminases [8]. In a myriad of cell line studies, decitabine was shown to hypomethylate and derepress numerous TSGs, some of the most commonly studied being *p16*, *APC*, *RASSF1A*, *hMLH1*, *PTEN*, and *DAPK* [6, 38, 39]. Several of these (and other) genes encode protein constituents of apoptosis pathways, and thus (like aza-dC) in addition to differentiation, 5-aza-dC can robustly induce apoptosis [52, 53]. Preclinical studies have now firmly established 5-aza-dC activity against hematologic cancers, including acute myeloid leukemia (AML), chronic myeloid leukemia, acute lymphocytic leukemia, and MDS (Fig. 14.1) [54–57], and may also elicit senescence and autophagy [58]. Although clinical studies of 5-aza-dC have yet to demonstrate substantial activity against solid tumors (see below), preclinical studies have convincingly shown proof-of-principle for antitumor efficacy [59–62]. Moreover, in two studies, an indirect apoptotic role was found in that 5-aza-dC hypomethylated and upregulated

microRNA-181, a regulator of *NOTCH4* and *KRAS*, while in liver cancer cells, 5-aza-dC induced the tumor suppressor microRNAs 124 and 203 [63, 64]. As noted above, epigenetic alterations in cancer often hyperactivate specific oncogenic pathways; 5-aza-dC is now known to antagonize several of those pathways, while upregulating tumor suppressive signaling. Examples of oncogenic signal blockade by 5-aza-dC was demonstrated by its upregulation of the endogenous Wnt pathway inhibitor DKK, resulting in significant xenograft tumor growth inhibition [65].

In addition to 5-aza-dC and 5-aza-C, various other compounds have been shown to elicit DNA demethylation. As decitabine is subject to intracellular deamination and aqueous instability (resulting in loss of hypomethylating activity), a more stable dinucleotide, 5-aza-dC-dG (SGI-110, Astex Pharmaceuticals, Cambridge, UK), was shown to resist cytidine deaminase, while also demonstrating potent antigrowth effects against bladder cancer cells and mouse xenografts, with negligible toxicity [66, 67]. Likewise, an elaidic acid analog of 5-aza-C, CP-4200, possessed enhanced stability and much higher tumoricidal activity than the parent compound (aza-C), possibly due to its independence from nucleoside uptake transporters [68]. Using a different (genetic) approach, short inhibitory RNAs against DNMTs 1 and 3b elicited DNA demethylation and gene derepression similar to (or greater than) deoxycytosine analogs [69]. In addition to decitabine, we have also studied another cytidine analog DNMTI, zebularine, demonstrating that this agent hypomethylates TSGs and allows for the chemosensitization of platinum-resistant ovarian cancer cells lines [70]. Other zebularine studies have demonstrated its greater stability than 5-aza-dC, demethylation in tumors *in vivo*, and colon cancer chemoprevention in a widely used mouse model [71, 72]. Like 5-aza-C, however, zebularine is a ribonucleotide and thus its potency is limited by inefficient reduction prior to incorporation into DNA [73]. Toward rectifying that limitation, deoxyzebularine phosphoramidate prodrugs were recently demonstrated as more potent hypomethylating agents in vitro, while also exhibiting antineoplastic activity against pancreatic cancer cell lines [74].

Several non-nucleoside compounds have also demonstrated DNA-hypomethylating activity. These include two previously FDA-approved agents, the antihypertensive hydralazine and the antiarrhythmic procainamide [75]. However, these compounds were found much less potent than 5-aza-dC [75, 76]. A mushroom-derived antibiotic, Verticullin A, likewise displayed DNMTI activity against SW620 colon cancer cells, upregulating several genes concordant with demethylation of their respective promoters, while also resensitizing those cells to the apoptosis-inducing, "death receptor" ligand TRAIL [77]. More recently, various "rationally designed," non-nucleoside DNMT inhibitors (thus influencing enzyme activity without DNA incorporation) have also demonstrated potent downregulation of methyltransferase activity. Two of these, SGI-1027 and RG108, facilitated reexpression of silenced TSGs, while also negatively affecting growth of colon and hepatocellular carcinoma cells [78–81]. Likewise, various high-throughput screens, using various reporter assays and virtual "docking" computational approaches, are now in widespread use for the identification of non-nucleoside DNA methyltransferase [82, 83]. These approaches will almost certainly lead to the identification of novel DNA methylation inhibitors.

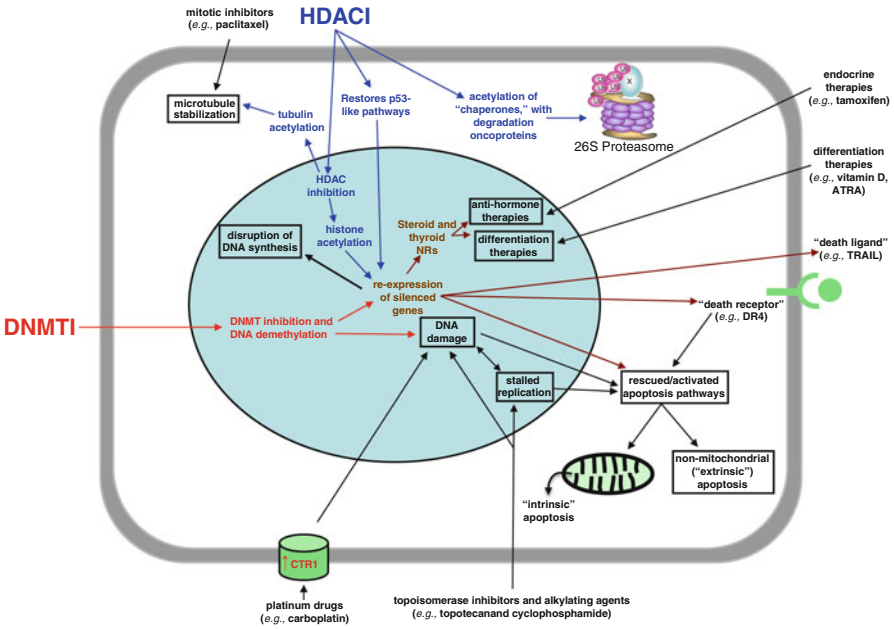

Fig. 14.2 Therapy response signals potentially affected by HDACIs and DNMTIs. Possible therapy-sensitization mechanisms by HDACIs and/or DNMTs. *Red* text denotes proteins/pathways impacted predominantly by DNMTIs, *blue* text indicates HDACI targets, and *brown* text designates possible alteration by either agent (and/or DNMTI/HDACI co-augmentation). *Black boxes* indicate pathway intersections where therapy sensitization may occur following DNMT/HDACI treatment. *ATRA* all-trans retinoic acid; *CTR1* copper transporter-1; *DNMTI* DNA methyltransferase inhibitor; *DR4* death receptor-4; *HDAC* histone deacetylase; *HDACI* histone deacetylase inhibitor; *TRAIL* TNF-related apoptosis-inducing ligand

While DNMTIs have shown success as monotherapies for hematologic cancers, accumulating evidence suggests they will be most effective when combined with conventional or targeted chemotherapies, likely via chemosensitization of resistant tumor subpopulations [8, 22, 84]. Such chemosensitization is hypothesized to result from DNMTI-mediated derepression of gene members of drug response pathways or inhibition of pro-survival pathways [8, 9, 85]. As shown in Fig. 14.2, multiple preclinical studies have now demonstrated that DNMTIs can resensitize resistant malignancies to numerous chemotherapeutics, via upregulation of pro-apoptosis pathways (both extrinsic and intrinsic), while also inhibiting oncogenic signaling cascades such as Wnt, PI3K/Akt, hedgehog, and Notch [65, 86–89]. In two early studies of the L1210 mouse leukemia models, cytoxicity of 5-aza-C was augmented by coadministration with another nucleoside analog, cytarabine; the hypothesized mechanism of action of this combination was inhibition of DNA synthesis [90]. Likewise, 5-aza-C antileukemic activity was also enhanced by a cytidine deaminase inhibitor [91]. More recently, in a study of aggressive prostate cancer, 5-aza-C caused potent but well-tolerated resensitization of tumor xenografts to docetaxel and cisplatin, concomitant with upregulation of a number of TSGs [92].

Like 5-aza-C, chemosensitization by 5-aza-dC is now well established. In one early study, 5-aza-dC combined with the topoisomerase-1 inhibitor topotecan, was synergistically cytotoxic to mouse colorectal adenocarcinomas [93]. Later, it was demonstrated that 5-aza-dC could resensitize platinum-resistant ovarian cancer cells and mouse xenografts to cisplatin, due to promoter demethylation and reexpression of the mismatch repair enzyme gene *hMLH1* [94, 95]. In two colon cancer studies, 5-aza-dC was found to be synergistically tumoricidal when combined with 5-fluorouracil (an antimetabolite) and the antineoplastic hormone irinotecan [96, 97]. Likewise, a study of colon cancer cells revealed that 5-aza-dC treatment resulted in upregulation of ten interferon pathway-associated genes, likely via induction of IFN-alpha2a and activation of STATs 1, 2, and 3 [98]. In endocrine cancers, DNMTIs have also been demonstrated to sensitize cancer cells to antihormonal therapies. For example, 5-aza-dC was shown to upregulate the DNA-methylation-repressed TSG *PTEN*, an inhibitor of the PI3K/Akt pathway, suppressing the growth of tamoxifen-resistant breast cancer cell xenografts and restoring responsiveness to antiestrogens [99]. The latter finding is further supported by a correlation of epigenetic aberrations and PI3K/Akt oncogenic signaling in breast cancer cells; those aberrations were reversible by a 5-aza-dC/PI3K inhibitor combination, which also cooperatively inhibited the growth of mouse xenografts [100]. Restoration of antiestrogen sensitivity in breast cancer is believed to be due (at least in part) to reexpression of the estrogen receptors alpha and/or beta [101, 102]. Similarly, in prostate cancer, androgen receptor silencing has been linked to both histone deacetylation and DNA methylation [103, 104]. In other prostate cancer studies, 5-aza-dC could sensitize both androgen-dependent and -independent prostate cancer cells to paclitaxel, while both DNMTIs and HDACIs cooperatively upregulated estrogen receptor-beta and delayed androgen independence in a common mouse model [105–107].

Preclinical cancer studies of histone deacetylase inhibitors (HDACIs). As histone deacetylation is another epigenetic modification repressive of TSGs, histone deacetylase inhibitors (HDACIs) also represent promising antineoplastics. Interestingly, the first HDAC inhibitor was the common organic solvent dimethylsulfoxide (DMSO), as discovered by Charlotte Friend to elicit differentiation of erythroleukemia cells [108]. Following that discovery, numerous other hybrid polar compounds were synthesized and similarly screened for differentiating activity, but whose mechanism of action (deacetylase inhibition, resulting in enhanced protein acetylation) remained unknown for over 20 years [109]. Numerous HDACIs, which antagonize the action of zinc-dependent histone deacetylases by chelation of the metal cation, have been shown to induce differentiation and apoptosis in tumor, but not normal, cells (reviewed in [110]). One proposed mechanism for cancer cell-specific HDACI toxicity is the induction of cell cycle checkpoints [111]; one such effect (G2 arrest followed by apoptosis) was also demonstrated in platinum-resistant ovarian cancer cells [112]. Of note, while HDACIs potently induce histone hyperacetylation, their effects on non-histone protein acetylation (including transcription factors, molecular chaperones, cargo transporters, and cytoskeletal proteins) may play an even greater role in their antineoplastic activity [110, 113]. In ovarian cancer in particular, several HDACIs induced cytodifferentiation and apop-

tosis of cultured cells and mouse xenografts [112, 114–117]. Newer studies suggest that HDACI repression of telomerase (*hTERT*) represents another anticancer mechanism of action (reviewed in [118]). Alternative non-epigenetic, HDACI antineoplastic effects include oncoprotein destabilization by acetylation of "chaperone" proteins (suggesting synergism with HSP inhibitors), diminished processing of "aggresomes" of misfolded proteins (suggesting synergism with proteasome inhibitors), acetylation of transcription factors, and reconstitution of p53-like tumor suppressive pathways (reviewed in [30, 110, 119], and see Fig. 14.2).

Similar to DNMTIs, preclinical studies have shown HDACIs to be most effective in combination with standard therapies, suggesting HDACI upregulation of drug response (apoptotic) or cellular differentiation pathways. In ovarian cancer preclinical studies, vorinostat alone was found effective against paclitaxel-resistant ovarian cancer cells; however, its antitumor activity was far greater in combination with paclitaxel [120–122]. Other HDACIs have similarly sensitized ovarian cancer cells to retinoids and the widely used chemotherapy cisplatin [115, 123, 124]. Similarly, our group demonstrated that a rationally designed HDACI, AR42, possessed greater cisplatin-resensitizing activity than vorinostat in chemoresistant ovarian cancer cells and mouse xenografts, enhancing both epithelial differentiation and apoptotic potential [125]. One specific example supporting HDACI-associated differentiation in therapy sensitization was that cholangiocarcinoma cells treated with the HDACI valproate upregulated numerous genes associated with differentiation, during sensitization to gemcitabine [126]. In similar studies, the HDACI Trichostatin A augmented UV-induced apoptosis over threefold in colon cancer RKO cells [127], and also sensitized osteosarcoma cells to a potentially antineoplastic, natural product geninstein [128].

Several HDACIs have also been demonstrated to upregulate "death receptor" apoptosis pathways, allowing resensitization of resistant cancer cells to death receptor ligands (Fig. 14.2). One report showed the HDACI MS-275 (entitostat) to resensitize aggressive MDA-MB-468 breast cancer cells to the death ligand TRAIL, both in cell culture and in mouse xenografts, while downregulating genes associated with the metastasis-related epithelial-to-mesenchymal transition [129]. In addition, HDACI-associated TRAIL sensitization (via reexpression of caspase-8) was markedly augmented by combination with interferon-gamma in meduloblastoma cells [130]. TRAIL sensitization by the HDACI valproate was also demonstrated in pancreatic cancer cell lines, via inhibition of HDAC2 and the restoration of extrinsic apoptosis pathways [131], while in hepatocellular carcinoma cell lines, the HDACIs valproic acid and ITF2357 both effected sensitization to TRAIL [132].

HDACIs have also shown activity against hormone-resistant neoplasms, including breast, uterine, and prostate cancers. Similar to DNMTI/antiestrogen studies HDACIs enhanced tamoxifen induction of both autophagy and apoptosis in tamoxifen-resistant breast cancer cells; that effect was further enhanced by inhibitors of autophagy [133]. In endometrial cancer studies, TSA/paclitaxel-combined treatment of mice bearing cancer cell tumor xenografts reduced tumor masses by >50% [134]. Moreover, another xenograft study showed that the HDACI apicidin reduced tumor size and repressed the angiogenesis-mediating oncoprotein VEGF [135]. Interestingly, it appears that in endometrial cancer, HDACIs may exert antigrowth

effects through repression of estrogen receptor-target genes, coincident with induction of genes targeted by the glucocorticoid receptor [136].

Preclinical studies of DNMTI/HDACI combinations. While HDACIs and DNMTIs have demonstrated clinical activity as single agent therapies for hematopoietic malignancies, DNA methylation and histone deacetylation also cooperatively inhibit gene transcription (often in multiple-repressor protein complexes), and relief of both silencing mechanisms may be necessary for maximal gene derepression [8, 137]. In ovarian cancer cells, a DNMTI/HDACI combination synergistically upregulated the pro-apoptotic gene *TMS1*/*ASC*, in contrast to either epigenetic agent alone [138], while a 5-aza-dC/vorinostat regimen induced various imprinted genes and also inhibited tumor xenograft growth [139]. Similarly, 5-aza-C combined with the HDACI Trichostatin A facilitated derepression of the progesterone receptor-B gene in endometrial cancer cells [140], while 5-aza-C plus entitostat cooperatively upregulated several pro-apoptosis genes and reduced tumor xenograft sizes by >75% in a mouse lung cancer model [141]. A newer preclinical study showed 5-aza-dC combined with the HDACI valproate was cancer-chemopreventive in a mouse medulloblastoma/rhabdosarcoma model, while each agent alone was not [142]. Interestingly, one compound, UVI5008, was found to be a "triple epigenetic inhibitor," concordantly inhibiting zinc-dependent HDACs, the DNA methyltransferase DNMT3A, and another family of HDACs that require a NAD^+ cofactor (rather than zinc), the sirtuins [143]. In that study, UV15008 potently induced apoptosis in breast cancer cells/xenografts via ROS production and activation of death receptor (i.e., extrinsic), mitochondria-independent, apoptosis [144].

It has also been reported that HDACIs and DNMTIs may actually mimic the epigenetic effects of one another. For example, it has been reported that several HDACIs can demethylate DNA, including Trichostatin A, valproate, and MS-275 (entitostat, SNDX-275) [145–148], possibly via transcriptional downregulation of DNMT-coding genes, as demonstrated in a study of human endometrial cancer cells [149]. Analogously, 5-aza-dC was also found to effect gene-specific, but not global, histone acetylation [150, 151]. However, a phase I study of AML or MDS patients examining 5-aza-C (5–14 days) followed by phenylbutyrate (5 days) demonstrated that 5-aza-C treatment alone resulted in histone acetylation in peripheral blood cells; phenylbutyrate, however, did not prevent remethylation of the cyclin-dependent kinase inhibitor gene *p15* (*CDKN2B*) [152]. Even so, these reciprocal epigenetic modifications, between HDACIs and DNMTIs, appear to be quite rare and context-dependent in nature.

While DNMTI/HDACI combinations often result in greater gene alterations than each agent in isolation, pairing of these epigenetic therapies will likely be even more effective in coordination with conventional cancer therapies [8–10]. For example, while caspase-8 gene reexpression in small cell lung cancer cells required a DNMTI/HDACI combination (thus restoring a functional apoptosis pathway), the induction of apoptosis still required the death receptor ligand TRAIL [153]. Similarly, combined treatment of decitabine and belinostat demonstrated significantly greater cisplatin sensitization of platinum-resistant ovarian cancer cell xenografts, in tumor-bearing mice, than either epigenetic therapy alone [154].

14.2 Clinical Studies of DNA Hypomethylating Agents and HDAC Inhibitors for Overcoming Drug Resistance

Four epigenetic derepressive agents are now FDA approved for two hematologic malignancies, MDS treatment with DNMTIs 5-aza-C (Vidaza) and 5-aza-dC (decitabine), and cutaneous T-cell lymphoma therapy using the HDACIs vorinostat and romidepsin [8, 9, 155]. While other hematologic malignancies will likely gain approval for monotherapy DNMTIs and HDACIs, including peripheral T-cell lymphoma and Hodgkin's disease, single-agent clinical studies of various solid tumors have proved fairly disappointing. For the latter, epigenetic drugs will likely prove most beneficial when combined with long-established approaches such as conventional cytotoxic chemotherapies, endocrine therapies, differentiation therapy, and radiotherapy [156, 157].

Studies of DNA methyltransferase inhibitors. In addition to incorporation into DNA, the ribonucleoside analog 5-aza-C is also incorporated into several RNA species, resulting in greater toxicity and lower stability than 5-aza-dC. Consequently, a more recent clinical studies have focused on 5-aza-dC (decitabine), although 5-aza-C remains widely used. An early Vidaza study of patients with acute leukemia, administered at 37–81 total mg/m^2, given over 30–60 h, resulted in some clinical benefit in 89% of patients, although substantial hematologic toxicity was observed in all patients [158]. A separate trial of 21 elderly patients with high-risk MDS, treatment with decitabine at 50 mg/m^2/day for three consecutive days, yielded a response rate of 54% (15 of 21), although significant myelotoxicity caused the death of 5/21 (17%) patients [159]. Another MDS phase I study, using an overall similar drug exposure (45 mg/m^2 b.i.d. for 3 days), yielded an overall response rate of 49%, but similarly resulted in moderate-to-severe toxicity (predominantly myelodepression), resulting in the death in 7% of the enrolled patients [160].

To possibly ameliorate the high toxicity and limited benefit of extended decitabine infusions (previously using regimens approaching its maximum tolerated dose), lower dose schedules were examined. In phase I/II sickle cell anemia studies of hydroxyurea-resistant patients, low-dose (0.3 mg/kg), repetitive doses (5 days/week for 2 weeks) of decitabine were found sufficient for demethylation and reexpression of fetal hemoglobin with little or no neutropenia [161, 162]. Such low-dose treatments were largely based on a mouse embryonic fibroblast study showing myotube differentiation and hypomethylation at low decitabine doses (1–5 μM), with cytotoxicity and increased methylation at higher (>5 μM) doses [29]. Subsequently, one MDS clinical trial examined a variety of repetitive low decitabine doses, with 1-h administration daily over longer durations (10–20 days) [163]. The results of that landmark study demonstrated that 15 mg/m^2 decitabine, administered over ten consecutive days, resulted in a response rate of 83% and was well tolerated, as compared to previous studies using >5-fold higher doses [163]. That pioneering work resulted in the widespread adoption of low-dose hypomethylating agents, both as monotherapies and in combination with other agents.

While single-agent decitabine demonstrated significant efficacy for MDS and other hematologic malignancies, solid tumor studies have been fairly disappointing, motivating studies of 5-aza-dC in combination with other conventional agents. Early combination studies, however, demonstrated minimal-to-moderate activity, with substantial toxicity. In a phase II study of non-small cell lung cancer, a maximum tolerated decitabine dose of 67 mg/m^2, given concurrently with 33 mg/m^2 cisplatin over a 2-h period for 3 consecutive days of a 21-day cycle, resulted in no objective responses and significant hematologic toxicity [164]. Similarly, a phase II trial of squamous cell cervical cancer, with decitabine administered continuously at 50 mg/m^2/day for 3 days, concurrent with 30 mg/m^2 cisplatin, resulted in eight partial and five stable disease responses; however, unacceptable toxicity was observed, resulting in one patient death [165]. However, based on the low-dose MDS efficacy study by Issa et al., newer trials have examined lower doses of decitabine in various combined regimens. One recent phase I/II combinatorial ovarian cancer study, of decitabine paired with carboplatin, demonstrated no significant improvement over carboplatin alone [166, 167]. By contrast, a separate phase IIa clinical trial of 5-aza-cytidine (Vidaza) and carboplatin resulted in one complete, three partial, and ten stable disease responses (of 29 total patients), with a 7.5-month average duration of response [168]. Likewise, our group recently completed a phase I trial of low-dose decitabine (five consecutive-day regimen), in combination with carboplatin in platinum-resistant ovarian cancer patients, revealing acceptable tolerability of the regimen [169]. Biological activity in vivo was also demonstrated, as assessed by hypomethylation of genome-wide repetitive elements (in peripheral blood cells) and specific ovarian cancer-associated genes (in plasma, ascites, or tumor) [169], resulting in one complete, six stable, and four (6-month) disease progression-free responses [169]. The successful phase II component of that study was recently described [170], and the results are promising. Other clinical studies combining 5-aza-dC with the EGFR antagonist erlotinib showed responses in 4 of 11 patients with advanced tumors [171]. However, a neuroblastoma trial of 5-aza-dC combined with cyclophosphamide or doxorubicin showed toxicity at the 5-aza-dC doses required for disease response [172]. In a 13-patient AML phase I study, decitabine combined with arsenic trioxide and/or ascorbic acid resulted in one complete remission and five patients with stable disease [173]. While chemosensitization by DNMTIs is believed to largely result from the restoration of apoptosis pathways, one recent phase II study of refractory solid tumors and lymphomas showed patient response to correlate with both DNA hypomethylation and expression of the copper transporter CTR1, a protein that facilitates platinum drug uptake [174] (Fig. 14.2).

Studies of histone deacetylase inhibitors. Like DNMTIs, despite successful studies of hematologic malignancies, solid tumor clinical trials of monotherapeutic HDACIs suggest similarly limited clinical activity. In ovarian cancer, two monotherapeutic phase I/II trials of the HDACIs vorinostat and belinostat proved tolerable but showed only moderate clinical activity [175, 176]. One recent phase II trial of the HDACI romidepsin in androgen-independent prostate cancer, although well tolerated, likewise showed minimal antineoplastic activity [177]. Another belinostat trial for metastatic renal cancer also yielded no patient responses [178]. Consequently,

it is now widely believed that these agents will be most effective in combination with conventional chemotherapies ([8, 9, 34] and see following sections).

For ovarian cancer, two recent ovarian cancer trials pairing belinostat with paclitaxel/carboplatin, and vorinostat with carboplatin, demonstrated safety and moderate clinical activity [179, 180], while planned clinical trials include HDACIs in combination with inhibitors of the DNA repair enzyme PARP or inhibitors of the embryonic signal mediator Hedgehog [181, 182]. Another phase II study of the HDACI vorinostat combined with the antiestrogen tamoxifen, in hormone-refractory breast cancer patients, yielded a clinical benefit rate (response or stable disease for over 24 weeks) of 40%, although toxicity necessitated dose adjustment in several patients [183]. Similarly, a 12-patient phase I trial combining the HDACI panobinostat with the angiogenesis inhibitor bevacizumab resulted in three partial responses and seven cases of stable disease [184].

Clinical studies of combined DNA methyltransferase and histone deacetylase inhibitors. DNMTI/HDACI combinations have also now been established to exert additive/synergistic effects on gene expression in vitro. However, success in clinical trials has been, similar to single-agent regimens, largely restricted to hematologic malignancies. For example, a phase II study of the DNMTI/HDACI combination of hydralazine and valproate for MDS showed an overall response rate of 50% [185]. Most solid tumor studies, however, have shown less efficacy. Nonetheless, one phase I clinical trial combining the HDACI valproic acid and the DNMTI azacytidine for various solid tumors demonstrated safety, in vivo biological activity, and stable disease in 25% of the enrolled patients, although no partial or complete responses were observed [186]. Likewise, a recent phase I study of 5-aza-dC/vorinostat combination resulted in 29% of non-Hodgkin's lymphoma and various solid tumors [187]. By analogy, a recent phase I/II trial of an azacytidine/entitostat combination in non-small cell lung cancer yielded major objective responses in 4 of 19 patients, and demethylation of a four-gene panel correlated with improved progression-free and overall survival [188].

Based on the above mentioned in vivo findings, it is speculated that chromatin depressive agents (singly or combined) alone may be only marginally efficient for eradicating cancer cells, thus motivating studies of their combination with conventional therapeutics [6, 8, 94]. For example, while apoptosis pathway function may be restored by epigenetic derepression, it is possible that epigenetic drugs remain inadequate as cancer cell stressors capable of provoking programmed cell death. In one phase III ovarian cancer trial (NCT00533299), the DNMTI hydralazine is being combined with the HDACI valproic acid, with or without the topoisomerase inhibitor topotecan, while a previous phase II trial of the same combination (hydralazine/valproate), coincident with four different chemotherapy regimens, yielded three partial and four stable disease responses (as assessed by the ovarian cancer marker CA-125) [189]. In various leukemias, a phase I trial of 5-aza-dC combined with valproic acid demonstrated acceptable patient tolerability and an objective response rate of 22% [190], while a melanoma trial combining 5-aza-dC and intravenous bolus interleukin-2 was well-tolerated and yielded a 31% objective response rate [191].

5-aza-C is also being examined in a phase I/II ovarian cancer trial (NCT00529022) in combination with valproic acid and carboplatin.

In addition to reactivation of TSGs (and possible chemotherapy response cascades), DNMTIs and HDACIs have also been found to induce various cancer/testis antigens (CTAs, components of the "tumor recognition complex") [34]. CTA proteins, expressed in male germ cells but normally silenced in adult tissues, are expressed in various malignancies as antigenic peptides copresented with HLA Class I/II molecules and thus may represent immunotherapy targets [192]. However, as CTA expression is often variable, due to epigenetic repression, more consistent reexpression can be achieved by DNMT and/or HDAC inhibitors [191]. Consequently, an ongoing phase I ovarian cancer trial (NCT00887796) is investigating decitabine combined with liposomal doxorubicin and peptide vaccination for the CTA NY-ESO-1, while two other trials (NCT00701298, NCT00886457, for unspecified cancers) are combining decitabine with interferon-α2b. These trials were based on the preclinical studies by Karpf et al. [98, 193] mentioned earlier. Thus, in addition to tumor suppressor reactivation, epigenetic therapies may also hold promise in immunotherapy.

14.3 Future Directions for the Use of Epigenetic Therapies for Overcoming Chemotherapy Resistance

One current focus within cancer epigenetic research is the design of specific inhibitors of enzymes facilitating other epigenetic repressive modifications, including the gene-repressive histone methyltransferases (HMTs) EZH2, which trimethylates histone H3, lysine 27 (H3K27me3), and DOT1L, which trimethylates H3K79 [194, 195]. Consistent with epigenetic gene repression in cancer, one DOT1L inhibitor, EPZ004777, showed activity against mixed lineage leukemia cells [196]. Similarly, one EZH2 inhibitor, DZNep, an *S*-adenosylmethionine (SAM) analog that also inhibits methylation of H4K20, resulting in upregulation of numerous previously silenced TSGs [194, 197]. DZNep has also shown anticancer activity against mouse prostate tumors and breast cancer, AML, and neuroblastoma cells [194, 197–199]. Similar to DNMTIs, DZNep induction of apoptosis was also augmented by HDACIs [200, 201], and recent studies of DZNep suggest possible negative effects toward CSCs [199, 202]. High-throughput approaches continue to identify various novel epigenetic therapies, including inhibitors of the Jarid family of H3K4me3 histone demethylases, the repressive HMT G9a (which trimethylates H3K9), isoform-specific HDACs, and various histone acetyltransferases [203–207]. In addition, tumor-suppressive microRNAs have been successfully delivered to tumors in mouse models of liver (miR-26a), colon (miRs-145 and -33a), and prostate cancers, using adeno-associated viruses, polyethylenimine conjugation, and rhabdomyosarcoma (miRs-1 and −206) [208–210]. Taken together it is likely that these emerging epigenetic therapeutics could be used for the much anticipated therapeutic approach of

"personalized medicine," based not only on patients' genomic/gene expression profiles, but also on their epigenetic profiles.

14.4 Summary/Conclusions

It is now well established that epigenetics is a principle mediator of mammalian development. To successfully carry out tissue/organ differentiation, genomic DNA expression is precisely regulated by a host of epigenetic modifications. It is thus not surprising that aberrant chromatin modifications result in defective differentiation states, a hallmark of cancer cells. It has also been recently shown that even highly aggressive cancer cells can revert to their original, tissue-specific differentiation state, and that epigenetic therapies may facilitate this phenomenon. Consequently, chromatin-altering agents hold promise for the treatment of numerous malignant diseases, particular when complemented with other (traditional or pathway-targeted) antineoplastic therapies.

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References

1. Gralow J, Ozols RF, Bajorin DF, Cheson BD, Sandler HM, Winer EP, Bonner J, Demetri GD, Curran W Jr, Ganz PA, Kramer BS, Kris MG, Markman M, Mayer RJ, Raghavan D, Ramsey S, Reaman GH, Sawaya R, Schuchter LM, Sweetenham JW, Vahdat LT, Davidson NE, Schilsky RL, Lichter AS (2008) Clinical cancer advances 2007: major research advances in cancer treatment, prevention, and screening—a report from the American Society of Clinical Oncology. J Clin Oncol 26(2):313–325
2. Jemal A, Siegel R, Xu J, Ward E (2010) Cancer statistics, 2010. CA Cancer J Clin 60(5):277–300
3. Longley DB, Johnston PG (2005) Molecular mechanisms of drug resistance. J Pathol 205(2):275–292
4. Raguz S, Yague E (2008) Resistance to chemotherapy: new treatments and novel insights into an old problem. Br J Cancer 99(3):387–391
5. Tredan O, Galmarini CM, Patel K, Tannock IF (2007) Drug resistance and the solid tumor microenvironment. J Natl Cancer Inst 99(19):1441–1454
6. Balch C, Huang TH, Brown R, Nephew KP (2004) The epigenetics of ovarian cancer drug resistance and resensitization. Am J Obstet Gynecol 191(5):1552–1572
7. Barton CA, Clark SJ, Hacker NF, O'Brien PM (2008) Epigenetic markers of ovarian cancer. Adv Exp Med Biol 622:35–51
8. Jones PA, Baylin SB (2007) The epigenomics of cancer. Cell 128(4):683–692
9. Esteller M (2008) Epigenetics in cancer. N Engl J Med 358(11):1148–1159

10. Rodriguez-Paredes M, Esteller M (2011) Cancer epigenetics reaches mainstream oncology. Nat Med 17(3):330–339
11. Lu L, Katsaros D, de la Longrais IA, Sochirca O, Yu H (2007) Hypermethylation of let-7a-3 in epithelial ovarian cancer is associated with low insulin-like growth factor-II expression and favorable prognosis. Cancer Res 67(21):10117–10122
12. Wiley A, Katsaros D, Chen H, Rigault de la Longrais IA, Beeghly A, Puopolo M, Singal R, Zhang Y, Amoako A, Zelterman D, Yu H (2006) Aberrant promoter methylation of multiple genes in malignant ovarian tumors and in ovarian tumors with low malignant potential. Cancer 107(2):299–308
13. Daley GQ (2008) Common themes of dedifferentiation in somatic cell reprogramming and cancer. Cold Spring Harb Symp Quant Biol 73:171–174
14. Dimri GP (2005) What has senescence got to do with cancer? Cancer Cell 7(6):505–512
15. Dalerba P, Cho RW, Clarke MF (2007) Cancer stem cells: models and concepts. Annu Rev Med 58:267–284
16. Jordan CT (2009) Cancer stem cells: controversial or just misunderstood? Cell Stem Cell 4(3):203–205
17. Von Hoff DD, Slavik M, Muggia FM (1976) 5-Azacytidine. A new anticancer drug with effectiveness in acute myelogenous leukemia. Ann Intern Med 85(2):237–245
18. Delva L, Zelent A, Naoe T, Fenaux P, Waxman S, Degos L, Chomienne C (2007) Meeting report: the 11th International Conference on Differentiation Therapy and Innovative Therapeutics in Oncology. Cancer Res 67(22):10635–10637
19. Ma WW, Adjei AA (2009) Novel agents on the horizon for cancer therapy. CA Cancer J Clin 59(2):111–137
20. Vincent A, Van Seuningen I (2009) Epigenetics, stem cells and epithelial cell fate. Differentiation 78(2–3):99–107
21. Scaffidi P, Misteli T (2010) Cancer epigenetics: from disruption of differentiation programs to the emergence of cancer stem cells. Cold Spring Harb Symp Quant Biol 75:251–258
22. Lotem J, Sachs L (2006) Epigenetics and the plasticity of differentiation in normal and cancer stem cells. Oncogene 25(59):7663–7672
23. Djuric U, Ellis J (2010) Epigenetics of induced pluripotency, the seven-headed dragon. Stem Cell Res Ther 1(1):3
24. Hochedlinger K, Plath K (2009) Epigenetic reprogramming and induced pluripotency. Development 136(4):509–523
25. Costa FF, Seftor EA, Bischof JM, Kirschmann DA, Strizzi L, Arndt K, de Fatima Bonaldo M, Soares MB, Hendrix MJ (2009) Epigenetically reprogramming metastatic tumor cells with an embryonic microenvironment. Epigenomics 1(2):387–398
26. Hendrix MJ, Seftor EA, Seftor RE, Kasemeier-Kulesa J, Kulesa PM, Postovit LM (2007) Reprogramming metastatic tumour cells with embryonic microenvironments. Nat Rev Cancer 7(4):246–255
27. Goldin A, Sandberg JS, Henderson ES, Newman JW, Frei E III, Holland JF (1971) The chemotherapy of human and animal acute leukemia. Cancer Chemother Pharmacol 55(4):309–505
28. Ney PA, D'Andrea AD (2000) Friend erythroleukemia revisited. Blood 96(12):3675–3680
29. Jones PA, Taylor SM (1980) Cellular differentiation, cytidine analogs and DNA methylation. Cell 20(1):85–93
30. Lane AA, Chabner BA (2009) Histone deacetylase inhibitors in cancer therapy. J Clin Oncol 27(32):5459–5468
31. Yang X, Lay F, Han H, Jones PA (2010) Targeting DNA methylation for epigenetic therapy. Trends Pharmacol Sci 31(11):536–546
32. Issa JP (2007) DNA methylation as a therapeutic target in cancer. Clin Cancer Res 13(6):1634–1637
33. Ewald B, Sampath D, Plunkett W (2008) Nucleoside analogs: molecular mechanisms signaling cell death. Oncogene 27(50):6522–6537
34. Yoo CB, Jones PA (2006) Epigenetic therapy of cancer: past, present and future. Nat Rev Drug Discov 5(1):37–50

35. Jabbour E, Issa JP, Garcia-Manero G, Kantarjian H (2008) Evolution of decitabine development: accomplishments, ongoing investigations, and future strategies. Cancer 112(11):2341–2351
36. Piskala A, Sorm F (1964) Nucleic acids components and the analogues. LI. Synthesis of 1-glycosyl derivatives of 5-azauracil and 5-azacytosine. Collect Czech Chem Commun 29:2060–2076
37. Shutt RH, Krueger RG (1972) The effect of actinomycin D and 5-azacytidine on macromolecular synthesis in murine myeloma tumor cells. J Immunol 108(3):819–830
38. Takai N, Kawamata N, Walsh CS, Gery S, Desmond JC, Whittaker S, Said JW, Popoviciu LM, Jones PA, Miyakawa I, Koeffler HP (2005) Discovery of epigenetically masked tumor suppressor genes in endometrial cancer. Mol Cancer Res 3(5):261–269
39. Sasaki M, Kaneuchi M, Fujimoto S, Tanaka Y, Dahiya R (2003) Hypermethylation can selectively silence multiple promoters of steroid receptors in cancers. Mol Cell Endocrinol 202(1–2):201–207
40. Nguyen CT, Weisenberger DJ, Velicescu M, Gonzales FA, Lin JC, Liang G, Jones PA (2002) Histone H3-lysine 9 methylation is associated with aberrant gene silencing in cancer cells and is rapidly reversed by 5-aza-2′-deoxycytidine. Cancer Res 62(22):6456–6461
41. Abbosh PH, Montgomery JS, Starkey JA, Novotny M, Zuhowski EG, Egorin MJ, Moseman AP, Golas A, Brannon KM, Balch C, Huang TH, Nephew KP (2006) Dominant-negative histone H3 lysine 27 mutant derepresses silenced tumor suppressor genes and reverses the drug-resistant phenotype in cancer cells. Cancer Res 66(11):5582–5591
42. Vesely J (1982) Synergistic effect of cis-dichlorodiammineplatinum and 5-aza-2′-deoxycytidine on mouse leukemic cells in vivo and in vitro. Int J Cancer 29(1):81–85
43. Taylor SM, Jones PA (1982) Mechanism of action of eukaryotic DNA methyltransferase. Use of 5-azacytosine-containing DNA. J Mol Biol 162(3):679–692
44. Nakahara Y, Northcott PA, Li M, Kongkham PN, Smith C, Yan H, Croul S, Ra YS, Eberhart C, Huang A, Bigner D, Grajkowska W, Van Meter T, Rutka JT, Taylor MD (2010) Genetic and epigenetic inactivation of Kruppel-like factor 4 in medulloblastoma. Neoplasia 12(1):20–27
45. Mahesh S, Saxena A, Qiu X, Perez-Soler R, Zou Y (2010) Intratracheally administered 5-azacytidine is effective against orthotopic human lung cancer xenograft models and devoid of important systemic toxicity. Clin Lung Cancer 11(6):405–411
46. Walker C, Shay JW (1984) 5-Azacytidine induced myogenesis in a differentiation defective cell line. Differentiation 25(3):259–263
47. Liu L, Harrington M, Jones PA (1986) Characterization of myogenic cell lines derived by 5-azacytidine treatment. Dev Biol 117(2):331–336
48. Hustad CM, Jones PA (1991) Effect of myogenic determination on tumorigenicity of chemically transformed 10T1/2 cells. Mol Carcinog 4(2):153–161
49. Schneider-Stock R, Diab-Assef M, Rohrbeck A, Foltzer-Jourdainne C, Boltze C, Hartig R, Schonfeld P, Roessner A, Gali-Muhtasib H (2005) 5-Aza-cytidine is a potent inhibitor of DNA methyltransferase 3a and induces apoptosis in HCT-116 colon cancer cells via Gadd45- and p53-dependent mechanisms. J Pharmacol Exp Ther 312(2):525–536
50. Wang XM, Wang X, Li J, Evers BM (1998) Effects of 5-azacytidine and butyrate on differentiation and apoptosis of hepatic cancer cell lines. Ann Surg 227(6):922–931
51. Burrows JF, Chanduloy S, McIlhatton MA, Nagar H, Yeates K, Donaghy P, Price J, Godwin AK, Johnston PG, Russell SE (2003) Altered expression of the septin gene, SEPT9, in ovarian neoplasia. J Pathol 201(4):581–588
52. Balch C, Montgomery JS, Paik HI, Kim S, Huang TH, Nephew KP (2005) New anti-cancer strategies: epigenetic therapies and biomarkers. Front Biosci 10:1897–1931
53. Momparler RL (2005) Epigenetic therapy of cancer with 5-aza-2′-deoxycytidine (decitabine). Semin Oncol 32(5):443–451
54. Wilson VL, Jones PA, Momparler RL (1983) Inhibition of DNA methylation in L1210 leukemic cells by 5-aza-2′-deoxycytidine as a possible mechanism of chemotherapeutic action. Cancer Res 43(8):3493–3496

55. Momparler RL, Bouchard J, Samson J (1985) Induction of differentiation and inhibition of DNA methylation in HL-60 myeloid leukemic cells by 5-AZA-2′-deoxycytidine. Leuk Res 9(11):1361–1366
56. Limonta M, Colombo T, Damia G, Catapano CV, Conter V, Gervasoni M, Masera G, Liso V, Specchia G, Giudici G et al (1993) Cytotoxic activity and mechanism of action of 5-Aza-2′-deoxycytidine in human CML cells. Leuk Res 17(11):977–982
57. Corn PG, Kuerbitz SJ, van Noesel MM, Esteller M, Compitello N, Baylin SB, Herman JG (1999) Transcriptional silencing of the p73 gene in acute lymphoblastic leukemia and Burkitt's lymphoma is associated with 5′ CpG island methylation. Cancer Res 59(14):3352–3356
58. Schnekenburger M, Grandjenette C, Ghelfi J, Karius T, Foliguet B, Dicato M, Diederich M (2011) Sustained exposure to the DNA demethylating agent, 2′-deoxy-5-azacytidine, leads to apoptotic cell death in chronic myeloid leukemia by promoting differentiation, senescence, and autophagy. Biochem Pharmacol 81(3):364–378
59. Obata T, Toyota M, Satoh A, Sasaki Y, Ogi K, Akino K, Suzuki H, Murai M, Kikuchi T, Mita H, Itoh F, Issa JP, Tokino T, Imai K (2003) Identification of HRK as a target of epigenetic inactivation in colorectal and gastric cancer. Clin Cancer Res 9(17):6410–6418
60. Alcazar O, Achberger S, Aldrich W, Hu Z, Negrotto S, Saunthararajah Y, Triozzi P (2012) Epigenetic regulation by decitabine of melanoma differentiation in vitro and in vivo. Int J Cancer 131(1):18–29
61. Chen W, Gao N, Shen Y, Cen JN (2010) Hypermethylation downregulates Runx3 gene expression and its restoration suppresses gastric epithelial cell growth by inducing p27 and caspase3 in human gastric cancer. J Gastroenterol Hepatol 25(4):823–831
62. Tseng RC, Lee SH, Hsu HS, Chen BH, Tsai WC, Tzao C, Wang YC (2010) SLIT2 attenuation during lung cancer progression deregulates beta-catenin and E-cadherin and associates with poor prognosis. Cancer Res 70(2):543–551
63. Furuta M, Kozaki KI, Tanaka S, Arii S, Imoto I, Inazawa J (2010) miR-124 and miR-203 are epigenetically silenced tumor-suppressive microRNAs in hepatocellular carcinoma. Carcinogenesis 31(5):766–776
64. Hashimoto Y, Akiyama Y, Otsubo T, Shimada S, Yuasa Y (2010) Involvement of epigenetically silenced microRNA-181c in gastric carcinogenesis. Carcinogenesis 31(5):777–784
65. Aguilera O, Fraga MF, Ballestar E, Paz MF, Herranz M, Espada J, Garcia JM, Munoz A, Esteller M, Gonzalez-Sancho JM (2006) Epigenetic inactivation of the Wnt antagonist DICKKOPF-1 (DKK-1) gene in human colorectal cancer. Oncogene 25(29):4116–4121
66. Chuang JC, Warner SL, Vollmer D, Vankayalapati H, Redkar S, Bearss DJ, Qiu X, Yoo CB, Jones PA (2010) S110, a 5-Aza-2′-deoxycytidine-containing dinucleotide, is an effective DNA methylation inhibitor in vivo and can reduce tumor growth. Mol Cancer Ther 9(5):1443–1450
67. Yoo CB, Jeong S, Egger G, Liang G, Phiasivongsa P, Tang C, Redkar S, Jones PA (2007) Delivery of 5-aza-2′-deoxycytidine to cells using oligodeoxynucleotides. Cancer Res 67(13):6400–6408
68. Brueckner B, Rius M, Markelova MR, Fichtner I, Hals PA, Sandvold ML, Lyko F (2010) Delivery of 5-azacytidine to human cancer cells by elaidic acid esterification increases therapeutic drug efficacy. Mol Cancer Ther 9(5):1256–1264
69. Leu YW, Rahmatpanah F, Shi H, Wei SH, Liu JC, Yan PS, Huang TH (2003) Double RNA interference of DNMT3b and DNMT1 enhances DNA demethylation and gene reactivation. Cancer Res 63(19):6110–6115
70. Balch C, Yan P, Craft T, Young S, Skalnik DG, Huang TH, Nephew KP (2005) Antimitogenic and chemosensitizing effects of the methylation inhibitor zebularine in ovarian cancer. Mol Cancer Ther 4(10):1505–1514
71. Yoo CB, Chuang JC, Byun HM, Egger G, Yang AS, Dubeau L, Long T, Laird PW, Marquez VE, Jones PA (2008) Long-term epigenetic therapy with oral zebularine has minimal side effects and prevents intestinal tumors in mice. Cancer Prev Res 1(4):233–240
72. Cheng JC, Matsen CB, Gonzales FA, Ye W, Greer S, Marquez VE, Jones PA, Selker EU (2003) Inhibition of DNA methylation and reactivation of silenced genes by zebularine. J Natl Cancer Inst 95(5):399–409

73. Cheng JC, Yoo CB, Weisenberger DJ, Chuang J, Wozniak C, Liang G, Marquez VE, Greer S, Orntoft TF, Thykjaer T, Jones PA (2004) Preferential response of cancer cells to zebularine. Cancer Cell 6(2):151–158
74. Yoo CB, Valente R, Congiatu C, Gavazza F, Angel A, Siddiqui MA, Jones PA, McGuigan C, Marquez VE (2008) Activation of p16 gene silenced by DNA methylation in cancer cells by phosphoramidate derivatives of 2′-deoxyzebularine. J Med Chem 51(23):7593–7601
75. Segura-Pacheco B, Trejo-Becerril C, Perez-Cardenas E, Taja-Chayeb L, Mariscal I, Chavez A, Acuna C, Salazar AM, Lizano M, Duenas-Gonzalez A (2003) Reactivation of tumor suppressor genes by the cardiovascular drugs hydralazine and procainamide and their potential use in cancer therapy. Clin Cancer Res 9(5):1596–1603
76. Chuang JC, Yoo CB, Kwan JM, Li TW, Liang G, Yang AS, Jones PA (2005) Comparison of biological effects of non-nucleoside DNA methylation inhibitors versus 5-aza-2′-deoxycytidine. Mol Cancer Ther 4(10):1515–1520
77. Liu F, Liu Q, Yang D, Bollag WB, Robertson K, Wu P, Liu K (2011) Verticillin A overcomes apoptosis resistance in human colon carcinoma through DNA methylation-dependent upregulation of BNIP3. Cancer Res 71(21):6807–6816
78. Brueckner B, Garcia Boy R, Siedlecki P, Musch T, Kliem HC, Zielenkiewicz P, Suhai S, Wiessler M, Lyko F (2005) Epigenetic reactivation of tumor suppressor genes by a novel small-molecule inhibitor of human DNA methyltransferases. Cancer Res 65(14):6305–6311
79. Datta J, Ghoshal K, Denny WA, Gamage SA, Brooke DG, Phiasivongsa P, Redkar S, Jacob ST (2009) A new class of quinoline-based DNA hypomethylating agents reactivates tumor suppressor genes by blocking DNA methyltransferase 1 activity and inducing its degradation. Cancer Res 69(10):4277–4285
80. Medina-Franco JL, Caulfield T (2011) Advances in the computational development of DNA methyltransferase inhibitors. Drug Discov Today 16:418–425
81. Siedlecki P, Garcia Boy R, Musch T, Brueckner B, Suhai S, Lyko F, Zielenkiewicz P (2006) Discovery of two novel, small-molecule inhibitors of DNA methylation. J Med Chem 49(2):678–683
82. Medina-Franco JL, Caulfield T (2011) Advances in the computational development of DNA methyltransferase inhibitors. Drug Discov Today 16(9–10):418–425
83. Castellano S, Kuck D, Viviano M, Yoo J, Lopez-Vallejo F, Conti P, Tamborini L, Pinto A, Medina-Franco JL, Sbardella G (2011) Synthesis and biochemical evaluation of delta(2)-isoxazoline derivatives as DNA methyltransferase 1 inhibitors. J Med Chem 54(21):7663–7677
84. Balch C, Nephew KP (2010) The role of chromatin, microRNAs, and tumor stem cells in ovarian cancer. Cancer Biomark 8(4):203–221
85. Wood TE, Dalili S, Simpson CD, Sukhai MA, Hurren R, Anyiwe K, Mao X, Suarez Saiz F, Gronda M, Eberhard Y, MacLean N, Ketela T, Reed JC, Moffat J, Minden MD, Batey RA, Schimmer AD (2010) Selective inhibition of histone deacetylases sensitizes malignant cells to death receptor ligands. Mol Cancer Ther 9(1):246–256
86. Hirata H, Hinoda Y, Nakajima K, Kawamoto K, Kikuno N, Ueno K, Yamamura S, Zaman MS, Khatri G, Chen Y, Saini S, Majid S, Deng G, Ishii N, Dahiya R (2011) Wnt antagonist DKK1 acts as a tumor suppressor gene that induces apoptosis and inhibits proliferation in human renal cell carcinoma. Int J Cancer 128(8):1793–1803
87. Liu T, Zhang X, So CK, Wang S, Wang P, Yan L, Myers R, Chen Z, Patterson AP, Yang CS, Chen X (2007) Regulation of Cdx2 expression by promoter methylation, and effects of Cdx2 transfection on morphology and gene expression of human esophageal epithelial cells. Carcinogenesis 28(2):488–496
88. Xu J, Zhou JY, Tainsky MA, Wu GS (2007) Evidence that tumor necrosis factor-related apoptosis-inducing ligand induction by 5-Aza-2′-deoxycytidine sensitizes human breast cancer cells to adriamycin. Cancer Res 67(3):1203–1211
89. Chun SG, Zhou W, Yee NS (2009) Combined targeting of histone deacetylases and hedgehog signaling enhances cytoxicity in pancreatic cancer. Cancer Biol Ther 8(14):1328–1339
90. Neil GL, Berger AE, Bhuyan BK, DeSante DC (1976) Combination chemotherapy of L1210 leukemia with 1-beta-D-arabinofuranosylcytosine and 5-azacytidine. Cancer Res 36(3):1114–1120

91. Neil GL, Moxley TE, Kuentzel SL, Manak RC, Hanka LJ (1975) Enhancement by tetrahydrouridine (NSC-112907) of the oral activity of 5-azacytidine (NSC-102816) in L1210 leukemic mice. Cancer Chemother Pharmacol 59(3):459–465
92. Festuccia C, Gravina GL, D'Alessandro AM, Muzi P, Millimaggi D, Dolo V, Ricevuto E, Vicentini C, Bologna M (2009) Azacitidine improves antitumor effects of docetaxel and cisplatin in aggressive prostate cancer models. Endocr Relat Cancer 16(2):401–413
93. Anzai H, Frost P, Abbruzzese JL (1992) Synergistic cytotoxicity with 2′-deoxy-5-azacytidine and topotecan in vitro and in vivo. Cancer Res 52(8):2180–2185
94. Balch C, Montgomery JS, Paik HI, Kim S, Kim S, Huang TH, Nephew KP (2005) New anticancer strategies: epigenetic therapies and biomarkers. Front Biosci 10:1897–1931
95. Plumb JA, Strathdee G, Sludden J, Kaye SB, Brown R (2000) Reversal of drug resistance in human tumor xenografts by 2′-deoxy-5-azacytidine-induced demethylation of the hMLH1 gene promoter. Cancer Res 60(21):6039–6044
96. Morita S, Iida S, Kato K, Takagi Y, Uetake H, Sugihara K (2006) The synergistic effect of 5-aza-2′-deoxycytidine and 5-fluorouracil on drug-resistant tumors. Oncology 71(5–6):437–445
97. Ishiguro M, Iida S, Uetake H, Morita S, Makino H, Kato K, Takagi Y, Enomoto M, Sugihara K (2007) Effect of combined therapy with low-dose 5-aza-2′-deoxycytidine and irinotecan on colon cancer cell line HCT-15. Ann Surg Oncol 14(5):1752–1762
98. Karpf AR, Peterson PW, Rawlins JT, Dalley BK, Yang Q, Albertsen H, Jones DA (1999) Inhibition of DNA methyltransferase stimulates the expression of signal transducer and activator of transcription 1, 2, and 3 genes in colon tumor cells. Proc Natl Acad Sci USA 96(24):14007–14012
99. Phuong NT, Kim SK, Lim SC, Kim HS, Kim TH, Lee KY, Ahn SG, Yoon JH, Kang KW (2011) Role of PTEN promoter methylation in tamoxifen-resistant breast cancer cells. Breast Cancer Res Treat 130(1):73–83
100. Zuo T, Liu TM, Lan X, Weng YI, Shen R, Gu F, Huang YW, Liyanarachchi S, Deatherage DE, Hsu PY, Taslim C, Ramaswamy B, Shapiro CL, Lin HJ, Cheng AS, Jin VX, Huang TH (2011) Epigenetic silencing mediated through activated PI3K/AKT signaling in breast cancer. Cancer Res 71(5):1752–1762
101. Stearns V, Zhou Q, Davidson NE (2007) Epigenetic regulation as a new target for breast cancer therapy. Cancer Invest 25(8):659–665
102. Sharma D, Saxena NK, Davidson NE, Vertino PM (2006) Restoration of tamoxifen sensitivity in estrogen receptor-negative breast cancer cells: tamoxifen-bound reactivated ER recruits distinctive corepressor complexes. Cancer Res 66(12):6370–6378
103. Gao L, Alumkal J (2010) Epigenetic regulation of androgen receptor signaling in prostate cancer. Epigenetics 5(2):100–104
104. Nelson WG, Yegnasubramanian S, Agoston AT, Bastian PJ, Lee BH, Nakayama M, De Marzo AM (2007) Abnormal DNA methylation, epigenetics, and prostate cancer. Front Biosci 12:4254–4266
105. Shang D, Liu Y, Liu Q, Zhang F, Feng L, Lv W, Tian Y (2009) Synergy of 5-aza-2′-deoxycytidine (DAC) and paclitaxel in both androgen-dependent and -independent prostate cancer cell lines. Cancer Lett 278(1):82–87
106. Zorn CS, Wojno KJ, McCabe MT, Kuefer R, Gschwend JE, Day ML (2007) 5-aza-2′-deoxycytidine delays androgen-independent disease and improves survival in the transgenic adenocarcinoma of the mouse prostate mouse model of prostate cancer. Clin Cancer Res 13(7):2136–2143
107. Walton TJ, Li G, Seth R, McArdle SE, Bishop MC, Rees RC (2008) DNA demethylation and histone deacetylation inhibition co-operate to re-express estrogen receptor beta and induce apoptosis in prostate cancer cell-lines. Prostate 68(2):210–222
108. Friend C, Scher W, Holland JG, Sato T (1971) Hemoglobin synthesis in murine virus-induced leukemic cells in vitro: stimulation of erythroid differentiation by dimethyl sulfoxide. Proc Natl Acad Sci USA 68(2):378–382
109. Marks PA, Breslow R (2007) Dimethyl sulfoxide to vorinostat: development of this histone deacetylase inhibitor as an anticancer drug. Nat Biotechnol 25(1):84–90

110. Minucci S, Pelicci PG (2006) Histone deacetylase inhibitors and the promise of epigenetic (and more) treatments for cancer. Nat Rev Cancer 6(1):38–51
111. Qiu L, Burgess A, Fairlie DP, Leonard H, Parsons PG, Gabrielli BG (2000) Histone deacetylase inhibitors trigger a G2 checkpoint in normal cells that is defective in tumor cells. Mol Biol Cell 11(6):2069–2083
112. Strait KA, Warnick CT, Ford CD, Dabbas B, Hammond EH, Ilstrup SJ (2005) Histone deacetylase inhibitors induce G2-checkpoint arrest and apoptosis in cisplatinum-resistant ovarian cancer cells associated with overexpression of the Bcl-2-related protein Bad. Mol Cancer Ther 4(4):603–611
113. Choudhary C, Kumar C, Gnad F, Nielsen ML, Rehman M, Walther TC, Olsen JV, Mann M (2009) Lysine acetylation targets protein complexes and co-regulates major cellular functions. Science 325(5942):834–840
114. Plumb JA, Finn PW, Williams RJ, Bandara MJ, Romero MR, Watkins CJ, La Thangue NB, Brown R (2003) Pharmacodynamic response and inhibition of growth of human tumor xenografts by the novel histone deacetylase inhibitor PXD101. Mol Cancer Ther 2(8):721–728
115. Qian X, LaRochelle WJ, Ara G, Wu F, Petersen KD, Thougaard A, Sehested M, Lichenstein HS, Jeffers M (2006) Activity of PXD101, a histone deacetylase inhibitor, in preclinical ovarian cancer studies. Mol Cancer Ther 5(8):2086–2095
116. Uchida H, Maruyama T, Nagashima T, Asada H, Yoshimura Y (2005) Histone deacetylase inhibitors induce differentiation of human endometrial adenocarcinoma cells through upregulation of glycodelin. Endocrinology 146(12):5365–5373
117. Takai N, Desmond JC, Kumagai T, Gui D, Said JW, Whittaker S, Miyakawa I, Koeffler HP (2004) Histone deacetylase inhibitors have a profound antigrowth activity in endometrial cancer cells. Clin Cancer Res 10(3):1141–1149
118. Rahman R, Grundy R (2011) Histone deacetylase inhibition as an anticancer telomerase-targeting strategy. Int J Cancer 129(12):2765–2774
119. Xu WS, Parmigiani RB, Marks PA (2007) Histone deacetylase inhibitors: molecular mechanisms of action. Oncogene 26(37):5541–5552
120. Cooper AL, Greenberg VL, Lancaster PS, van Nagell JR Jr, Zimmer SG, Modesitt SC (2007) In vitro and in vivo histone deacetylase inhibitor therapy with suberoylanilide hydroxamic acid (SAHA) and paclitaxel in ovarian cancer. Gynecol Oncol 104:596–601
121. Dietrich CS III, Greenberg VL, DeSimone CP, Modesitt SC, van Nagell JR, Craven R, Zimmer SG (2010) Suberoylanilide hydroxamic acid (SAHA) potentiates paclitaxel-induced apoptosis in ovarian cancer cell lines. Gynecol Oncol 116(1):126–130
122. Sonnemann J, Gange J, Pilz S, Stotzer C, Ohlinger R, Belau A, Lorenz G, Beck JF (2006) Comparative evaluation of the treatment efficacy of suberoylanilide hydroxamic acid (SAHA) and paclitaxel in ovarian cancer cell lines and primary ovarian cancer cells from patients. BMC Cancer 6:183
123. Zuco V, Benedetti V, De Cesare M, Zunino F (2010) Sensitization of ovarian carcinoma cells to the atypical retinoid ST1926 by the histone deacetylase inhibitor, RC307: enhanced DNA damage response. Int J Cancer 126(5):1246–1255
124. Son DS, Wilson AJ, Parl AK, Khabele D (2010) The effects of the histone deacetylase inhibitor romidepsin (FK228) are enhanced by aspirin (ASA) in COX-1 positive ovarian cancer cells through augmentation of p21. Cancer Biol Ther 9(11):928–935
125. Yang YT, Balch C, Kulp SK, Mand MR, Nephew KP, Chen CS (2009) A rationally designed histone deacetylase inhibitor with distinct antitumor activity against ovarian cancer. Neoplasia 11(6):552–563; 553 p following 563
126. Iwahashi S, Shimada M, Utsunomiya T, Morine Y, Imura S, Ikemoto T, Mori H, Hanaoka J, Saito Y (2011) Histone deacetylase inhibitor enhances the anti-tumor effect of gemcitabine: a special reference to gene-expression microarray analysis. Oncol Rep 26(5):1057–1062
127. Kim MS, Baek JH, Chakravarty D, Sidransky D, Carrier F (2005) Sensitization to UV-induced apoptosis by the histone deacetylase inhibitor trichostatin A (TSA). Exp Cell Res 306(1):94–102
128. Roh MS, Kim CW, Park BS, Kim GC, Jeong JH, Kwon HC, Suh DJ, Cho KH, Yee SB, Yoo YH (2004) Mechanism of histone deacetylase inhibitor Trichostatin A induced apoptosis in human osteosarcoma cells. Apoptosis 9(5):583–589

129. Srivastava RK, Kurzrock R, Shankar S (2010) MS-275 sensitizes TRAIL-resistant breast cancer cells, inhibits angiogenesis and metastasis, and reverses epithelial-mesenchymal transition in vivo. Mol Cancer Ther 9(12):3254–3266
130. Hacker S, Dittrich A, Mohr A, Schweitzer T, Rutkowski S, Krauss J, Debatin KM, Fulda S (2009) Histone deacetylase inhibitors cooperate with IFN-gamma to restore caspase-8 expression and overcome TRAIL resistance in cancers with silencing of caspase-8. Oncogene 28(35):3097–3110
131. Schuler S, Fritsche P, Diersch S, Arlt A, Schmid RM, Saur D, Schneider G (2010) HDAC2 attenuates TRAIL-induced apoptosis of pancreatic cancer cells. Mol Cancer Ther 9:80
132. Pathil A, Armeanu S, Venturelli S, Mascagni P, Weiss TS, Gregor M, Lauer UM, Bitzer M (2006) HDAC inhibitor treatment of hepatoma cells induces both TRAIL-independent apoptosis and restoration of sensitivity to TRAIL. Hepatology 43(3):425–434
133. Thomas S, Thurn KT, Bicaku E, Marchion DC, Munster PN (2011) Addition of a histone deacetylase inhibitor redirects tamoxifen-treated breast cancer cells into apoptosis, which is opposed by the induction of autophagy. Breast Cancer Res Treat 130(2):437–447
134. Dowdy SC, Jiang S, Zhou XC, Hou X, Jin F, Podratz KC, Jiang SW (2006) Histone deacetylase inhibitors and paclitaxel cause synergistic effects on apoptosis and microtubule stabilization in papillary serous endometrial cancer cells. Mol Cancer Ther 5(11):2767–2776
135. Ahn MY, Chung HY, Choi WS, Lee BM, Yoon S, Kim HS (2010) Anti-tumor effect of apicidin on Ishikawa human endometrial cancer cells both in vitro and in vivo by blocking histone deacetylase 3 and 4. Int J Oncol 36(1):125–131
136. Bali P, Pranpat M, Swaby R, Fiskus W, Yamaguchi H, Balasis M, Rocha K, Wang HG, Richon V, Bhalla K (2005) Activity of suberoylanilide hydroxamic Acid against human breast cancer cells with amplification of her-2. Clin Cancer Res 11(17):6382–6389
137. Morey L, Brenner C, Fazi F, Villa R, Gutierrez A, Buschbeck M, Nervi C, Minucci S, Fuks F, Di Croce L (2008) MBD3, a component of the NuRD complex, facilitates chromatin alteration and deposition of epigenetic marks. Mol Cell Biol 28(19):5912–5923
138. Terasawa K, Sagae S, Toyota M, Tsukada K, Ogi K, Satoh A, Mita H, Imai K, Tokino T, Kudo R (2004) Epigenetic inactivation of TMS1/ASC in ovarian cancer. Clin Cancer Res 10(6):2000–2006
139. Chen MY, Liao WS, Lu Z, Bornmann WG, Hennessey V, Washington MN, Rosner GL, Yu Y, Ahmed AA, Bast RC Jr (2011) Decitabine and suberoylanilide hydroxamic acid (SAHA) inhibit growth of ovarian cancer cell lines and xenografts while inducing expression of imprinted tumor suppressor genes, apoptosis, G2/M arrest, and autophagy. Cancer 117(19):4424–4438
140. Xiong Y, Dowdy SC, Gonzalez Bosquet J, Zhao Y, Eberhardt NL, Podratz KC, Jiang SW (2005) Epigenetic-mediated upregulation of progesterone receptor B gene in endometrial cancer cell lines. Gynecol Oncol 99(1):135–141
141. Belinsky SA, Grimes MJ, Picchi MA, Mitchell HD, Stidley CA, Tesfaigzi Y, Channell MM, Liu Y, Casero RA Jr, Baylin SB, Reed MD, Tellez CS, March TH (2011) Combination therapy with vidaza and entinostat suppresses tumor growth and reprograms the epigenome in an orthotopic lung cancer model. Cancer Res 71(2):454–462
142. Ecke I, Petry F, Rosenberger A, Tauber S, Monkemeyer S, Hess I, Dullin C, Kimmina S, Pirngruber J, Johnsen SA, Uhmann A, Nitzki F, Wojnowski L, Schulz-Schaeffer W, Witt O, Hahn H (2009) Antitumor effects of a combined 5-aza-2′deoxycytidine and valproic acid treatment on rhabdomyosarcoma and medulloblastoma in Ptch mutant mice. Cancer Res 69(3):887–895
143. Herranz D, Serrano M (2010) SIRT1: recent lessons from mouse models. Nat Rev Cancer 10(12):819–823
144. Nebbioso A, Pereira R, Khanwalkar H, Matarese F, Garcia-Rodriguez J, Miceli M, Logie C, Kedinger V, Ferrara F, Stunnenberg HG, de Lera AR, Gronemeyer H, Altucci L (2011) Death receptor pathway activation and increase of ROS production by the triple epigenetic inhibitor, UVI5008. Mol Cancer Ther 10(12):2394–2404

145. Milutinovic S, D'Alessio AC, Detich N, Szyf M (2007) Valproate induces widespread epigenetic reprogramming which involves demethylation of specific genes. Carcinogenesis 28(3):560–571
146. Dong E, Guidotti A, Grayson DR, Costa E (2007) Histone hyperacetylation induces demethylation of reelin and 67-kDa glutamic acid decarboxylase promoters. Proc Natl Acad Sci USA 104(11):4676–4681
147. Arzenani MK, Zade AE, Ming Y, Vijverberg SJ, Zhang Z, Khan Z, Sadique S, Kallenbach L, Hu L, Vukojevic V, Ekstrom TJ (2011) Genomic DNA hypomethylation by histone deacetylase inhibition implicates DNMT1 nuclear dynamics. Mol Cell Biol 31(19):4119–4128
148. Ou JN, Torrisani J, Unterberger A, Provencal N, Shikimi K, Karimi M, Ekstrom TJ, Szyf M (2007) Histone deacetylase inhibitor Trichostatin A induces global and gene-specific DNA demethylation in human cancer cell lines. Biochem Pharmacol 73(9):1297–1307
149. Xiong Y, Dowdy SC, Podratz KC, Jin F, Attewell JR, Eberhardt NL, Jiang SW (2005) Histone deacetylase inhibitors decrease DNA methyltransferase-3B messenger RNA stability and down-regulate de novo DNA methyltransferase activity in human endometrial cells. Cancer Res 65(7):2684–2689
150. Scott SA, Dong WF, Ichinohasama R, Hirsch C, Sheridan D, Sanche SE, Geyer CR, Decoteau JF (2006) 5-Aza-2′-deoxycytidine (decitabine) can relieve p21WAF1 repression in human acute myeloid leukemia by a mechanism involving release of histone deacetylase 1 (HDAC1) without requiring p21WAF1 promoter demethylation. Leuk Res 30(1):69–76
151. Egger G, Aparicio AM, Escobar SG, Jones PA (2007) Inhibition of histone deacetylation does not block resilencing of p16 after 5-aza-2′-deoxycytidine treatment. Cancer Res 67(1):346–353
152. Gore SD, Baylin S, Sugar E, Carraway H, Miller CB, Carducci M, Grever M, Galm O, Dauses T, Karp JE, Rudek MA, Zhao M, Smith BD, Manning J, Jiemjit A, Dover G, Mays A, Zwiebel J, Murgo A, Weng LJ, Herman JG (2006) Combined DNA methyltransferase and histone deacetylase inhibition in the treatment of myeloid neoplasms. Cancer Res 66(12):6361–6369
153. Kaminskyy VO, Surova OV, Vaculova A, Zhivotovsky B (2011) Combined inhibition of DNA methyltransferase and histone deacetylase restores caspase-8 expression and sensitizes SCLC cells to TRAIL. Carcinogenesis 32(10):1450–1458
154. Steele N, Finn P, Brown R, Plumb JA (2009) Combined inhibition of DNA methylation and histone acetylation enhances gene re-expression and drug sensitivity in vivo. Br J Cancer 100(5):758–763
155. Matei DE, Nephew KP (2010) Epigenetic therapies for chemoresensitization of epithelial ovarian cancer. Gynecol Oncol 116(2):195–201
156. Kristensen LS, Nielsen HM, Hansen LL (2009) Epigenetics and cancer treatment. Eur J Pharmacol 625(1–3):131–142
157. Sigalotti L, Fratta E, Coral S, Cortini E, Covre A, Nicolay HJ, Anzalone L, Pezzani L, Di Giacomo AM, Fonsatti E, Colizzi F, Altomonte M, Calabro L, Maio M (2007) Epigenetic drugs as pleiotropic agents in cancer treatment: biomolecular aspects and clinical applications. J Cell Physiol 212(2):330–344
158. Momparler RL, Rivard GE, Gyger M (1985) Clinical trial on 5-aza-2′-deoxycytidine in patients with acute leukemia. Pharmacol Ther 30(3):277–286
159. Wijermans PW, Krulder JW, Huijgens PC, Neve P (1997) Continuous infusion of low-dose 5-Aza-2′-deoxycytidine in elderly patients with high-risk myelodysplastic syndrome. Leukemia 11(suppl 1):S19–S23
160. Wijermans P, Lubbert M, Verhoef G, Bosly A, Ravoet C, Andre M, Ferrant A (2000) Low-dose 5-aza-2′-deoxycytidine, a DNA hypomethylating agent, for the treatment of high-risk myelodysplastic syndrome: a multicenter phase II study in elderly patients. J Clin Oncol 18(5):956–962
161. DeSimone J, Koshy M, Dorn L, Lavelle D, Bressler L, Molokie R, Talischy N (2002) Maintenance of elevated fetal hemoglobin levels by decitabine during dose interval treatment of sickle cell anemia. Blood 99(11):3905–3908

162. Koshy M, Dorn L, Bressler L, Molokie R, Lavelle D, Talischy N, Hoffman R, van Overveld W, DeSimone J (2000) 2-deoxy 5-azacytidine and fetal hemoglobin induction in sickle cell anemia. Blood 96(7):2379–2384
163. Issa JP, Garcia-Manero G, Giles FJ, Mannari R, Thomas D, Faderl S, Bayar E, Lyons J, Rosenfeld CS, Cortes J, Kantarjian HM (2004) Phase 1 study of low-dose prolonged exposure schedules of the hypomethylating agent 5-aza-2′-deoxycytidine (decitabine) in hematopoietic malignancies. Blood 103(5):1635–1640
164. Invest New DrugsSchwartsmann G, Schunemann H, Gorini CN, Filho AF, Garbino C, Sabini G, Muse I, DiLeone L, Mans DR (2000) A phase I trial of cisplatin plus decitabine, a new DNA-hypomethylating agent, in patients with advanced solid tumors and a follow-up early phase II evaluation in patients with inoperable non-small cell lung cancer. Invest New Drugs 18(1):83–91
165. Pohlmann P, DiLeone LP, Cancella AI, Caldas AP, Dal Lago L, Campos O Jr, Monego E, Rivoire W, Schwartsmann G (2002) Phase II trial of cisplatin plus decitabine, a new DNA hypomethylating agent, in patients with advanced squamous cell carcinoma of the cervix. Am J Clin Oncol 25(5):496–501
166. Appleton K, Mackay HJ, Judson I, Plumb JA, McCormick C, Strathdee G, Lee C, Barrett S, Reade S, Jadayel D, Tang A, Bellenger K, Mackay L, Setanoians A, Schatzlein A, Twelves C, Kaye SB, Brown R (2007) Phase I and pharmacodynamic trial of the DNA methyltransferase inhibitor decitabine and carboplatin in solid tumors. J Clin Oncol 25(29):4603–4609
167. Glasspool RM, Gore M, Rustin G, McNeish I, Wilson R, Pledge S, Paul J, Mackean M, Halford S, Kaye S (2009) Randomized phase II study of in combination with carboplatin compared with carboplatin alone in patients with recurrent advanced ovarian cancer. J Clin Oncol 26(15S (May 20 suppl)):Abstract 5562
168. Fu S, Hu W, Iyer R, Kavanagh JJ, Coleman RL, Levenback CF, Sood AK, Wolf JK, Gershenson DM, Markman M, Hennessy BT, Kurzrock R, Bast RC Jr (2011) Phase 1b-2a study to reverse platinum resistance through use of a hypomethylating agent, azacitidine, in patients with platinum-resistant or platinum-refractory epithelial ovarian cancer. Cancer 117(8):1661–1669
169. Fang F, Balch C, Schilder J, Breen T, Zhang S, Shen C, Li L, Kulesavage C, Snyder AJ, Nephew KP, Matei DE (2010) A phase 1 and pharmacodynamic study of decitabine in combination with carboplatin in patients with recurrent, platinum-resistant, epithelial ovarian cancer. Cancer 116(17):4043–4053
170. Matei D, Fang F, Shen C, Schilder J, Arnold A, Zeng Y, Berry WA, Huang T, Nephew KP (2012). Epigenetic resensitization to platinum in ovarian cancer. Cancer Res 72(9): 2197–2205
171. Bauman J, Verschraegen C, Belinsky S, Muller C, Rutledge T, Fekrazad M, Ravindranathan M, Lee SJ, Jones D (2012) A phase I study of 5-azacytidine and erlotinib in advanced solid tumor malignancies. Cancer Chemother Pharmacol 69(2):547–554
172. George RE, Lahti JM, Adamson PC, Zhu K, Finkelstein D, Ingle AM, Reid JM, Krailo M, Neuberg D, Blaney SM, Diller L (2010) Phase I study of decitabine with doxorubicin and cyclophosphamide in children with neuroblastoma and other solid tumors: a Children's Oncology Group study. Pediatr Blood Cancer 55(4):629–638
173. Welch JS, Klco JM, Gao F, Procknow E, Uy GL, Stockerl-Goldstein KE, Abboud CN, Westervelt P, DiPersio JF, Hassan A, Cashen AF, Vij R (2011) Combination decitabine, arsenic trioxide, and ascorbic acid for the treatment of myelodysplastic syndrome and acute myeloid leukemia: a phase I study. Am J Hematol 86(9):796–800
174. Stewart DJ, Issa JP, Kurzrock R, Nunez MI, Jelinek J, Hong D, Oki Y, Guo Z, Gupta S, Wistuba II (2009) Decitabine effect on tumor global DNA methylation and other parameters in a phase I trial in refractory solid tumors and lymphomas. Clin Cancer Res 15(11): 3881–3888
175. Modesitt SC, Sill M, Hoffman JS, Bender DP (2008) A phase II study of vorinostat in the treatment of persistent or recurrent epithelial ovarian or primary peritoneal carcinoma: a Gynecologic Oncology Group study. Gynecol Oncol 109(2):182–186

176. Mackay HJ, Hirte H, Colgan T, Covens A, MacAlpine K, Grenci P, Wang L, Mason J, Pham PA, Tsao MS, Pan J, Zwiebel J, Oza AM (2010) Phase II trial of the histone deacetylase inhibitor belinostat in women with platinum resistant epithelial ovarian cancer and micropapillary (LMP) ovarian tumours. Eur J Cancer 46(9):1573–1579
177. Molife LR, Attard G, Fong PC, Karavasilis V, Reid AH, Patterson S, Riggs CE Jr, Higano C, Stadler WM, McCulloch W, Dearnaley D, Parker C, de Bono JS (2010) Phase II, two-stage, single-arm trial of the histone deacetylase inhibitor (HDACi) romidepsin in metastatic castration-resistant prostate cancer (CRPC). Ann Oncol 21(1):109–113
178. Hainsworth JD, Infante JR, Spigel DR, Arrowsmith ER, Boccia RV, Burris HA (2011) A phase II trial of panobinostat, a histone deacetylase inhibitor, in the treatment of patients with refractory metastatic renal cell carcinoma. Cancer Invest 29(7):451–455
179. Takai N, Narahara H (2010) Histone deacetylase inhibitor therapy in epithelial ovarian cancer. J Oncol 2010:458431
180. Thurn KT, Thomas S, Moore A, Munster PN (2011) Rational therapeutic combinations with histone deacetylase inhibitors for the treatment of cancer. Future Oncol 7(2):263–283
181. Rodon J, Iniesta MD, Papadopoulos K (2009) Development of PARP inhibitors in oncology. Expert Opin Investig Drugs 18(1):31–43
182. Teicher BA (2010) Combinations of PARP, hedgehog and HDAC inhibitors with standard drugs. Curr Opin Pharmacol 10(4):397–404
183. Munster PN, Thurn KT, Thomas S, Raha P, Lacevic M, Miller A, Melisko M, Ismail-Khan R, Rugo H, Moasser M, Minton SE (2011) A phase II study of the histone deacetylase inhibitor vorinostat combined with tamoxifen for the treatment of patients with hormone therapy-resistant breast cancer. Br J Cancer 104(12):1828–1835
184. Drappatz J, Lee EQ, Hammond S, Grimm SA, Norden AD, Beroukhim R, Gerard M, Schiff D, Chi AS, Batchelor TT, Doherty LM, Ciampa AS, Lafrankie DC, Ruland S, Snodgrass SM, Raizer JJ, Wen PY (2012) Phase I study of panobinostat in combination with bevacizumab for recurrent high-grade glioma. J Neurooncol 107(1):133–138
185. Candelaria M, Herrera A, Labardini J, Gonzalez-Fierro A, Trejo-Becerril C, Taja-Chayeb L, Perez-Cardenas E, de la Cruz-Hernandez E, Arias-Bofill D, Vidal S, Cervera E, Duenas-Gonzalez A (2011) Hydralazine and magnesium valproate as epigenetic treatment for myelodysplastic syndrome. Preliminary results of a phase-II trial. Ann Hematol 90(4):379–387
186. Braiteh F, Soriano AO, Garcia-Manero G, Hong D, Johnson MM, Silva Lde P, Yang H, Alexander S, Wolff J, Kurzrock R (2008) Phase I study of epigenetic modulation with 5-azacytidine and valproic acid in patients with advanced cancers. Clin Cancer Res 14(19):6296–6301
187. Stathis A, Hotte SJ, Chen EX, Hirte HW, Oza AM, Moretto P, Webster S, Laughlin A, Stayner LA, McGill S, Wang L, Zhang WJ, Espinoza-Delgado I, Holleran JL, Egorin MJ, Siu LL (2011) Phase I study of decitabine in combination with vorinostat in patients with advanced solid tumors and non-Hodgkin's lymphomas. Clin Cancer Res 17(6):1582–1590
188. Juergens RA, Wrangle J, Vendetti FP, Murphy SC, Zhao M, Coleman B, Sebree R, Rodgers K, Hooker CM, Franco N, Lee BH, Tsai S, Delgado IE, Rudek MA, Belinsky SA, Herman JG, Baylin SB, Brock MV, Rudin CM (2011) Combination epigenetic therapy has efficacy in patients with refractory advanced non-small cell lung cancer. Cancer Discov 1:598–607
189. Candelaria M, Gallardo-Rincon D, Arce C, Cetina L, Aguilar-Ponce JL, Arrieta O, Gonzalez-Fierro A, Chavez-Blanco A, de la Cruz-Hernandez E, Camargo MF, Trejo-Becerril C, Perez-Cardenas E, Perez-Plasencia C, Taja-Chayeb L, Wegman-Ostrosky T, Revilla-Vazquez A, Duenas-Gonzalez A (2007) A phase II study of epigenetic therapy with hydralazine and magnesium valproate to overcome chemotherapy resistance in refractory solid tumors. Ann Oncol 18(9):1529–1538
190. Garcia-Manero G, Kantarjian HM, Sanchez-Gonzalez B, Yang H, Rosner G, Verstovsek S, Rytting M, Wierda WG, Ravandi F, Koller C, Xiao L, Faderl S, Estrov Z, Cortes J, O'Brien S, Estey E, Bueso-Ramos C, Fiorentino J, Jabbour E, Issa JP (2006) Phase 1/2 study of the combination of 5-aza-2′-deoxycytidine with valproic acid in patients with leukemia. Blood 108(10):3271–3279
191. Gollob JA, Sciambi CJ, Peterson BL, Richmond T, Thoreson M, Moran K, Dressman HK, Jelinek J, Issa JP (2006) Phase I trial of sequential low-dose 5-aza-2′-deoxycytidine plus

high-dose intravenous bolus interleukin-2 in patients with melanoma or renal cell carcinoma. Clin Cancer Res 12(15):4619–4627
192. Simpson AJ, Caballero OL, Jungbluth A, Chen YT, Old LJ (2005) Cancer/testis antigens, gametogenesis and cancer. Nat Rev Cancer 5(8):615–625
193. Karpf AR (2006) A potential role for epigenetic modulatory drugs in the enhancement of cancer/germ-line antigen vaccine efficacy. Epigenetics 1(3):116–120
194. Tan J, Yang X, Zhuang L, Jiang X, Chen W, Lee PL, Karuturi RK, Tan PB, Liu ET, Yu Q (2007) Pharmacologic disruption of Polycomb-repressive complex 2-mediated gene repression selectively induces apoptosis in cancer cells. Genes Dev 21(9):1050–1063
195. Yao Y, Chen P, Diao J, Cheng G, Deng L, Anglin JL, Prasad BV, Song Y (2011) Selective inhibitors of histone methyltransferase DOT1L: design, synthesis, and crystallographic studies. J Am Chem Soc 133(42):16746–16749
196. Daigle SR, Olhava EJ, Therkelsen CA, Majer CR, Sneeringer CJ, Song J, Johnston LD, Scott MP, Smith JJ, Xiao Y, Jin L, Kuntz KW, Chesworth R, Moyer MP, Bernt KM, Tseng JC, Kung AL, Armstrong SA, Copeland RA, Richon VM, Pollock RM (2011) Selective killing of mixed lineage leukemia cells by a potent small-molecule DOT1L inhibitor. Cancer Cell 20(1):53–65
197. Wang C, Liu Z, Woo CW, Li Z, Wang L, Wei JS, Marquez VE, Bates SE, Jin Q, Khan J, Ge K, Thiele CJ (2012) EZH2 mediates epigenetic silencing of neuroblastoma suppressor genes CASZ1, CLU, RUNX3 and NGFR. Cancer Res 72(1):315–324
198. Zhou J, Bi C, Cheong LL, Mahara S, Liu SC, Tay KG, Koh TL, Yu Q, Chng WJ (2011) The histone methyltransferase inhibitor, DZNep, up-regulates TXNIP, increases ROS production, and targets leukemia cells in AML. Blood 118(10):2830–2839
199. Crea F, Hurt EM, Mathews LA, Cabarcas SM, Sun L, Marquez VE, Danesi R, Farrar WL (2011) Pharmacologic disruption of Polycomb Repressive Complex 2 inhibits tumorigenicity and tumor progression in prostate cancer. Mol Cancer 10:40
200. Fiskus W, Wang Y, Sreekumar A, Buckley KM, Shi H, Jillella A, Ustun C, Rao R, Fernandez P, Chen J, Balusu R, Koul S, Atadja P, Marquez VE, Bhalla KN (2009) Combined epigenetic therapy with the histone methyltransferase EZH2 inhibitor 3-deazaneplanocin A and the histone deacetylase inhibitor panobinostat against human AML cells. Blood 114(13):2733–2743
201. Hayden A, Johnson PW, Packham G, Crabb SJ (2011) S-adenosylhomocysteine hydrolase inhibition by 3-deazaneplanocin A analogues induces anti-cancer effects in breast cancer cell lines and synergy with both histone deacetylase and HER2 inhibition. Breast Cancer Res Treat 127(1):109–119
202. Suva ML, Riggi N, Janiszewska M, Radovanovic I, Provero P, Stehle JC, Baumer K, Le Bitoux MA, Marino D, Cironi L, Marquez VE, Clement V, Stamenkovic I (2009) EZH2 is essential for glioblastoma cancer stem cell maintenance. Cancer Res 69(24):9211–9218
203. Quinn AM, Allali-Hassani A, Vedadi M, Simeonov A (2010) A chemiluminescence-based method for identification of histone lysine methyltransferase inhibitors. Mol Biosyst 6(5):782–788
204. King ON, Li XS, Sakurai M, Kawamura A, Rose NR, Ng SS, Quinn AM, Rai G, Mott BT, Beswick P, Klose RJ, Oppermann U, Jadhav A, Heightman TD, Maloney DJ, Schofield CJ, Simeonov A (2010) Quantitative high-throughput screening identifies 8-hydroxyquinolines as cell-active histone demethylase inhibitors. PLoS One 5(11):e15535
205. Tang W, Luo T, Greenberg EF, Bradner JE, Schreiber SL (2011) Discovery of histone deacetylase 8 selective inhibitors. Bioorg Med Chem Lett 21(9):2601–2605
206. Vedadi M, Barsyte-Lovejoy D, Liu F, Rival-Gervier S, Allali-Hassani A, Labrie V, Wigle TJ, Dimaggio PA, Wasney GA, Siarheyeva A, Dong A, Tempel W, Wang SC, Chen X, Chau I, Mangano TJ, Huang XP, Simpson CD, Pattenden SG, Norris JL, Kireev DB, Tripathy A, Edwards A, Roth BL, Janzen WP, Garcia BA, Petronis A, Ellis J, Brown PJ, Frye SV, Arrowsmith CH, Jin J (2011) A chemical probe selectively inhibits G9a and GLP methyltransferase activity in cells. Nat Chem Biol 7(8):566–574
207. Cole PA (2008) Chemical probes for histone-modifying enzymes. Nat Chem Biol 4(10): 590–597

208. Kota J, Chivukula RR, O'Donnell KA, Wentzel EA, Montgomery CL, Hwang HW, Chang TC, Vivekanandan P, Torbenson M, Clark KR, Mendell JR, Mendell JT (2009) Therapeutic microRNA delivery suppresses tumorigenesis in a murine liver cancer model. Cell 137(6):1005–1017
209. Ibrahim AF, Weirauch U, Thomas M, Grunweller A, Hartmann RK, Aigner A (2011) MicroRNA replacement therapy for miR-145 and miR-33a is efficacious in a model of colon carcinoma. Cancer Res 71(15):5214–5224
210. Taulli R, Bersani F, Foglizzo V, Linari A, Vigna E, Ladanyi M, Tuschl T, Ponzetto C (2009) The muscle-specific microRNA miR-206 blocks human rhabdomyosarcoma growth in xenotransplanted mice by promoting myogenic differentiation. J Clin Invest 119(8):2366–2378
211. Avramis VI, Mecum RA, Nyce J, Steele DA, Holcenberg JS (1989) Pharmacodynamic and DNA methylation studies of high-dose 1-beta-D-arabinofuranosyl cytosine before and after in vivo 5-azacytidine treatment in pediatric patients with refractory acute lymphocytic leukemia. Cancer Chemother Pharmacol 24(4):203–210
212. Belinsky SA, Klinge DM, Stidley CA, Issa JP, Herman JG, March TH, Baylin SB (2003) Inhibition of DNA methylation and histone deacetylation prevents murine lung cancer. Cancer Res 63(21):7089–7093
213. Goldberg J, Gryn J, Raza A, Bennett J, Browman G, Bryant J, Grunwald H, Larson R, Vogler R, Preisler H (1993) Mitoxantrone and 5-azacytidine for refractory/relapsed ANLL or CML in blast crisis: a leukemia intergroup study. Am J Hematol 43(4):286–290
214. Hakami N, Look AT, Steuber PC, Krischer J, Castleberry R, Harris R, Ravindranath Y, Vietti TJ (1987) Combined etoposide and 5-azacitidine in children and adolescents with refractory or relapsed acute nonlymphocytic leukemia: a Pediatric Oncology Group Study. J Clin Oncol 5(7):1022–1025
215. Huang Y, Nayak S, Jankowitz R, Davidson NE, Oesterreich S (2011) Epigenetics in breast cancer: what's new? Breast Cancer Res 13(6):225
216. Kim MS, Blake M, Baek JH, Kohlhagen G, Pommier Y, Carrier F (2003) Inhibition of histone deacetylase increases cytotoxicity to anticancer drugs targeting DNA. Cancer Res 63(21):7291–7300
217. Leshin M (1985) 5-Azacytidine and sodium butyrate induce expression of aromatase in fibroblasts from chickens carrying the henny feathering trait but not from wild-type chickens. Proc Natl Acad Sci USA 82(9):3005–3009
218. Liu WH, Yung BY (1998) Mortalization of human promyelocytic leukemia HL-60 cells to be more susceptible to sodium butyrate-induced apoptosis and inhibition of telomerase activity by down-regulation of nucleophosmin/B23. Oncogene 17(23):3055–3064
219. Momparler RL, Bouffard DY, Momparler LF, Dionne J, Belanger K, Ayoub J (1997) Pilot phase I-II study on 5-aza-2′-deoxycytidine (Decitabine) in patients with metastatic lung cancer. Anticancer Drugs 8(4):358–368
220. Pollyea DA, Kohrt HE, Gallegos L, Figueroa ME, Abdel-Wahab O, Zhang B, Bhattacharya S, Zehnder J, Liedtke M, Gotlib JR, Coutre S, Berube C, Melnick A, Levine R, Mitchell BS, Medeiros BC (2012) Safety, efficacy and biological predictors of response to sequential azacitidine and lenalidomide for elderly patients with acute myeloid leukemia. Leukemia 26(5):893–901
221. Schwartsmann G, Fernandes MS, Schaan MD, Moschen M, Gerhardt LM, Di Leone L, Loitzembauer B, Kalakun L (1997) Decitabine (5-Aza-2′-deoxycytidine; DAC) plus daunorubicin as a first line treatment in patients with acute myeloid leukemia: preliminary observations. Leukemia 11(suppl 1):S28–S31
222. Willemze R, Archimbaud E, Muus P (1993) Preliminary results with 5-aza-2′-deoxycytidine (DAC)-containing chemotherapy in patients with relapsed or refractory acute leukemia. The EORTC Leukemia Cooperative Group. Leukemia 7(suppl 1):49–50

Chapter 15
Methods for Cancer Epigenome Analysis

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Abstract Accurate detection of epimutations in tumor cells is crucial for understanding the molecular pathogenesis of cancer. Alterations in DNA methylation in cancer are functionally important and clinically relevant, but even this well-studied area is continually re-evaluated in light of unanticipated results, such as the strong association between aberrant DNA methylation in adult tumors and polycomb group profiles in embryonic stem cells, cancer-associated genetic mutations in epigenetic regulators such as *DNMT3A* and *TET* family genes, and the discovery of altered 5-hydroxymethylcytosine, a product of TET proteins acting on 5-methylcytosine, in human tumors with TET mutations. The abundance and distribution of covalent histone modifications in primary cancer tissues relative to normal cells is an important but largely uncharted area, although there is good evidence for a mechanistic role of cancer-specific alterations in histone modifications in tumor etiology, drug response, and tumor progression. Meanwhile, the discovery of new epigenetic marks continues, and there are many useful methods for epigenome analysis applicable to primary tumor samples, in addition to cancer cell lines. For DNA methylation and hydroxymethylation, next-generation sequencing allows increasingly inexpensive and quantitative whole-genome profiling. Similarly, the refinement and maturation of chromatin immunoprecipitation with next-generation sequencing (ChIP-seq) has made possible genome-wide mapping of histone modifications, open chromatin, and transcription factor binding sites. Computational tools have been developed apace with these epigenome methods to better enable accurate interpretation of the profiling data.

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Abbreviations

5MC	5-methylcytosine
5HMC	5-hydroxymethylcytosine
ChIP-seq	Chromatin immunoprecipitation-sequencing
MBD	Methyl binding domain
MeDIP	Methyl DNA immunoprecipitation
MRE	Methyl-sensitive restriction enzyme
RRBS	Reduced representation bisulfite sequencing

15.1 Introduction

DNA methylation is required for genome function through its roles in maintenance of chromatin structure, chromosome stability, and transcription [1–4]. 5-methylcytosine (5MC) is found at a subset of 5′-CpG-3′ dinucleotides and is also sometimes observed at CpNpG, notably in embryonic stem cells [5–7] but also in adult tissues [8]. The modified DNA base 5-hydroxymethylcytosine (5HMC) is also present in mammalian genomes, albeit at a much lower levels compared to 5MC [9, 10]. TET proteins catalyze the hydroxylation of 5MC to generate 5HMC, and can act further on 5HMC to yield 5-formylcytosine and carboxylcytosine [10–12].

The N-terminal tails of histone proteins are modified by acetylation, methylation, phosphorylation, ubiquitylation, crotonylation [13], and other covalent modifications. At some histone residues, such as histone H3 lysine 4 (H3K4), methylation can be mono-, di-, or tri-methyl. Furthermore, multiple types of modifications can exist on a single histone molecule. In addition to DNA methylation and histone modifications, there are other interrelated, potentially epigenetic mechanisms including specific deposition of histone variants, noncoding RNAs, chromatin remodeling, and nuclear organization, which are not discussed here. Current epigenomic methods, especially those making use of next-generation sequencing, provide powerful tools to map 5MC, 5HMC, and histone modifications at high resolution across the genome. However, there are many considerations for selecting the most suitable method, including ease of use, cost, resolution, specificity, quantitation, and availability of computational methods to analyze the data. We describe current epigenomic methods below, focusing primarily on genome-scale mapping methods that use next-generation sequencing.

15.2 Methods for Measurement of DNA Methylation and Hydroxymethylation

There are three main approaches to detect 5MC and 5HMC. Methyl-sensitive restriction enzymes (MRE) cut DNA based on methylation status of cytosines within their recognition sequences (Fig. 15.1a). A second approach includes

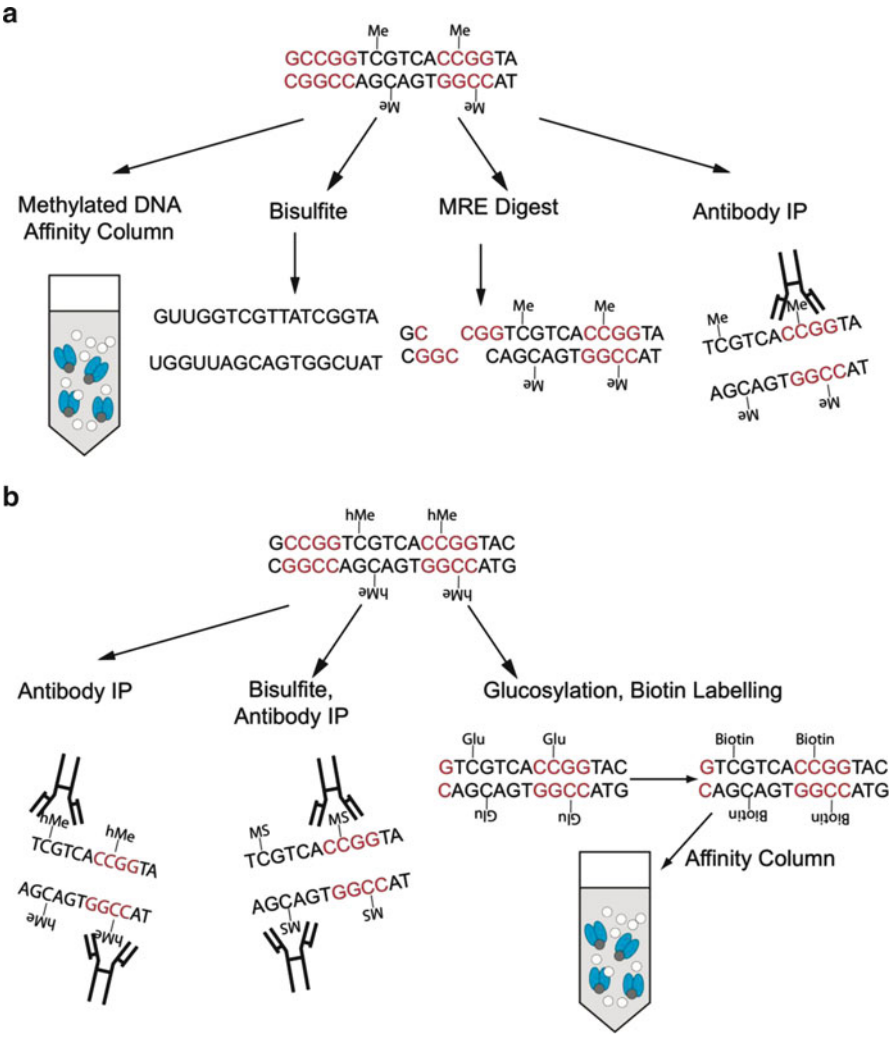

Fig. 15.1 A summary of methods for direct detection of cytosine methylation and hydroxymethylation. (**a**) Methylated DNA can be detected with methyl-sensitive restriction enzymes (MRE), the use of antibodies specific for 5-methylcytosine (5MC), by binding to affinity columns that contain methylated DNA binding domains or by the conversion of DNA with sodium bisulfite. It is important to note that some MRE are also sensitive to hydroxymethylation. (**b**) Several methods have been developed to detect 5 hydroxymethylcytosine (5HMC). These include the addition of a biotin tag to 5HMC through glucosylation and subsequent chemical steps which is followed by an affinity pulldown of the biotin tag, the use of antibodies specific for 5HMC and conversion of 5HMC to 5-cytosine methylenesulfonate (MS) which is then immunoprecipitated with an antibody specific to 5CMS. *Me* methylated cytosine; *hMe* hydroxymethylated cytosine; *Glu* glucosylated cytosine

differential chemical conversion or enzymatic modification of cytosine according to methylation/hydroxymethylation status, such as sodium bisulfite conversion and 5HMC-specific glucosylation. Third, enrichment methods include methyl DNA immunoprecipitation (MeDIP), hydroxyMeDIP (hMeDIP), and methyl binding domain (MBD) affinity purification that are used to enrich for methylated or hydroxymethylated regions. These approaches can be applied to investigate a single locus, hundreds of thousands of loci, or to all mappable sites genome-wide.

15.2.1 Overview of DNA Methylation Reagents

MRE have been used widely for precise, reliable, and inexpensive methylation detection. MRE only assay CpGs within their recognition sites but when multiple non-redundant and frequent-cutting MRE are used in parallel, this limitation is less problematic. There are approximately 50 unique MRE, though only a few have a methylation-insensitive isoschizomer. MRE can resolve the methylation status regionally or at individual CpGs, depending on the platform used following MRE digestion. Some MRE are inhibited by methylation or hydroxymethylation, for example, *Hpa*II [10]. The reliability of MRE enables their straightforward application to next-generation sequencing (MRE-seq) allowing analysis of greater than one million CpGs.

Antibodies against 5MC and 5HMC, and columns containing methylated DNA-binding proteins (domains of MBD2 or MeCP2 alone, or MBD2b combination with MBD3L) allow enrichment for 5MC/5HMC independent of DNA sequence (Fig. 15.1a, b) [14–17]. Enrichment is greater for regions with higher methylated CpG content relative to fully methylated regions with lower CpG content. These reagents are simple to use and many are commercially available. The lower-limit of resolution is determined initially by the size range of DNA prior to enrichment, generally 100–300 bp, and subsequently by the platform used to assess the enrichment, commonly oligonucleotide arrays and next-generation sequencing.

Chemicals including sodium bisulfite and hydrazine react differentially with unmethylated vs. methylated cytosine and allow DNA methylation mapping at single base resolution (Fig. 15.1a) [18–20]. Of these, sodium bisulfite is the most commonly used as it results in a positive display of methylation, among other advantages. Sodium bisulfite initiates conversion of cytosine to uracil, which is replaced by thymine during PCR amplification. In contrast, methylated cytosines are non-reactive, and remain as cytosine after bisulfite treatment. Sequencing of individual clones of the PCR product allows assessment of methylation status of contiguous CpGs derived from a single genomic DNA fragment. Bisulfite has many advantages, including single CpG resolution, detection of strand and allele-specific methylation, and detection of non-CpG cytosine methylation. Unlike other methylation-detection reagents, bisulfite provides estimates of absolute rather than relative DNA methylation levels, depending on the platform used. The reduced sequence complexity of the genome following bisulfite treatment complicates its

application to oligonucleotide arrays [21], but is not a major issue when a sequencing platform is used. Hydroxymethylated cytosines are resistant to conversion to uracil and are indistinguishable from 5MC in bisulfite sequencing. The reaction of 5HMC with bisulfite yields cytosine methylenesulfonate, which can be specifically detected with an affinity method [22]. Alternatively, the hydroxyl group of 5HMC can be enzymatically glucosylated and biotin labeled to detect 5HMC [22, 23].

15.2.2 Methyl-Sensitive Restriction Enzyme Methods

The HTF (*Hpa*II tiny fragments) enrichment by ligation-mediated PCR, or HELP assay, uses the methyl-sensitive *Hpa*II along with its methylation-insensitive isoschizomer *Msp*I to identify unmethylated CpG sites within the sequence 5′-CCGG-3′ [24]. Genomic DNA digested separately with each enzyme is size-selected to capture small DNA fragments. Custom adaptors complementary to digest ends are ligated and the adaptor-ligated molecules are amplified by PCR. The amplification products can be analyzed using a variety of platforms, including next-generation sequencing on the Illumina platform (HELP-seq) [25]. Methyl-seq is a second Illumina sequencing-based assay that uses *Hpa*II/*Msp*I [26]. Similar to HELP, the protocol involves separate *Hpa*II and *Msp*I digests, adaptor ligation, and Illumina sequencing. Approximately 65% of the CpG islands (CGIs) in the human genome are sampled using Methyl-seq. MRE methods are generally biased to CGIs, which constitute 1–2% of the genome and 7% of all CpGs in the genome. Methyl-seq is similarly biased, though non-CGI sites account for ~61% of the regions assayed, including a variety of genomic sequences such as promoters, exons, introns, and intergenic regions.

Ball et al. reported a third variation of MRE-seq, using *Hpa*II/*Msp*I digestion with Illumina sequencing to analyze DNA methylation in the PGP1 EBV-transformed B-lymphocyte cell line [27]. This approach, termed methyl-sensitive cut counting (MSCC), assayed ~1.4 million unique *Hpa*II sites. Using MSCC and a complementary method, bisulfite padlock probe sequencing (BSPP) to assay the methylation status of approximately 10,000 CpGs, highly expressed genes were found to be associated with high gene-body methylation and low promoter methylation. MSCC read counts were linearly related to BSPP percent methylation at 381 CpG sites that were assayed with both methods, suggesting that MSCC allows relative quantification of methylation levels.

DNA methylation has also been assessed through traditional Sanger sequencing combined with MRE in digital karyotyping [28, 29]. Using a combination of MRE that recognize 6–8 bp sites and methylation insensitive restriction enzymes, a library of short sequence tags is generated. The number of tags sequenced reflects the level of methylation at each recognition site, with lower tag counts representing greater methylation levels. In this method, the number of sites analyzed depends on the MRE used—use of *Asc*I, for example, can generate over 5,000 unique tags that correspond to >4,000 genes.

These sequencing-based methods demonstrate the utility of MRE for analysis of DNA methylation. The single CpG resolution and ability to assay a significant portion of the methylome with next-generation sequencing, including most CGIs, makes this a powerful, accurate, and straightforward way to assess methylation across the genome. When used alone, the MRE-seq methods enable relative rather than absolute methylation levels to be estimated. An integrative method [30, 31] that combines MRE-seq in parallel with MeDIP-seq to increase resolution, CpG coverage, and accuracy in quantitation is discussed below.

15.2.3 *Mcr*BC and CHARM

The methylation-dependent restriction enzyme *Mcr*BC recognizes methylated DNA and cuts near its recognition sequence. *Mcr*BC recognizes $R^mC(N)_{55–103}R^mC$ and cuts once between each pair of half-sites, close to one half-site or the other. The cuts can be distributed over several base pairs and approximately 30 base pairs distant from the methylated base, generating a distribution of DNA ends rather than precisely defined DNA ends. *Mcr*BC is useful to size-separate methylated DNA from unmethylated DNA, since the unmethylated DNA remains high-molecular weight after digestion. *Mcr*BC was initially applied to microarrays [32].

The "comprehensive high-throughput arrays for relative methylation" (CHARM) method is an array-based technique for methylation profiling using *Mcr*BC [33]. To improve specificity and sensitivity, probes were optimized based on location and CpG density on custom arrays. Because neighboring CpG sites tend to have a highly correlated methylation status, neighboring probe signals are averaged to reduce background noise without loss of sensitivity or specificity, though modestly reducing resolution. By comparing CHARM to MeDIP or *Hpa*II on arrays, Irizarry et al. showed that *Mcr*BC yields better methylome coverage than *Hpa*II and less bias for CpG density than MeDIP. Using CHARM, aberrant DNA methylation was found in colon cancer at sequences up to 2 kb flanking CGIs, referred to as CGI shores [34]. These data demonstrate the utility of *Mcr*BC-based methylation detection, and the new biological insights afforded by the CHARM method.

15.2.4 Methyl DNA Immunoprecipitation

In addition to MRE and *Mcr*BC, methylation can be assessed by immunoprecipitation of methylated DNA with a monoclonal antibody against 5-methylcytidine (MeDIP) [14]. This antibody does not recognize 5HMC [35], which can be specifically immunoprecipitated with an anti-5HMC antibody [36–39]. A major advantage of MeDIP-based detection is that it is not limited to a specific restriction site and theoretically any fragment with a methylated cytosine is immunoprecipitated. One approach involves the coupling of MeDIP with DNA

microarrays to obtain relative methylation levels at the loci represented on the array [14, 40–44].

MeDIP combined with next-generation sequencing (MeDIP-seq) can be used to interrogate the majority of mappable CpG and non-CpG cytosines in the genome. In a step forward from array-based methods, MeDIP-seq allows analysis of monoallelic methylation and methylation in a significant number of repeat sequences. Most protocols generate a MeDIP sequencing library by sonicating DNA followed by end-repair, adaptor ligation, immunoprecipitation with the anti-methylcytidine antibody and PCR amplification. The methylation-enriched library is sequenced and the reads are mapped back to a reference genome. A specific genomic region shows higher read density when methylated in one sample compared to when the same region is unmethylated in another sample, although read density between different regions is affected by the density of methylated CpGs, DNA copy number, and potentially other factors (discussed in Robinson et al. [45, 46]). These considerations are also important for MBD affinity-based approaches. MeDIP-seq has been applied to a variety of sample types from multiple organisms including human cancer [30, 31, 47–53].

Several computational methods have been specifically designed for analyzing MeDIP data while addressing local density of methylated CpGs. MEDME (modeling experimental data with MeDIP enrichment) is a combination of analytical and experimental methodologies that improve the interpretation of MeDIP-chip data, and addresses the non-linear relationship between enrichment signal and CpG density that is particular to MeDIP-chip [54]. A second analytical method for MeDIP-chip and also MeDIP–seq data called Bayesian tool for methylation analysis (BATMAN) uses a CpG density-derived coupling factor to quantify methylation levels across a range of CpG densities [47]. MEDIPS is a third approach that, like BATMAN, uses a CpG density coupling factor and in addition provides a framework for evaluating quality control parameters, estimating absolute methylation and comparing samples to detect regions of statistically significant differential methylation [51]. MeDIP-chip and MeDIP-seq are lower resolution compared to bisulfite-based methods. On the other hand, MeDIP-seq provides comprehensive methylome coverage at a fraction of the cost of shotgun bisulfite sequencing. Experimental and computational advances should enable increased resolution and quantitation of methylation levels using MeDIP-seq alone or in combination with MRE-seq.

15.2.5 Affinity-Based Enrichment Using Methyl Binding Domains

The Methylated CpG Island Recovery Assay (MIRA) is an alternative to MeDIP for selecting/enriching for methylated DNA, particularly at CGIs [15–17]. MIRA involves size fractionation of DNA, either by sonication or with *Mse*I which recognizes 5′-TTAA, a site that is typically found outside of CGIs. After digestion, adaptors are ligated to the DNA followed by selective binding of methylated fragments

on a column with full-length MBD2b and MBD3L1 proteins. MBD2b is a methyl-binding protein that exhibits a high affinity for methylated DNA relative to unmethylated DNA [15]. MBD3L1 lacks a methyl-CpG binding domain but can interact with MBD2b and improves enrichment of methylated DNA [15]. The methylated DNA eluted from the column is amplified by PCR, fluorescently labeled and hybridized to a microarray.

There are several similar approaches that combine affinity enrichment with Illumina sequencing. In MethylCap-seq, the MBD of MeCP2 is used to capture methylated DNA fragments after sonication [52, 55]. Binding occurs at low salt concentration and then a step-wise elution of captured DNA is performed by increasing the salt concentration, allowing collection of fractions with differing methylated CpG density, with highly methylated, CpG-dense fragments eluting at the higher salt concentrations. The eluates can be sequenced separately or pooled. The MBD2 MBD alone can be used for enrichment followed by Illumina sequencing, called MBD-isolated Genome Sequencing (MiGS) [56]. In this protocol, a single elution is performed. MBD2 enrichment with serial elution in increasing salt has been called MBD-seq [31, 57] or MBDCap-seq [45].

Several studies have directly compared MeDIP-seq with MBD affinity-based sequencing. Harris et al. found that MeDIP-seq and MBD-seq were 99% concordant using binary methylation calls in 200 bp windows or 1,000 bp windows [31]. MeDIP-seq enriched more at regions of low methylated CpG density compared to MBD-seq. Also, MeDIP-seq appeared to detect non-CpG methylation (i.e., at CpNpG) but MBD-seq did not, as predicted. Bock et al. compared MeDIP-seq with MethylCap-seq and observed similar levels of accuracy in quantifying methylation when comparing each to Infinium 27 K data. In both of these studies, MeDIP-seq and MBD affinity-based sequencing performed well in comparison with bisulfite next-generation sequencing.

15.2.6 Integrative MeDIP- and MRE-seq

MeDIP-seq and other affinity-based methods provide a positive display of methylated loci, and the absence of signal usually represents unmethylated loci, but also could be a result of regions that are difficult to PCR amplify or sequence, or insufficient sequencing depth. A method that combines MeDIP-seq with MRE-seq leverages their complementarity [30, 31, 58]. Independent MeDIP-seq and MRE-seq libraries are generated from the same DNA sample and sequenced separately. For MRE-seq, three to five parallel digests are performed using the MRE *Hpa*II, *Aci*I, *Hin*61, *Bsh*1236I, and *Hpy*CH4IV; the digests are size-selected and combined into a single library. Because the restriction sites from these enzymes are non-overlapping, each additional enzyme greatly increases coverage of unique CpG sites. At a moderate sequencing depth integrated MeDIP- and 3 enzyme MRE-seq together interrogate either uniquely or as multimapping sites ~22 million of the ~29 million

CpGs in the haploid human genome [31]. The integrative method is useful for detecting intermediate methylation, including regions of allelic methylation that overlap with monoallelic histone modifications and monoallelic gene expression [31]. This illustrates another significant advantage of sequencing-based epigenome analyses—the ability to assign an epigenetic state to a given genetic allele. For extensive DNA methylation profiles of human cells and tissues, see http://vizhub.wustl.edu/.

15.2.7 Indirect Methylation Detection with Demethylating Agents and Expression Arrays

Genetic or chemical inhibition of DNA methylation followed by expression array analysis can identify genes that may have been silenced by DNA methylation [59–63]. siRNA or shRNA can be used to knock down the DNA methyltransferases, or cell lines can be treated with demethylating agents such as 5-aza-2′deoxycytidine (5-aza) alone, or 5-aza in combination with histone deacetylase inhibitors. 5-aza is a cytidine analog that is incorporated into DNA and covalently binds and inhibits DNA methyltransferase, resulting in passive demethylation. 5-aza treatment results in activation of genes that were silenced by DNA methylation, provided that the appropriate transcription factors are present. However, interpretation of this indirect assessment of methylation is complicated by the fact that genes lacking promoter methylation may also exhibit an increase in expression following 5-aza treatment [64]. Presumably this results from demethylation at other loci within the same gene or in genes upstream that are required for its expression, though direct effects on unmethylated regulatory elements cannot be ruled out. Furthermore, this approach is best applied to cells grown in culture such as cell lines or early passage primary cells [65], as 5-aza requires replication to induce passive demethylation. The application of this approach to cultured tumor cells is complicated by epigenetic silencing that results from long-term culturing, rather than cancer or cell type-specificity.

15.2.8 Reduced Representation Bisulfite Sequencing

Bisulfite treatment converts unmethylated cytosines to uracil but methylcytosine and hydroxymethylcytosine are resistant to conversion. When followed by cloning and Sanger sequencing, this approach yields quantitative, allelic, contiguous, and base resolution of cytosine methylation. However, the shotgun bisulfite approach has been quite expensive for mammalian methylomes. It is important to note that hydroxymethylcytosine and methylcytosine cannot be distinguished by bisulfite sequencing as both block conversion.

To retain the advantages of methylation detection by bisulfite while reducing the cost of shotgun bisulfite sequencing, Meissner et al. developed a technique that interrogates DNA fragments from a reduced representation of the bisulfite-treated genome [66–68]. The reduction comes from DNA digestion with methylation-insensitive restriction enzyme *Msp*I and fragment size selection. After digestion, the ends of the DNA are filled-in with dGTP and methylated dCTP, followed by the addition of an A overhang to enable adaptor ligation. The adaptors used for this assay are methylated at cytosine residues to prevent conversion during bisulfite treatment. The adaptor-ligated DNA is then size selected on a gel and two fractions are excised—the sizes of which depend on the organism. For mouse DNA, approximately 300,000 *Msp*I fragments that span 40–220 bp are analyzed, which corresponds to nearly 1.4 million CpG sites analyzed at the nucleotide level [67]. These fragments are then bisulfite treated, PCR amplified, and size selected again to generate a sequencing library. Several factors must be considered with this approach. First, the choice of a restriction enzyme to fractionate the DNA will bias the portion of the genome that is represented. A second consideration is the process of mapping reads of bisulfite-converted DNA to the genome. Several mapping algorithms for "bisulfite genomes" have been developed [67, 69–71]. Compared to other sequencing methods, reduced representation bisulfite sequencing (RRBS) provides an efficient way to generate absolute quantification of methylation of more than one million CpG sites at single base pair resolution. Methylation at non-CpG cytosines can also be assessed by RRBS [8]. RRBS has been successfully applied to nanogram quantities of genomic DNA [72] and to large numbers of human cell and tissue types (http://vizhub.wustl.edu/).

15.2.9 Shotgun Bisulfite Sequencing

Shotgun sequencing of bisulfite-treated DNA has been successfully applied to several organisms, including humans [7, 69, 70, 73–78] and provides comprehensive, single cytosine quantification of methylation level when sequence coverage is sufficiently deep. A single-CpG-resolution shotgun bisulfite experiment on human DNA requires hundreds of millions of sequencing reads, with the exact number varying depending on the desired sequencing depth and on read lengths [78]. Many regions >200 bp in the mammalian genome do not contain CpGs and thus a large number of sequence reads may be uninformative, at least for CpG methylation. Prior selection of sequences, for example, through sequence capture methodology, or enrichment of methylated DNA or unmethylated DNA followed by shotgun sequencing could increase the efficiency and decrease the cost of this approach. Bisulfite sequencing that first employs selective "reduction" of the genome (e.g., RRBS) is far less expensive. Nevertheless, the cost of sequencing full DNA methylomes has decreased 20-fold since the first human methylome [7]. Shotgun bisulfite methylomes have been generated for a breast cancer cell line and primary human

mammary epithelial cells [79] and primary colorectal cancer and adjacent normal colon tissue [80].

RRBS and shotgun bisulfite sequencing require algorithms that are tailored to mapping the sequence reads from bisulfite-treated DNA back onto the genome. Several algorithms have been developed for this computationally intensive problem [67, 69–71, 81, 82]. The reduction in base complexity from the bisulfite conversion and the fact that a CpG can be methylated or unmethylated are issues that are addressable though complex when aligning bisulfite reads. Due to the bisulfite conversion process, the forward and reverse strands of DNA are no longer complementary and the sequence reads therefore are aligned to four different bisulfite-converted genomes: forward BS, forward BS reverse complement, reverse BS, reverse BS reverse complement). Thus, for this mapping there is increased search space along with a reduction of sequence complexity, requiring significant computation time for the read mapping [31].

15.2.10 Other Bisulfite Methods

Illumina Infinium methylation assays are mid-range platforms using bisulfite conversion and bead arrays to quantify methylation levels at individual CpGs. The HumanMethylation27 and HumanMethylation450 formats interrogate 27,578 and >450,000 CpGs, respectively. Bead-bound oligonucleotides corresponding to the methylated and unmethylated states of a single CpG site are hybridized to bisulfite-converted DNA and differentially labeled with Cy3 or Cy5. The methylation level is determined by the ratio of Cy3 and Cy5 fluorescence on the bead array. The HumanMethylation27 BeadChip interrogates 12 samples at a time and includes probes from 1,000 cancer-related genes and from putative promoters of 110 miRNA, among others. While there are on average 2 CpG sites assayed per gene for the majority of genes, 150 genes known to exhibit aberrant tumor-specific methylation are assayed at 5–10 CpGs each. The vast majority of 27 K probes are located in promoters. The 450 K platform expands the genomic regions that are assayed by Infinium. Genes are broadly profiled, with probes in the promoter, 5′ UTR, first exon, gene body, and 3′ UTR. Ninety nine percent of CGIs have probes, and the CGI shores, 2 kb regions flanking CGIs, and regions flanking shores, called "shelves," are also examined for most CGIs. Like the 27 K assay, a single 450 BeadChip can assay 12 samples. Both versions require 500 ng of DNA prior to bisulfite conversion. These methods do not assess multiple closely apposed CpGs individually, and such regions are generally avoided in the assay development. This bias is likely to impact biological insights drawn from this data.

Another bisulfite-based method, the Sequenom EpiTyper assay, utilizes MALDI-TOF mass spectrometry to analyze RNA cleavage fragments derived from post-bisulfite PCR products that contain a promoter to drive transcription [83, 84]. This unique assay allows high-throughput quantitative methylation analysis at hundreds

of loci, usually at single CpG resolution, and is quite useful for candidate loci in hundreds of samples, or as a follow-up to genome-wide profiling.

BSPPs are molecular inversion probes designed to target and capture specific CpG sites from bisulfite-converted DNA [27, 85]. The strategy is similar to RRBS in that a subset of CpG sites are analyzed by bisulfite sequencing to reduce the genomic space that must be covered, but with the advantage that particular CpGs can be assayed, instead of only those located within a set of restriction fragments. Tens of thousands of BSPPs can be amplified in single reaction and sequenced on the Illumina platform. Deng et al. were able to assay ~66,000 CpG sites, primarily in CGIs [85]. A prominent advantage of this technology is that it is customizable and can target a specific set of CpG sites of interest to the investigator.

15.3 Detection of 5-Hydroxymethylcytosine

5HMC is abundant in mammalian genomes. The tissue-specificity, genomic distribution, and functional significance of 5HMC are under investigation. Pre-existing 5MC is hydroxylated by the TET family of dioxygenases (TET1, TET2, and TET3) to yield 5HMC [10, 86]. TET proteins can further modify 5HMC resulting in formylmethylcytosine, carboxymethylcytosine, and possibly through steps mediated by base excision repair, unmodified cytosine [11, 12]. *TET1* is an *MLL* translocation partner in acute myeloid leukemia [87, 88] and *TET2* mutations occur in myeloid malignancies associated with decreased 5HMC [89], suggesting that dysregulation of 5HMC plays a role in cancer.

Detecting and quantifying 5HMC is challenging because many reagents used for detecting 5MC do not distinguish 5HMC from 5MC. Like 5MC, 5HMC is resistant to C-to-U transition following bisulfite treatment [90], and these bases are indistinguishable by bisulfite cloning and sequencing or other bisulfite-based methods. In addition, 5HMC reacts with bisulfite to yield cytosine 5-methylenesulfonate (CMS) and DNA with dense CMS is inefficiently amplified during PCR due to *Taq* polymerase stalling at CMS sites [90]. As a result, quantification of hydroxymethylation in regions of dense 5HMC, if they exist in some biological contexts, may be underestimated with bisulfite-based methods. MRE-based methods also do not distinguish 5MC from 5HMC, depending on the enzymes used, such as *Hpa*II, which is inhibited by 5MC or 5HMC in its recognition sequence [10]. Finally, affinity-based 5MC methods (MeDIP-seq, MBD-seq, etc.) are specific to 5MC and do not detect 5HMC directly, but could indirectly enrich for regions with 5HMC when it occurs on the same DNA fragment as 5MC [35].

Global quantification of 5HMC levels (measuring the relative or absolute amount of 5HMC present within a DNA sample) can be assayed by thin layer chromatography (TLC) [9, 10] and high-performance liquid chromatography-mass spectrometry (HPLC-MS) [9, 91]. Recently, a profusion of 5HMC mapping techniques have also been developed, many of which can be employed for genome-wide analysis.

15.3.1 5HMC Glucosylation Methods

There are several methods based on in vitro glucosylation of 5HMC in DNA that can be used for global quantification or mapping of 5HMC. These methods use bacteriophage T4 beta-glucosyltransferase (BGT) to catalyze the addition of a glucose moiety to the hydroxyl group of 5HMC. For global quantification, a radiolabeled substrate (uridine 5′-diphosphate-[^{3}H]-glucose) is used in the BGT-catalyzed reaction. The amount of labeled substrate incorporated is compared to standards, allowing absolute quantification [92]. A mapping method called GLIB (glucosylation, periodate oxidation, biotinylation) combines glucosylation by BGT with subsequent chemical reactions, resulting in the addition of two biotin molecules to each 5HMC [22]. The biotin-tagged 5HMC DNA is then pulled down with streptavidin and sequenced on the Helicos single molecule platform. GLIB has high sensitivity, with 90% recovery of DNA fragments containing a single 5HMC molecule. Song et al. present a second mapping method, in which a chemically engineered glucose containing an azide group is transferred to 5HMC by BGT [23]. The azide group is then chemically tagged with biotin and affinity enriched, with global quantification performed using avidin-horseradish peroxidase and genome-wide mapping through Illumina sequencing. Finally, a method has been developed utilizing the restriction endonuclease *Msp*I, which cuts C^{m}CGG and C^{hm}CGG, but not C^{gluc}CGG sites. Locus-specific 5HMC can be estimated using *Msp*I digestion on BGT-modified DNA followed by quantitative PCR across the cleavage site [36, 93].

15.3.2 5HMC Affinity Enrichment Methods

There are two enrichment methods for 5HMC based on antibodies that detect 5HMC itself or 5-cytosine methylenesulfonate (CMS), the product of reacting 5HMC with sodium bisulfite. The 5HMC antibody with sequencing approach, hMeDIP-seq [36–39], is similar to MeDIP-seq, and informatic tools originally developed for MeDIP-seq data have been employed in hMeDIP-seq. Monoclonal and polyclonal anti-5HMC antibodies are commercially available, but their 5HMC-density dependence [22, 89], along with the relatively low genomic abundance of 5HMC in some tissues, might result in inefficient pulldown of 5HMC-sparse regions. The anti-CMS antibody sequencing approach was developed as a more sensitive, less density-dependent alternative to hMeDIP-seq [22]. CMS pulldown had lower background and decreased density dependence compared to commercial anti-5HMC antibodies. CMS-enriched libraries were sequenced on the Illumina platform. Since Illumina library construction protocols usually require at least one PCR step, the tendency of *Taq* polymerase to stall at regions of dense CMS could be problematic.

The rapid development of methods for the detection and quantification of 5HMC has paralleled the exciting pace of discovery of the distribution and potential

functional roles of this "sixth base." Computational tools that are specific for hMeDIP-seq and CMS-pulldown have not been reported yet. For hMeDIP-seq, tools developed for MeDIP-seq, such as MEDIPS [51] have been adapted [38]. Stroud et al. used SICER, which was originally developed for analyzing chromatin immunoprecipitation-sequencing (ChIP-seq) data for diffusely distributed histone modifications, to define regions of 5HMC enrichment [39]. The next generation of genome-wide mapping methods for 5HMC may involve direct detection of the modified base by single molecule sequencing [23, 94].

15.4 Chromatin Immunoprecipitation-Sequencing

Alterations in histone modification patterns and transcription factor binding impact gene expression and have been implicated in tumorigenesis, cancer cell stemness, metastasis, and drug resistance [95–98]. Chromatin immunoprecipitation coupled with next-generation sequencing (ChIP-seq) has become the gold standard to study histone modifications and transcription factor binding genome-wide. It provides higher resolution, improved signal-to-noise ratios, and when using indexed libraries, it is less expensive than coupling ChIP with microarrays (ChIP-chip) [99]. Fresh or fresh frozen tissue or cells are either kept native (N-ChIP) [100] or formaldehyde cross-linked to preserve weaker DNA–protein interactions (X-ChIP) [101], followed by cell lysis (Fig. 15.2). N-ChIP is primarily used for histone modifications, where the DNA histone interactions are inherently strong [99]. Antibody specificity and immunoprecipitation are more efficient with N-ChIP as epitopes can be disrupted by formaldehyde [100], however, N-ChIP cannot be applied to proteins with lower DNA binding affinities such as transcription factors. Cross-linking ameliorates this problem, and minimizes stochastic nucleosome movement that can occur during N-ChIP [100], however, it also may fix transient non-functional interactions and reacts at lysines which may create biases. Native or cross-linked chromatin is then fragmented by sonication or micrococcal nuclease (MNase) digestion. Both methods impart bias in downstream sequencing [102]. MNase creates higher resolution, primarily mononucleosome (~146 bp) fragments, but is less efficient at cutting between G and C bases, creating greater fragmentation bias [103, 104]. In contrast, sonication provides decreased resolution (200–600 bp) but is more uniform [99]. Fragmented chromatin is immunoprecipitated with an antibody that specifically recognizes the epitope of interest. The success of ChIP reactions is dependent on antibody quality. Polyclonal antibodies are advantageous for X-ChIP experiments, as they reduce the chance of cross-linking destroying antibody interactions [101], but may have increased cross-reactivity. Relative enrichment of ChIP DNA is assayed via qPCR. Enrichment varies greatly with the protein of interest, antibody quality, and positive and negative control regions of the genome that are used. To minimize the number of reads contributing to background noise, it is common to require greater enrichment in ChIP-seq (5–50-fold) when compared to single locus ChIP-PCR [102]. Purified ChIP DNA sequencing libraries are constructed by end

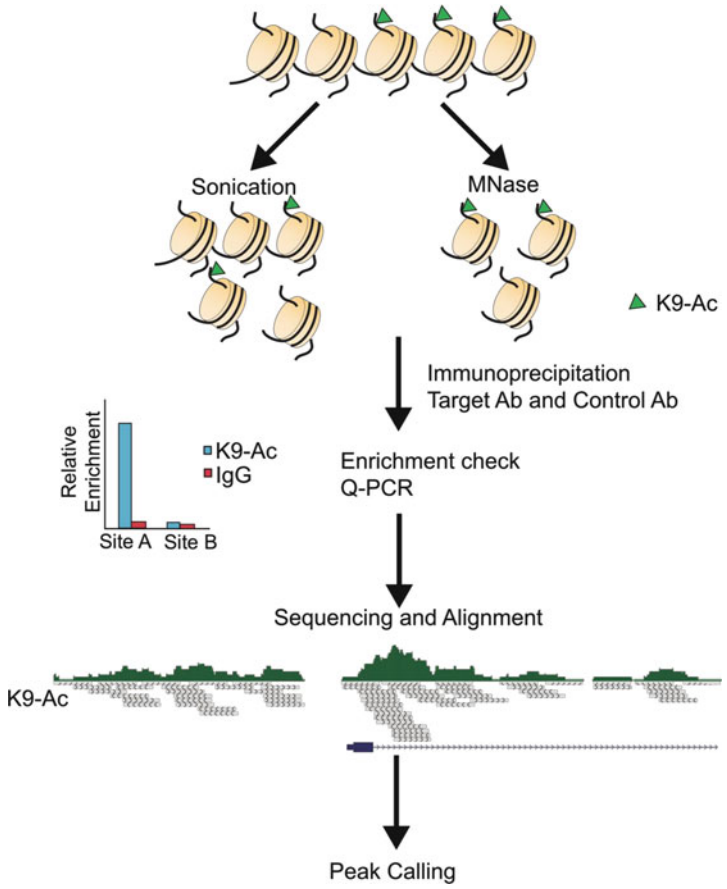

Fig. 15.2 Overview of chromatin immunoprecipitation-sequencing. DNA is fractionated via sonication (~200–600 bp) or with micrococcal nuclease (~146 bp). The fractionated DNA is then immunoprecipitated (IP) with a target antibody and an isotype control antibody. The efficiency of the immunoprecipitation is assayed by quantitative PCR, testing regions that are known to be bound (site A, positive control) or not bound (site B, negative control). The enriched DNA is then used to generate a DNA sequencing library, which is sequenced and reads are aligned to the appropriate genome. Each read is depicted as a *grey line*, the read densities are displayed above in *green* and a gene is shown in *blue*. Finally, the aligned reads are used to generate peaks that mark regions of statistically significant enrichment of reads for the IP of the histone mark or chromatin protein of interest

repair, A base addition, adapter ligation, PCR amplification and size selection. Additional bias may occur during library construction and PCR amplification, as both GC-rich and GC-poor regions are underrepresented [99, 102]. The total number of sequence reads required depends on the quality of ChIP enrichment, the expected number of peaks and peak size, but sequencing multiple-indexed ChIP libraries in a single lane is common practice.

15.4.1 ChIP-seq Data Analysis

Transforming the millions of sequencing reads generated by ChIP-seq into biologically interpretable data is a computationally demanding, multi-step process for which a variety of tools have been developed. While many tools address the same problem, each tool is different and can impact the final result. The first and most resource-intensive step is aligning the sequence reads to the genome. Most sequencing platforms come with alignment pipelines, however, third-party aligners are commonly used, such as MAQ [105], Bowtie [106], BWA [107], SOAP [108, 109], and PASH [110]. These packages differ by alignment algorithm, as well as how multi-aligning reads and gapped vs. un-gapped alignments are handled, resulting in differences in sensitivity and specificity. For most cancer samples a gapped aligner is preferred to allow for the variety of genetic aberrations accumulated in the tumor. Aligned reads are then analyzed to find enriched areas or "peaks" in the genome, for which a number of "peak calling" algorithms have been created [99, 111]. Though the exact method varies between programs, most shift tags based on chromatin fragment size to accumulate tags near the true binding site and increase peak resolution [111]. Regions of statistical enrichment of IP tags relative to a background control are calculated. The most commonly used control is input DNA isolated from the same chromatin batch as the ChIP [99]. This reduces false positives introduced from fragmentation and mappability biases, and controls for genetic differences such as copy number alterations that affect read density. Finally, peaks are filtered based on uneven distributions of sense and antisense tag accumulation [111]. Most current peak callers identify focal enrichments such as transcription factor binding sites, however, some have been developed for broader marks like histone modifications associated with heterochromatin [112–114]. Many groups are actively researching ways to reduce noise and increase true positives.

15.4.2 Application of ChIP-seq to Cancer Epigenomes

The network of transcription regulatory factor interactions and their effects on gene expression in cancer are under investigation. ChIP-seq was initially used to profile T-cells, and since then a main focus has been on embryonic stem cells and cell lines [115–117]. Recently, distinct chromatin states or "signatures" comprised of combinatorial histone marks have been linked to specific functional genomic elements by integrating multiple ChIP-seq data across human cell lines [118–120]. The combinatorial histone signatures identified in these studies have not been investigated in the context of tumor progression. Multidimensional epigenomic profiles of tumors also provide a novel means of sub-type classification, identifying prognostic markers, and insight into tumor cell of origin. ChIP-seq will also help the annotation and functional characterization of non-genic susceptibility loci, as has been recently performed in prostate cancer [121] and in GWAS studies [120]. New techniques are being developed to perform ChIP-seq on a small number of cells, creating an

opportunity to better analyze intratumoral heterogeneity of epigenomic patterns [122, 123]. Finally, chromosome conformation capture (3C) technology [124] and its high-throughput derivatives (4C [125], 5C [126], Hi-C [127], ChIP-Loop [128, 129], ChIA-PET [130]) detect distal DNA–DNA interactions (e.g., promoter-enhancer), but can also be used to identify complex genomic rearrangements in cancers [131]. Coupling ChIP with 3C technologies followed by sequencing will likely be a powerful way to study how both epigenetic patterns and associated structural interactions change during the process of tumorigenesis.

15.5 Future Directions

Recent unanticipated data offer new understanding of, and stimulate new investigations into aberrant epigenetic patterns in cancer. First, promoters with polycomb-mediated histone modifications in ES cells are among those commonly aberrantly hypermethylated in adult tumors [132–134]. Second, cancer-associated mutations occur in the DNA methyltransferase *DNMT3A* [135, 136], suggesting another possible origin of DNA methylation abnormalities, though this remains to be determined. Similarly, the occurrence of *TET1* translocation [87, 88] and *TET2* mutations in cancer points to an etiologic role for these epigenetic regulators and their marks. Finally, human tissues harbor abundant 5HMC, a product of TET proteins acting on 5MC, while cancers with TET mutations tend to have reduced 5HMC.

The future of cancer epigenomic methods will be shaped by two technological trends. First, the rapid pace of advances in next-generation sequencing will continue to improve 5MC/5HMC, histone modification, and chromatin conformation mapping. Genome-wide epigenomic experiments will become increasingly inexpensive and accessible, though paralleled with needs for increased computational power and data storage. Second, direct single molecule sequencing that distinguishes between modified bases without bisulfite conversion could revolutionize mapping of 5MC and 5HMC. For example, in single molecule real-time (SMRT) sequencing, fluorescently labeled nucleotides are incorporated by DNA polymerase on complementary DNA strands. Real-time monitoring of the kinetics of this process can identify both unmodified and modified bases, including N6-methyladenine, 5MC, and 5HMC [94]. SMRT sequencing has also been combined with selective glucosylation and cleavable biotin labeling of 5HMC to improve detection kinetics [23]. Similarly, the direct detection of modified bases via inexpensively produced nanopores, if they become amenable to high-throughput, could be technologically transformative [137].

References

1. Trasler JM, Trasler DG, Bestor TH, Li E, Ghibu F (1996) DNA methyltransferase in normal and Dnmtn/Dnmtn mouse embryos. Dev Dyn 206(3):239–247. doi:10.1002/(SICI)1097-0177(199607)206:3<239::AID-AJA2>3.0.CO;2-J [pii] 10.1002/(SICI)1097-0177(199607)206:3<239::AID-AJA2>3.0.CO;2-J

2. Chen RZ, Pettersson U, Beard C, Jackson-Grusby L, Jaenisch R (1998) DNA hypomethylation leads to elevated mutation rates. Nature 395(6697):89–93
3. Maraschio P, Zuffardi O, Dalla Fior T, Tiepolo L (1988) Immunodeficiency, centromeric heterochromatin instability of chromosomes 1, 9, and 16, and facial anomalies: the ICF syndrome. J Med Genet 25(3):173–180
4. Xu GL, Bestor TH, Bourc'his D, Hsieh CL, Tommerup N, Bugge M, Hulten M, Qu X, Russo JJ, Viegas-Pequignot E (1999) Chromosome instability and immunodeficiency syndrome caused by mutations in a DNA methyltransferase gene. Nature 402(6758):187–191. doi:10.1038/46052
5. Clark SJ, Harrison J, Frommer M (1995) CpNpG methylation in mammalian cells. Nat Genet 10(1):20–27. doi:10.1038/ng0595-20
6. Ramsahoye BH, Biniszkiewicz D, Lyko F, Clark V, Bird AP, Jaenisch R (2000) Non-CpG methylation is prevalent in embryonic stem cells and may be mediated by DNA methyltransferase 3a. Proc Natl Acad Sci USA 97(10):5237–5242. doi:97/10/5237 [pii]
7. Lister R, Pelizzola M, Dowen RH, Hawkins RD, Hon G, Tonti-Filippini J, Nery JR, Lee L, Ye Z, Ngo QM, Edsall L, Antosiewicz-Bourget J, Stewart R, Ruotti V, Millar AH, Thomson JA, Ren B, Ecker JR (2009) Human DNA methylomes at base resolution show widespread epigenomic differences. Nature 462(7271):315–322. doi:nature08514 [pii] 10.1038/nature08514
8. Ziller MJ, Muller F, Liao J, Zhang Y, Gu H, Bock C, Boyle P, Epstein CB, Bernstein BE, Lengauer T, Gnirke A, Meissner A (2011) Genomic distribution and inter-sample variation of non-CpG methylation across human cell types. PLoS Genet 7(12):e1002389. doi:10.1371/journal.pgen.1002389 PGENETICS-D-11-00694 [pii]
9. Kriaucionis S, Heintz N (2009) The nuclear DNA base 5-hydroxymethylcytosine is present in Purkinje neurons and the brain. Science 324(5929):929–930. doi:1169786 [pii] 10.1126/science.1169786
10. Tahiliani M, Koh KP, Shen Y, Pastor WA, Bandukwala H, Brudno Y, Agarwal S, Iyer LM, Liu DR, Aravind L, Rao A (2009) Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. Science 324(5929):930–935. doi:1170116 [pii] 10.1126/science.1170116
11. Ito S, Shen L, Dai Q, Wu SC, Collins LB, Swenberg JA, He C, Zhang Y (2011) Tet proteins can convert 5-methylcytosine to 5-formylcytosine and 5-carboxylcytosine. Science 333(6047):1300–1303. doi:science.1210597 [pii] 10.1126/science.1210597
12. He YF, Li BZ, Li Z, Liu P, Wang Y, Tang Q, Ding J, Jia Y, Chen Z, Li L, Sun Y, Li X, Dai Q, Song CX, Zhang K, He C, Xu GL (2011) Tet-mediated formation of 5-carboxylcytosine and its excision by TDG in mammalian DNA. Science 333(6047):1303–1307. doi:science.1210944 [pii] 10.1126/science.1210944
13. Tan M, Luo H, Lee S, Jin F, Yang JS, Montellier E, Buchou T, Cheng Z, Rousseaux S, Rajagopal N, Lu Z, Ye Z, Zhu Q, Wysocka J, Ye Y, Khochbin S, Ren B, Zhao Y (2011) Identification of 67 histone marks and histone lysine crotonylation as a new type of histone modification. Cell 146(6):1016–1028. doi:S0092-8674(11)00891-9 [pii] 10.1016/j.cell.2011.08.008
14. Weber M, Davies JJ, Wittig D, Oakeley EJ, Haase M, Lam WL, Schubeler D (2005) Chromosome-wide and promoter-specific analyses identify sites of differential DNA methylation in normal and transformed human cells. Nat Genet 37(8):853–862. doi:ng1598 [pii] 10.1038/ng1598
15. Rauch T, Li H, Wu X, Pfeifer GP (2006) MIRA-assisted microarray analysis, a new technology for the determination of DNA methylation patterns, identifies frequent methylation of homeodomain-containing genes in lung cancer cells. Cancer Res 66(16):7939–7947. doi:66/16/7939 [pii] 10.1158/0008-5472.CAN-06-1888
16. Rauch T, Wang Z, Zhang X, Zhong X, Wu X, Lau SK, Kernstine KH, Riggs AD, Pfeifer GP (2007) Homeobox gene methylation in lung cancer studied by genome-wide analysis with a microarray-based methylated CpG island recovery assay. Proc Natl Acad Sci USA 104(13):5527–5532. doi:0701059104 [pii] 10.1073/pnas.0701059104

17. Rauch TA, Wu X, Zhong X, Riggs AD, Pfeifer GP (2009) A human B cell methylome at 100-base pair resolution. Proc Natl Acad Sci USA 106(3):671–678. doi:0812399106 [pii] 10.1073/pnas.0812399106
18. Frommer M, McDonald LE, Millar DS, Collis CM, Watt F, Grigg GW, Molloy PL, Paul CL (1992) A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. Proc Natl Acad Sci USA 89(5):1827–1831
19. Pfeifer GP, Riggs AD (1996) Genomic sequencing by ligation-mediated PCR. Mol Biotechnol 5(3):281–288
20. Clark SJ, Statham A, Stirzaker C, Molloy PL, Frommer M (2006) DNA methylation: bisulphite modification and analysis. Nat Protoc 1(5):2353–2364. doi:nprot.2006.324 [pii] 10.1038/nprot.2006.324
21. Gitan RS, Shi H, Chen CM, Yan PS, Huang TH (2002) Methylation-specific oligonucleotide microarray: a new potential for high-throughput methylation analysis. Genome Res 12(1):158–164. doi:10.1101/gr.202801
22. Pastor WA, Pape UJ, Huang Y, Henderson HR, Lister R, Ko M, McLoughlin EM, Brudno Y, Mahapatra S, Kapranov P, Tahiliani M, Daley GQ, Liu XS, Ecker JR, Milos PM, Agarwal S, Rao A (2011) Genome-wide mapping of 5-hydroxymethylcytosine in embryonic stem cells. Nature 473(7347):394–397. doi:nature10102 [pii] 10.1038/nature10102
23. Song CX, Szulwach KE, Fu Y, Dai Q, Yi C, Li X, Li Y, Chen CH, Zhang W, Jian X, Wang J, Zhang L, Looney TJ, Zhang B, Godley LA, Hicks LM, Lahn BT, Jin P, He C (2011) Selective chemical labeling reveals the genome-wide distribution of 5-hydroxymethylcytosine. Nat Biotechnol 29(1):68–72. doi:nbt.1732 [pii] 10.1038/nbt.1732
24. Khulan B, Thompson RF, Ye K, Fazzari MJ, Suzuki M, Stasiek E, Figueroa ME, Glass JL, Chen Q, Montagna C, Hatchwell E, Selzer RR, Richmond TA, Green RD, Melnick A, Greally JM (2006) Comparative isoschizomer profiling of cytosine methylation: the HELP assay. Genome Res 16(8):1046–1055. doi:gr.5273806 [pii] 10.1101/gr.5273806
25. Oda M, Glass JL, Thompson RF, Mo Y, Olivier EN, Figueroa ME, Selzer RR, Richmond TA, Zhang X, Dannenberg L, Green RD, Melnick A, Hatchwell E, Bouhassira EE, Verma A, Suzuki M, Greally JM (2009) High-resolution genome-wide cytosine methylation profiling with simultaneous copy number analysis and optimization for limited cell numbers. Nucleic Acids Res 37(12):3829–3839. doi:gkp260 [pii] 10.1093/nar/gkp260
26. Brunner AL, Johnson DS, Kim SW, Valouev A, Reddy TE, Neff NF, Anton E, Medina C, Nguyen L, Chiao E, Oyolu CB, Schroth GP, Absher DM, Baker JC, Myers RM (2009) Distinct DNA methylation patterns characterize differentiated human embryonic stem cells and developing human fetal liver. Genome Res 19(6):1044–1056. doi:gr.088773.108 [pii] 10.1101/gr.088773.108
27. Ball MP, Li JB, Gao Y, Lee JH, LeProust EM, Park IH, Xie B, Daley GQ, Church GM (2009) Targeted and genome-scale strategies reveal gene-body methylation signatures in human cells. Nat Biotechnol 27(4):361–368. doi:nbt.1533 [pii] 10.1038/nbt.1533
28. Hu M, Yao J, Cai L, Bachman KE, van den Brule F, Velculescu V, Polyak K (2005) Distinct epigenetic changes in the stromal cells of breast cancers. Nat Genet 37(8):899–905. doi:ng1596 [pii] 10.1038/ng1596
29. Bloushtain-Qimron N, Yao J, Snyder EL, Shipitsin M, Campbell LL, Mani SA, Hu M, Chen H, Ustyansky V, Antosiewicz JE, Argani P, Halushka MK, Thomson JA, Pharoah P, Porgador A, Sukumar S, Parsons R, Richardson AL, Stampfer MR, Gelman RS, Nikolskaya T, Nikolsky Y, Polyak K (2008) Cell type-specific DNA methylation patterns in the human breast. Proc Natl Acad Sci USA 105(37):14076–14081. doi:0805206105 [pii] 10.1073/pnas.0805206105
30. Maunakea AK, Nagarajan RP, Bilenky M, Ballinger TJ, D'Souza C, Fouse SD, Johnson BE, Hong C, Nielsen C, Zhao Y, Turecki G, Delaney A, Varhol R, Thiessen N, Shchors K, Heine VM, Rowitch DH, Xing X, Fiore C, Schillebeeckx M, Jones SJ, Haussler D, Marra MA, Hirst M, Wang T, Costello JF (2010) Conserved role of intragenic DNA methylation in regulating alternative promoters. Nature 466(7303):253–257. doi:nature09165 [pii] 10.1038/nature09165

31. Harris RA, Wang T, Coarfa C, Nagarajan RP, Hong C, Downey SL, Johnson BE, Fouse SD, Delaney A, Zhao Y, Olshen A, Ballinger T, Zhou X, Forsberg KJ, Gu J, Echipare L, O'Geen H, Lister R, Pelizzola M, Xi Y, Epstein CB, Bernstein BE, Hawkins RD, Ren B, Chung WY, Gu H, Bock C, Gnirke A, Zhang MQ, Haussler D, Ecker JR, Li W, Farnham PJ, Waterland RA, Meissner A, Marra MA, Hirst M, Milosavljevic A, Costello JF (2010) Comparison of sequencing-based methods to profile DNA methylation and identification of monoallelic epigenetic modifications. Nat Biotechnol 28(10):1097–1105. doi:nbt.1682 [pii] 10.1038/nbt.1682
32. Rabinowicz PD, Schutz K, Dedhia N, Yordan C, Parnell LD, Stein L, McCombie WR, Martienssen RA (1999) Differential methylation of genes and retrotransposons facilitates shotgun sequencing of the maize genome. Nat Genet 23(3):305–308. doi:10.1038/15479
33. Irizarry RA, Ladd-Acosta C, Carvalho B, Wu H, Brandenburg SA, Jeddeloh JA, Wen B, Feinberg AP (2008) Comprehensive high-throughput arrays for relative methylation (CHARM). Genome Res 18(5):780–790. doi:gr.7301508 [pii] 10.1101/gr.7301508
34. Irizarry RA, Ladd-Acosta C, Wen B, Wu Z, Montano C, Onyango P, Cui H, Gabo K, Rongione M, Webster M, Ji H, Potash JB, Sabunciyan S, Feinberg AP (2009) The human colon cancer methylome shows similar hypo- and hypermethylation at conserved tissue-specific CpG island shores. Nat Genet 41(2):178–186. doi:ng.298 [pii] 10.1038/ng.298
35. Jin SG, Kadam S, Pfeifer GP (2010) Examination of the specificity of DNA methylation profiling techniques towards 5-methylcytosine and 5-hydroxymethylcytosine. Nucleic Acids Res 38(11):e125. doi:gkq223 [pii] 10.1093/nar/gkq223
36. Ficz G, Branco MR, Seisenberger S, Santos F, Krueger F, Hore TA, Marques CJ, Andrews S, Reik W (2011) Dynamic regulation of 5-hydroxymethylcytosine in mouse ES cells and during differentiation. Nature 473(7347):398–402. doi:nature10008 [pii] 10.1038/nature10008
37. Xu Y, Wu F, Tan L, Kong L, Xiong L, Deng J, Barbera AJ, Zheng L, Zhang H, Huang S, Min J, Nicholson T, Chen T, Xu G, Shi Y, Zhang K, Shi YG (2011) Genome-wide regulation of 5hmC, 5mC, and gene expression by Tet1 hydroxylase in mouse embryonic stem cells. Mol Cell 42(4):451–464. doi:S1097-2765(11)00283-8 [pii] 10.1016/j.molcel.2011.04.005
38. Williams K, Christensen J, Pedersen MT, Johansen JV, Cloos PA, Rappsilber J, Helin K (2011) TET1 and hydroxymethylcytosine in transcription and DNA methylation fidelity. Nature 473(7347):343–348. doi:nature10066 [pii] 10.1038/nature10066
39. Stroud H, Feng S, Morey Kinney S, Pradhan S, Jacobsen SE (2011) 5-Hydroxymethylcytosine is associated with enhancers and gene bodies in human embryonic stem cells. Genome Biol 12(6):R54. doi:gb-2011-12-6-r54 [pii] 10.1186/gb-2011-12-6-r54
40. Keshet I, Schlesinger Y, Farkash S, Rand E, Hecht M, Segal E, Pikarski E, Young RA, Niveleau A, Cedar H, Simon I (2006) Evidence for an instructive mechanism of de novo methylation in cancer cells. Nat Genet 38(2):149–153. doi:ng1719 [pii] 10.1038/ng1719
41. Fouse SD, Shen Y, Pellegrini M, Cole S, Meissner A, Van Neste L, Jaenisch R, Fan G (2008) Promoter CpG methylation contributes to ES cell gene regulation in parallel with Oct4/Nanog, PcG complex, and histone H3K4/K27 trimethylation. Cell Stem Cell 2(2):160–169. doi:S1934-5909(07)00327-X [pii] 10.1016/j.stem.2007.12.011
42. Shen Y, Matsuno Y, Fouse SD, Rao N, Root S, Xu R, Pellegrini M, Riggs AD, Fan G (2008) X-inactivation in female human embryonic stem cells is in a nonrandom pattern and prone to epigenetic alterations. Proc Natl Acad Sci USA 105(12):4709–4714. doi:0712018105 [pii] 10.1073/pnas.0712018105
43. Zhang X, Yazaki J, Sundaresan A, Cokus S, Chan SW, Chen H, Henderson IR, Shinn P, Pellegrini M, Jacobsen SE, Ecker JR (2006) Genome-wide high-resolution mapping and functional analysis of DNA methylation in arabidopsis. Cell 126(6):1189–1201. doi:S0092-8674(06)01018-X [pii] 10.1016/j.cell.2006.08.003
44. Zilberman D, Gehring M, Tran RK, Ballinger T, Henikoff S (2007) Genome-wide analysis of Arabidopsis thaliana DNA methylation uncovers an interdependence between methylation and transcription. Nat Genet 39(1):61–69. doi:ng1929 [pii] 10.1038/ng1929
45. Robinson MD, Stirzaker C, Statham AL, Coolen MW, Song JZ, Nair SS, Strbenac D, Speed TP, Clark SJ (2010) Evaluation of affinity-based genome-wide DNA methylation data: effects

of CpG density, amplification bias, and copy number variation. Genome Res 20(12):1719–1729. doi:gr.110601.110 [pii] 10.1101/gr.110601.110
46. Robinson MD, Statham AL, Speed TP, Clark SJ (2010) Protocol matters: which methylome are you actually studying? Epigenomics 2(4):587–598. doi:10.2217/epi.10.36
47. Down TA, Rakyan VK, Turner DJ, Flicek P, Li H, Kulesha E, Graf S, Johnson N, Herrero J, Tomazou EM, Thorne NP, Backdahl L, Herberth M, Howe KL, Jackson DK, Miretti MM, Marioni JC, Birney E, Hubbard TJ, Durbin R, Tavare S, Beck S (2008) A Bayesian deconvolution strategy for immunoprecipitation-based DNA methylome analysis. Nat Biotechnol 26(7):779–785. doi:nbt1414 [pii] 10.1038/nbt1414
48. Pomraning KR, Smith KM, Freitag M (2009) Genome-wide high throughput analysis of DNA methylation in eukaryotes. Methods 47(3):142–150. doi:S1046-2023(08)00182-5 [pii] 10.1016/j.ymeth.2008.09.022
49. Ruike Y, Imanaka Y, Sato F, Shimizu K, Tsujimoto G (2010) Genome-wide analysis of aberrant methylation in human breast cancer cells using methyl-DNA immunoprecipitation combined with high-throughput sequencing. BMC Genomics 11:137. doi:1471-2164-11-137 [pii] 10.1186/1471-2164-11-137
50. Li N, Ye M, Li Y, Yan Z, Butcher LM, Sun J, Han X, Chen Q, Zhang X, Wang J (2010) Whole genome DNA methylation analysis based on high throughput sequencing technology. Methods 52(3):203–212. doi:S1046-2023(10)00127-1 [pii] 10.1016/j.ymeth.2010.04.009
51. Chavez L, Jozefczuk J, Grimm C, Dietrich J, Timmermann B, Lehrach H, Herwig R, Adjaye J (2010) Computational analysis of genome-wide DNA methylation during the differentiation of human embryonic stem cells along the endodermal lineage. Genome Res 20(10):1441–1450. doi:gr.110114.110 [pii] 10.1101/gr.110114.110
52. Bock C, Tomazou EM, Brinkman AB, Muller F, Simmer F, Gu H, Jager N, Gnirke A, Stunnenberg HG, Meissner A (2010) Quantitative comparison of genome-wide DNA methylation mapping technologies. Nat Biotechnol 28(10):1106–1114. doi:nbt.1681 [pii] 10.1038/nbt.1681
53. Feber A, Wilson GA, Zhang L, Presneau N, Idowu B, Down TA, Rakyan VK, Noon LA, Lloyd AC, Stupka E, Schiza V, Teschendorff AE, Schroth GP, Flanagan A, Beck S (2011) Comparative methylome analysis of benign and malignant peripheral nerve sheath tumors. Genome Res 21(4):515–524. doi:gr.109678.110 [pii] 10.1101/gr.109678.110
54. Pelizzola M, Koga Y, Urban AE, Krauthammer M, Weissman S, Halaban R, Molinaro AM (2008) MEDME: an experimental and analytical methodology for the estimation of DNA methylation levels based on microarray derived MeDIP-enrichment. Genome Res 18(10):1652–1659. doi:gr.080721.108 [pii] 10.1101/gr.080721.108
55. Brinkman AB, Simmer F, Ma K, Kaan A, Zhu J, Stunnenberg HG (2010) Whole-genome DNA methylation profiling using MethylCap-seq. Methods 52(3):232–236. doi:S1046-2023(10)00166-0 [pii] 10.1016/j.ymeth.2010.06.012
56. Serre D, Lee BH, Ting AH (2010) MBD-isolated genome sequencing provides a high-throughput and comprehensive survey of DNA methylation in the human genome. Nucleic Acids Res 38(2):391–399. doi:gkp992 [pii] 10.1093/nar/gkp992
57. Lan X, Adams C, Landers M, Dudas M, Krissinger D, Marnellos G, Bonneville R, Xu M, Wang J, Huang TH, Meredith G, Jin VX (2011) High resolution detection and analysis of CpG dinucleotides methylation using MBD-Seq technology. PLoS One 6(7):e22226. doi:10.1371/journal.pone.0022226 PONE-D-11-02256 [pii]
58. Zhou X, Maricque B, Xie M, Li D, Sundaram V, Martin EA, Koebbe BC, Nielsen C, Hirst M, Farnham P, Kuhn RM, Zhu J, Smirnov I, Kent WJ, Haussler D, Madden PA, Costello JF, Wang T (2011) The human epigenome browser at washington university. Nat Methods 8(12):989–990. doi:10.1038/nmeth.1772 nmeth.1772 [pii]
59. Karpf AR, Peterson PW, Rawlins JT, Dalley BK, Yang Q, Albertsen H, Jones DA (1999) Inhibition of DNA methyltransferase stimulates the expression of signal transducer and activator of transcription 1, 2, and 3 genes in colon tumor cells. Proc Natl Acad Sci USA 96(24):14007–14012
60. Yamashita K, Upadhyay S, Osada M, Hoque MO, Xiao Y, Mori M, Sato F, Meltzer SJ, Sidransky D (2002) Pharmacologic unmasking of epigenetically silenced tumor suppressor

genes in esophageal squamous cell carcinoma. Cancer Cell 2(6):485–495. doi:S1535610802002155 [pii]
61. Foltz G, Yoon JG, Lee H, Ryken TC, Sibenaller Z, Ehrich M, Hood L, Madan A (2009) DNA methyltransferase-mediated transcriptional silencing in malignant glioma: a combined whole-genome microarray and promoter array analysis. Oncogene. doi:onc2009122 [pii] 10.1038/onc.2009.122
62. Cameron EE, Bachman KE, Myohanen S, Herman JG, Baylin SB (1999) Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. Nat Genet 21(1):103–107
63. Shames DS, Girard L, Gao B, Sato M, Lewis CM, Shivapurkar N, Jiang A, Perou CM, Kim YH, Pollack JR, Fong KM, Lam CL, Wong M, Shyr Y, Nanda R, Olopade OI, Gerald W, Euhus DM, Shay JW, Gazdar AF, Minna JD (2006) A genome-wide screen for promoter methylation in lung cancer identifies novel methylation markers for multiple malignancies. PLoS Med 3(12):e486. doi:06-PLME-RA-0315R2 [pii] 10.1371/journal.pmed.0030486
64. Gius D, Cui H, Bradbury CM, Cook J, Smart DK, Zhao S, Young L, Brandenburg SA, Hu Y, Bisht KS, Ho AS, Mattson D, Sun L, Munson PJ, Chuang EY, Mitchell JB, Feinberg AP (2004) Distinct effects on gene expression of chemical and genetic manipulation of the cancer epigenome revealed by a multimodality approach. Cancer Cell 6(4):361–371
65. Mueller W, Nutt CL, Ehrich M, Riemenschneider MJ, von Deimling A, van den Boom D, Louis DN (2007) Downregulation of RUNX3 and TES by hypermethylation in glioblastoma. Oncogene 26(4):583–593. doi:1209805 [pii] 10.1038/sj.onc.1209805
66. Meissner A, Gnirke A, Bell GW, Ramsahoye B, Lander ES, Jaenisch R (2005) Reduced representation bisulfite sequencing for comparative high-resolution DNA methylation analysis. Nucleic Acids Res 33(18):5868–5877. doi:33/18/5868 [pii] 10.1093/nar/gki901
67. Meissner A, Mikkelsen TS, Gu H, Wernig M, Hanna J, Sivachenko A, Zhang X, Bernstein BE, Nusbaum C, Jaffe DB, Gnirke A, Jaenisch R, Lander ES (2008) Genome-scale DNA methylation maps of pluripotent and differentiated cells. Nature 454(7205):766–770. doi:nature07107 [pii] 10.1038/nature07107
68. Smith ZD, Gu H, Bock C, Gnirke A, Meissner A (2009) High-throughput bisulfite sequencing in mammalian genomes. Methods 48(3):226–232. doi:S1046-2023(09)00111-X [pii] 10.1016/j.ymeth.2009.05.003
69. Cokus SJ, Feng S, Zhang X, Chen Z, Merriman B, Haudenschild CD, Pradhan S, Nelson SF, Pellegrini M, Jacobsen SE (2008) Shotgun bisulphite sequencing of the Arabidopsis genome reveals DNA methylation patterning. Nature 452(7184):215–219
70. Lister R, O'Malley RC, Tonti-Filippini J, Gregory BD, Berry CC, Millar AH, Ecker JR (2008) Highly integrated single-base resolution maps of the epigenome in Arabidopsis. Cell 133(3):523–536. doi:S0092-8674(08)00448-0 [pii] 10.1016/j.cell.2008.03.029
71. Xi Y, Li W (2009) BSMAP: whole genome bisulfite sequence MAPping program. BMC Bioinformatics 10:232. doi:1471-2105-10-232 [pii] 10.1186/1471-2105-10-232
72. Gu H, Smith ZD, Bock C, Boyle P, Gnirke A, Meissner A (2011) Preparation of reduced representation bisulfite sequencing libraries for genome-scale DNA methylation profiling. Nat Protoc 6(4):468–481. doi:nprot.2010.190 [pii] 10.1038/nprot.2010.190
73. Xiang H, Zhu J, Chen Q, Dai F, Li X, Li M, Zhang H, Zhang G, Li D, Dong Y, Zhao L, Lin Y, Cheng D, Yu J, Sun J, Zhou X, Ma K, He Y, Zhao Y, Guo S, Ye M, Guo G, Li Y, Li R, Zhang X, Ma L, Kristiansen K, Guo Q, Jiang J, Beck S, Xia Q, Wang W, Wang J (2010) Single base-resolution methylome of the silkworm reveals a sparse epigenomic map. Nat Biotechnol 28(5):516–520. doi:nbt.1626 [pii] 10.1038/nbt.1626
74. Schroeder DI, Lott P, Korf I, LaSalle JM (2011) Large-scale methylation domains mark a functional subset of neuronally expressed genes. Genome Res 21(10):1583–1591. doi:gr.119131.110 [pii] 10.1101/gr.119131.110
75. Feng S, Cokus SJ, Zhang X, Chen PY, Bostick M, Goll MG, Hetzel J, Jain J, Strauss SH, Halpern ME, Ukomadu C, Sadler KC, Pradhan S, Pellegrini M, Jacobsen SE (2010) Conservation and divergence of methylation patterning in plants and animals. Proc Natl Acad Sci USA 107(19):8689–8694. doi:1002720107 [pii] 10.1073/pnas.1002720107

76. Zemach A, McDaniel IE, Silva P, Zilberman D (2010) Genome-wide evolutionary analysis of eukaryotic DNA methylation. Science 328(5980):916–919. doi:science.1186366 [pii] 10.1126/science.1186366
77. Laurent L, Wong E, Li G, Huynh T, Tsirigos A, Ong CT, Low HM, Kin Sung KW, Rigoutsos I, Loring J, Wei CL (2010) Dynamic changes in the human methylome during differentiation. Genome Res 20(3):320–331. doi:gr.101907.109 [pii] 10.1101/gr.101907.109
78. Lister R, Pelizzola M, Kida YS, Hawkins RD, Nery JR, Hon G, Antosiewicz-Bourget J, O'Malley R, Castanon R, Klugman S, Downes M, Yu R, Stewart R, Ren B, Thomson JA, Evans RM, Ecker JR (2011) Hotspots of aberrant epigenomic reprogramming in human induced pluripotent stem cells. Nature 471(7336):68–73. doi:nature09798 [pii] 10.1038/nature09798
79. Hon GC, Hawkins RD, Caballero OL, Lo C, Lister R, Pelizzola M, Valsesia A, Ye Z, Kuan S, Edsall LE, Camargo AA, Stevenson BJ, Ecker JR, Bafna V, Strausberg RL, Simpson AJ, Ren B (2011) Global DNA hypomethylation coupled to repressive chromatin domain formation and gene silencing in breast cancer. Genome Res. doi:gr.125872.111 [pii] 10.1101/gr.125872.111
80. Berman BP, Weisenberger DJ, Aman JF, Hinoue T, Ramjan Z, Liu Y, Noushmehr H, Lange CP, van Dijk CM, Tollenaar RA, Van Den Berg D, Laird PW (2011) Regions of focal DNA hypermethylation and long-range hypomethylation in colorectal cancer coincide with nuclear lamina-associated domains. Nat Genet 44(1):40–46. doi:10.1038/ng.969 ng.969 [pii]
81. Krueger F, Andrews SR (2011) Bismark: a flexible aligner and methylation caller for Bisulfite-Seq applications. Bioinformatics 27(11):1571–1572. doi:btr167 [pii] 10.1093/bioinformatics/btr167
82. Xi Y, Bock C, Muller F, Sun D, Meissner A, Li W (2011) RRBSMAP: a fast, accurate and user-friendly alignment tool for reduced representation bisulfite sequencing. Bioinformatics. doi:btr668 [pii] 10.1093/bioinformatics/btr668
83. Ehrich M, Nelson MR, Stanssens P, Zabeau M, Liloglou T, Xinarianos G, Cantor CR, Field JK, van den Boom D (2005) Quantitative high-throughput analysis of DNA methylation patterns by base-specific cleavage and mass spectrometry. Proc Natl Acad Sci USA 102(44):15785–15790. doi:0507816102 [pii] 10.1073/pnas.0507816102
84. Raval A, Tanner SM, Byrd JC, Angerman EB, Perko JD, Chen SS, Hackanson B, Grever MR, Lucas DM, Matkovic JJ, Lin TS, Kipps TJ, Murray F, Weisenburger D, Sanger W, Lynch J, Watson P, Jansen M, Yoshinaga Y, Rosenquist R, de Jong PJ, Coggill P, Beck S, Lynch H, de la Chapelle A, Plass C (2007) Downregulation of death-associated protein kinase 1 (DAPK1) in chronic lymphocytic leukemia. Cell 129(5):879–890. doi:S0092-8674(07)00512-0 [pii] 10.1016/j.cell.2007.03.043
85. Deng J, Shoemaker R, Xie B, Gore A, LeProust EM, Antosiewicz-Bourget J, Egli D, Maherali N, Park IH, Yu J, Daley GQ, Eggan K, Hochedlinger K, Thomson J, Wang W, Gao Y, Zhang K (2009) Targeted bisulfite sequencing reveals changes in DNA methylation associated with nuclear reprogramming. Nat Biotechnol 27(4):353–360. doi:nbt.1530 [pii] 10.1038/nbt.1530
86. Ito S, D'Alessio AC, Taranova OV, Hong K, Sowers LC, Zhang Y (2010) Role of Tet proteins in 5mC to 5hmC conversion, ES-cell self-renewal and inner cell mass specification. Nature 466(7310):1129–1133. doi:nature09303 [pii] 10.1038/nature09303
87. Ono R, Taki T, Taketani T, Taniwaki M, Kobayashi H, Hayashi Y (2002) LCX, leukemia-associated protein with a CXXC domain, is fused to MLL in acute myeloid leukemia with trilineage dysplasia having t(10;11)(q22;q23). Cancer Res 62(14):4075–4080
88. Lorsbach RB, Moore J, Mathew S, Raimondi SC, Mukatira ST, Downing JR (2003) TET1, a member of a novel protein family, is fused to MLL in acute myeloid leukemia containing the t(10;11)(q22;q23). Leukemia 17(3):637–641. doi:10.1038/sj.leu.2402834 [pii]
89. Ko M, Huang Y, Jankowska AM, Pape UJ, Tahiliani M, Bandukwala HS, An J, Lamperti ED, Koh KP, Ganetzky R, Liu XS, Aravind L, Agarwal S, Maciejewski JP, Rao A (2010) Impaired hydroxylation of 5-methylcytosine in myeloid cancers with mutant TET2. Nature 468(7325):839–843. doi:nature09586 [pii] 10.1038/nature09586

90. Huang Y, Pastor WA, Shen Y, Tahiliani M, Liu DR, Rao A (2010) The behaviour of 5-hydroxymethylcytosine in bisulfite sequencing. PLoS One 5(1):e8888. doi:10.1371/journal.pone.0008888
91. Globisch D, Munzel M, Muller M, Michalakis S, Wagner M, Koch S, Bruckl T, Biel M, Carell T (2010) Tissue distribution of 5-hydroxymethylcytosine and search for active demethylation intermediates. PLoS One 5(12):e15367. doi:10.1371/journal.pone.0015367
92. Szwagierczak A, Bultmann S, Schmidt CS, Spada F, Leonhardt H (2010) Sensitive enzymatic quantification of 5-hydroxymethylcytosine in genomic DNA. Nucleic Acids Res 38(19):e181. doi:gkq684 [pii] 10.1093/nar/gkq684
93. Kinney SM, Chin HG, Vaisvila R, Bitinaite J, Zheng Y, Esteve PO, Feng S, Stroud H, Jacobsen SE, Pradhan S (2011) Tissue-specific distribution and dynamic changes of 5-hydroxymethylcytosine in mammalian genomes. J Biol Chem 286(28):24685–24693. doi:M110.217083 [pii] 10.1074/jbc.M110.217083
94. Flusberg BA, Webster DR, Lee JH, Travers KJ, Olivares EC, Clark TA, Korlach J, Turner SW (2010) Direct detection of DNA methylation during single-molecule, real-time sequencing. Nat Methods 7(6):461–465. doi:nmeth.1459 [pii] 10.1038/nmeth.1459
95. Ke X-S, Qu Y, Rostad K, Li W-C, Lin B, Halvorsen OJ, Haukaas SA, Jonassen I, Petersen K, Goldfinger N, Rotter V, Akslen LA, Oyan AM, Kalland K-H (2009) Genome-wide profiling of histone h3 lysine 4 and lysine 27 trimethylation reveals an epigenetic signature in prostate carcinogenesis. PLoS One 4:e4687
96. Fraga MF, Ballestar E, Villar-Garea A, Boix-Chornet M, Espada J, Schotta G, Bonaldi T, Haydon C, Ropero S, Petrie K, Iyer NG, Pérez-Rosado A, Calvo E, Lopez JA, Cano A, Calasanz MJ, Colomer D, Piris MA, Ahn N, Imhof A, Caldas C, Jenuwein T, Esteller M (2005) Loss of acetylation at Lys16 and trimethylation at Lys20 of histone H4 is a common hallmark of human cancer. Nat Genet 37:391–400
97. Varambally S, Dhanasekaran SM, Zhou M, Barrette TR, Kumar-Sinha C, Sanda MG, Ghosh D, Pienta KJ, Sewalt RGAB, Otte AP, Rubin MA, Chinnaiyan AM (2002) The polycomb group protein EZH2 is involved in progression of prostate cancer. Nature 419:624–629
98. Sharma SV, Lee DY, Li B, Quinlan MP, Takahashi F, Maheswaran S, McDermott U, Azizian N, Zou L, Fischbach MA (2010) A chromatin-mediated reversible drug-tolerant state in cancer cell subpopulations. Cell 141:69–80
99. Park PJ (2009) ChIP-seq: advantages and challenges of a maturing technology. Nat Rev Genet 10:669–680
100. O'Neill LP, Turner BM, Turner B (2003) ChIP with native chromatin: advantages and problems relative to methods using cross-linked material. Methods 31(1):76–82
101. Orlando V (2000) Mapping chromosomal proteins in vivo by formaldehyde-crosslinked-chromatin immunoprecipitation. Trends Biochem Sci 25:99–104
102. Barski A, Zhao K (2009) Genomic location analysis by ChIP-Seq. J Cell Biochem 107(107):11–18
103. Dingwall C, Lomonossoff GP, Laskey RA (1981) High sequence specificity of micrococcal nuclease. Nucleic Acids Res 9:2659–2673
104. Hörz W, Altenburger W (1981) Sequence specific cleavage of DNA by micrococcal nuclease. Nucleic Acids Res 9:2643–2658
105. Li H, Ruan J, Durbin R (2008) Mapping short DNA sequencing reads and calling variants using mapping quality scores. Genome Res 18:1851–1858
106. Langmead B, Trapnell C, Pop M, Salzberg SL (2009) Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol 10:R25
107. Li H, Durbin R (2010) Fast and accurate long-read alignment with Burrows-Wheeler transform. Bioinformatics 26:589–595
108. Li R, Yu C, Li Y, Lam T-W, Yiu S-M, Kristiansen K, Wang J (2009) SOAP2: an improved ultrafast tool for short read alignment. Bioinformatics 25:1966–1967
109. Li R, Li Y, Kristiansen K, Wang J (2008) SOAP: short oligonucleotide alignment program. Bioinformatics 24:713–714

110. Coarfa C, Yu F, Miller CA, Chen Z, Harris RA, Milosavljevic A (2010) Pash 3.0: a versatile software package for read mapping and integrative analysis of genomic and epigenomic variation using massively parallel DNA sequencing. BMC Bioinformatics 11:572. doi:1471-2105-11-572 [pii] 10.1186/1471-2105-11-572
111. Pepke S, Wold B, Mortazavi A (2009) Computation for ChIP-seq and RNA-seq studies. Nat Methods 6:S22–S32
112. Xu H, Handoko L, Wei X, Ye C, Sheng J, Wei C-L, Lin F, Sung W-K (2010) A signal-noise model for significance analysis of ChIP-seq with negative control. Bioinformatics 26:1199–1204
113. Xu H, Wei C-L, Lin F, Sung W-K (2008) An HMM approach to genome-wide identification of differential histone modification sites from ChIP-seq data. Bioinformatics 24:2344–2349
114. Zang C, Schones DE, Zeng C, Cui K, Zhao K, Peng W (2009) A clustering approach for identification of enriched domains from histone modification ChIP-Seq data. Bioinformatics 25:1952–1958
115. Barski A, Cuddapah S, Cui K, Roh T-Y, Schones DE, Wang Z, Wei G, Chepelev I, Zhao K (2007) High-resolution profiling of histone methylations in the human genome. Cell 129:823–837
116. Johnson DS, Mortazavi A, Myers RM, Wold B (2007) Genome-wide mapping of in vivo protein-DNA interactions. Science 316:1497–1502
117. Mikkelsen TS, Ku M, Jaffe DB, Issac B, Lieberman E, Giannoukos G, Alvarez P, Brockman W, Kim T-K, Koche RP, Lee W, Mendenhall E, O'Donovan A, Presser A, Russ C, Xie X, Meissner A, Wernig M, Jaenisch R, Nusbaum C, Lander ES, Bernstein BE (2007) Genome-wide maps of chromatin state in pluripotent and lineage-committed cells. Nature 448:553–560
118. Heintzman ND, Stuart RK, Hon G, Fu Y, Ching CW, Hawkins RD, Barrera LO, Van Calcar S, Qu C, Ching KA, Wang W, Weng Z, Green RD, Crawford GE, Ren B (2007) Distinct and predictive chromatin signatures of transcriptional promoters and enhancers in the human genome. Nat Genet 39:311–318
119. Heintzman ND, Hon GC, Hawkins RD, Kheradpour P, Stark A, Harp LF, Ye Z, Lee LK, Stuart RK, Ching CW, Ching KA, Antosiewicz-Bourget JE, Liu H, Zhang X, Green RD, Lobanenkov VV, Stewart R, Thomson JA, Crawford GE, Kellis M, Ren B (2009) Histone modifications at human enhancers reflect global cell-type-specific gene expression. Nature 459:108–112
120. Ernst J, Kheradpour P, Mikkelsen TS, Shoresh N, Ward LD, Epstein CB, Zhang X, Wang L, Issner R, Coyne M, Ku M, Durham T, Kellis M, Bernstein BE (2011) Mapping and analysis of chromatin state dynamics in nine human cell types. Nature 473:43–49
121. Wasserman NF, Aneas I, Nobrega MA (2010) An 8q24 gene desert variant associated with prostate cancer risk confers differential in vivo activity to a MYC enhancer. Genome Res 20:1191–1197
122. Adli M, Bernstein BE (2011) Whole-genome chromatin profiling from limited numbers of cells using nano-ChIP-seq. Nat Protoc 6:1656–1668
123. Goren A, Ozsolak F, Shoresh N, Ku M, Adli M, Hart C, Gymrek M, Zuk O, Regev A, Milos PM, Bernstein BE (2010) Chromatin profiling by directly sequencing small quantities of immunoprecipitated DNA. Nat Methods 7:47–49
124. Dekker J, Rippe K, Dekker M, Kleckner N (2002) Capturing chromosome conformation. Science 295:1306–1311
125. Zhao Z, Tavoosidana G, Sjölinder M, Göndör A, Mariano P, Wang S, Kanduri C, Lezcano M, Sandhu KS, Singh U, Pant V, Tiwari V, Kurukuti S, Ohlsson R (2006) Circular chromosome conformation capture (4C) uncovers extensive networks of epigenetically regulated intra- and interchromosomal interactions. Nat Genet 38:1341–1347
126. Dostie J, Richmond TA, Arnaout RA, Selzer RR, Lee WL, Honan TA, Rubio ED, Krumm A, Lamb J, Nusbaum C, Green RD, Dekker J (2006) Chromosome Conformation Capture Carbon Copy (5C): a massively parallel solution for mapping interactions between genomic elements. Genome Res 16:1299–1309

127. Lieberman-Aiden E, van Berkum NL, Williams L, Imakaev M, Ragoczy T, Telling A, Amit I, Lajoie BR, Sabo PJ, Dorschner MO, Sandstrom R, Bernstein B, Bender MA, Groudine M, Gnirke A, Stamatoyannopoulos J, Mirny LA, Lander ES, Dekker J (2009) Comprehensive mapping of long-range interactions reveals folding principles of the human genome. Science 326:289–293
128. Cai S, Lee CC, Kohwi-Shigematsu T (2006) SATB1 packages densely looped, transcriptionally active chromatin for coordinated expression of cytokine genes. Nat Genet 38:1278–1288
129. Simonis M, Kooren J, de Laat W (2007) An evaluation of 3C-based methods to capture DNA interactions. Nat Methods 4:895–901
130. Fullwood MJ, Wei C-L, Liu ET, Ruan Y (2009) Next-generation DNA sequencing of paired-end tags (PET) for transcriptome and genome analyses. Genome Res 19:521–532
131. Simonis M, Klous P, Homminga I, Galjaard R-J, Rijkers E-J, Grosveld F, Meijerink JPP, de Laat W (2009) High-resolution identification of balanced and complex chromosomal rearrangements by 4C technology. Nat Methods 6:837–842
132. Ohm JE, McGarvey KM, Yu X, Cheng L, Schuebel KE, Cope L, Mohammad HP, Chen W, Daniel VC, Yu W, Berman DM, Jenuwein T, Pruitt K, Sharkis SJ, Watkins DN, Herman JG, Baylin SB (2007) A stem cell-like chromatin pattern may predispose tumor suppressor genes to DNA hypermethylation and heritable silencing. Nat Genet 39(2):237–242. doi:ng1972 [pii] 10.1038/ng1972
133. Widschwendter M, Fiegl H, Egle D, Mueller-Holzner E, Spizzo G, Marth C, Weisenberger DJ, Campan M, Young J, Jacobs I, Laird PW (2007) Epigenetic stem cell signature in cancer. Nat Genet 39(2):157–158. doi:ng1941 [pii] 10.1038/ng1941
134. Schlesinger Y, Straussman R, Keshet I, Farkash S, Hecht M, Zimmerman J, Eden E, Yakhini Z, Ben-Shushan E, Reubinoff BE, Bergman Y, Simon I, Cedar H (2007) Polycomb-mediated methylation on Lys27 of histone H3 pre-marks genes for de novo methylation in cancer. Nat Genet 39(2):232–236. doi:ng1950 [pii] 10.1038/ng1950
135. Ley TJ, Ding L, Walter MJ, McLellan MD, Lamprecht T, Larson DE, Kandoth C, Payton JE, Baty J, Welch J, Harris CC, Lichti CF, Townsend RR, Fulton RS, Dooling DJ, Koboldt DC, Schmidt H, Zhang Q, Osborne JR, Lin L, O'Laughlin M, McMichael JF, Delehaunty KD, McGrath SD, Fulton LA, Magrini VJ, Vickery TL, Hundal J, Cook LL, Conyers JJ, Swift GW, Reed JP, Alldredge PA, Wylie T, Walker J, Kalicki J, Watson MA, Heath S, Shannon WD, Varghese N, Nagarajan R, Westervelt P, Tomasson MH, Link DC, Graubert TA, DiPersio JF, Mardis ER, Wilson RK (2010) DNMT3A mutations in acute myeloid leukemia. N Engl J Med 363(25):2424–2433. doi:10.1056/NEJMoa1005143
136. Yamashita Y, Yuan J, Suetake I, Suzuki H, Ishikawa Y, Choi YL, Ueno T, Soda M, Hamada T, Haruta H, Takada S, Miyazaki Y, Kiyoi H, Ito E, Naoe T, Tomonaga M, Toyota M, Tajima S, Iwama A, Mano H (2010) Array-based genomic resequencing of human leukemia. Oncogene 29(25):3723–3731. doi:onc2010117 [pii] 10.1038/onc.2010.117
137. Clarke J, Wu HC, Jayasinghe L, Patel A, Reid S, Bayley H (2009) Continuous base identification for single-molecule nanopore DNA sequencing. Nat Nanotechnol 4(4):265–270. doi:nnano.2009.12 [pii] 10.1038/nnano.2009.12

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Epigenetic Alterations in Oncogenesis

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Preface

Epigenetics refers to heritable changes in gene expression or genome function encoded by marks other than DNA base sequence; information literally "above" the level of genetics. Epigenetic marks include cytosine methylation and cytosine hydroxymethylation, histone tail modifications, histone variants, and nucleosome positional information, all of which are resident along the DNA duplex. Epigenetic marks frequently show interdependent relationships, for example, the close association of DNA methylation states with particular histone tail modifications and histone variants. From the standpoint of cell physiology, epigenetics provides a mechanism for cells to integrate environmental or intrinsic stimuli into heritable changes in genome function. From the standpoint of development, epigenetics provides a platform for cell differentiation and cell specialization, which in principle cannot simply be the consequence of DNA sequence. Most relevant to this book is the fact that changes in epigenetic states are now recognized to play a fundamental role in cancer development and progression. Cancer, almost uniquely among common human diseases, is characterized by natural selection for cellular variants with improved fitness, e.g., proliferative capacity and rate, evasion of cell death, invasive growth, migration to and proliferation at secondary sites, chemotherapy resistance, and a myriad of other naturally or artificially selected phenotypes. Epigenetic changes play a key role in this phenotypic selection, possibly to an equal to or even greater extent than do genetic mutations.

As a field, cancer epigenetics has now reached young adulthood. The observations that started the field were of DNA hypomethylation changes in cancer in the 1980s, followed by the discovery of DNA hypermethylation in cancer in the 1990s. In the last decade, additional alterations at other levels of epigenetic control (e.g., histone modifications) have also been discovered and characterized in cancer. Also, over the past few years rapid progress has been made in translating the findings of epigenetic alterations into new cancer biomarkers and therapeutic targets. One clear highlight in the field has been the FDA-approval of DNA methyltransferase (DNMT) inhibitors and histone deacetylase (HDAC) inhibitors to treat a select number of human malignancies.

The early work in cancer epigenetics was largely hypothesis or "candidate-gene" driven. More recent work using unbiased and global approaches (i.e., epigenomics)

have validated and greatly extended the early observations. Evidence now suggests that DNA hypomethylation is linked to oncogenic gene activation and genomic instability, and that DNA hypermethylation leads to tumor suppressor gene inactivation, including inactivation of DNA repair genes that also may promote genomic instability. Thus, epigenetic mutations (epimutations) appear to promote genetic mutations and genomic rearrangements in cancer. Intriguingly, a number of recent findings largely from cancer genome sequencing data suggest that genes involved in epigenetic control processes are commonly mutated in a variety of cancers, thus demonstrating that genetic changes can also promote epigenetic alterations in cancer. Taken together, the data now indicate that the roles of genetics and epigenetics in cancer development are highly intertwined.

Epigenetic Alterations in Oncogenesis comprises 15 chapters contributed by leading active researchers in the field. The book is divided into three sections that run the gamut from a description of the basic epigenetic mechanisms that regulate gene expression in human cancer, to how alterations in epigenetic marks contribute to cancer biology, and concluding with an account of the uses for epigenetic-targeted drugs to treat human cancer, as well as the analysis methods to decipher cancer epigenomes.

Part I, *Epigenetic Marks and Mechanisms*, provides an introduction to the major epigenetic marks and how these are altered during oncogenesis. The part begins with a discussion by Jin and Robertson in Chap. 1 on cytosine DNMTs and DNA hypermethylation in cancer, and focuses particularly on the silencing of genes involved in DNA repair, which are a frequent target of hypermethylation. In addition, the authors summarize important recent work showing that DNMTs themselves participate in DNA repair processes. In Chap. 2, Ehrlich and Lacey turn attention to the flip side of the coin, DNA hypomethylation, which was the original epigenetic alteration observed in cancer. The authors discuss the diverse genomic contexts in which DNA hypomethylation can occur and present possible mechanisms to explain DNA hypomethylation in cancer. An exciting recent development in epigenetics is the discovery of 5-hydroxymethylcytosine (5-hmC) as a novel epigenetic mark, which itself appears to be linked to DNA hypomethylation. The biological significance of 5-hmC as well as the enzymes that catalyze its formation (ten–eleven translocation or TET proteins, which can be mutated in cancer) is discussed by Kinney and Pradhan in Chap. 3. In Chap. 4, attention turns to altered histone modifications in cancer with a detailed discussion by Campbell and Turner on how posttranslational histone modifications are controlled under normal circumstances and the mechanisms driving their alteration in malignancy. A critical concept in epigenetics is that DNA methylation and histone modifications ultimately impact gene expression and genome function via their effects on nucleosomes; the important topic of altered nucleosome occupancy in cancer is covered by Andreu-Vieyra and Liang in Chap. 5.

Part III, *The Impact of Epigenetic Alterations on Cancer Biology*, discusses how epigenetic changes contribute to critically important cancer phenotypes. The section begins in Chap. 6, where Fabbri and colleagues discuss miRNA expression alterations in cancer caused by epigenetic changes, including DNA methylation, histone modifications, and Polycomb proteins. The importance of this concept is illustrated by the inherent capacity of altered miRNA expression to derange entire

transcriptional programs in cancer cells. A large family of genes known as cancer-testis or cancer-germ line genes encodes antigens that are a major target of cancer vaccines. Additionally, a number of these genes have emerging oncogenic functions. In Chap. 7, De Smet and Loriot discuss how epigenetic mechanisms, most prominently DNA hypomethylation, lead to the activation of these genes in many human malignancies. Andersen and Jones follow this with a discussion in Chap. 8 of how DNA methylation controls cell fate in the intestine and how, when the tumor suppressor gene adenomatous polyposis coli (APC) is lost, this promotes DNA hypomethylation and intestinal tumorigenesis. In Chap. 9, Futscher describes how tractable cell model systems are being used to discern the temporal epigenetic alterations that are linked to cell immortalization and transformation. It is now recognized that epigenetic regulation lies at the heart of stem cell maintenance and differentiation. In Chap. 10, Huang and colleagues discuss epigenetic regulation of mesenchymal stem cells (MSC) during tumorigenesis, and highlight recent work showing that targeted DNA methylation of tumor suppressor genes provides a model system to study MSC-driven tumorigenesis.

Part III, *Clinical Implications and Analysis Methods*, provides an overview of important topics related to the utility of epigenetic alterations as cancer biomarkers and therapeutic targets, and provides a detailed overview of the methods used to decipher cancer epigenomes. In the past few years, a major link between environmental toxicants, epigenetic changes, and cancer has become apparent. In Chap. 11, Pogribny and Rusyn discuss these developments as they pertain to chemical carcinogens such as arsenic, as well as other pharmaceutical and biological agents. While epigenetic alterations in cancer cells and tumor tissues is well established, emerging data suggest that systemic epigenetic changes (i.e., those affecting normal tissues) can also occur in cancer patients, as well as in individuals with elevated risk for cancer. Marsit and Christensen highlight the current research in this exciting and potentially high impact area in Chap. 12. Epigenetic therapies have entered the clinic and received their first widespread use in the context of myeloid malignancies, particularly myelodysplastic syndrome (MDS) and acute myelogenous leukemia (AML). In Chap. 13, Griffiths and Gore discuss the clinical work in this arena, with a focus on the FDA-approved azanucleosides 5-azacytidine (vidaza) and decitabine (dacogen), but also touching on HDAC inhibitors. In Chap. 14, Balch and Nephew discuss how epigenetic therapies may be particularly well suited for chemotherapy sensitization to overcome drug resistance, and review the extensive preclinical work and rapidly accumulating clinical knowledge in this area. Finally, in Chap. 15, Costello and colleagues review the approaches used for the analysis of cancer epigenomes. In particular, they discuss the methods appropriate for the analysis of cytosine methylation and hydroxymethylation, discuss next-generation sequencing approaches, and touch on the computational methods now being used to explore cancer epigenomes.

Omaha, Nebraska, USA Adam R. Karpf

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Part I
Epigenetic Marks and Mechanisms

Chapter 1
DNA Methyltransferases, DNA Damage Repair, and Cancer

Bilian Jin and Keith D. Robertson

Abstract The maintenance DNA methyltransferase (DNMT) 1 and the de novo methyltransferases DNMT3A and DNMT3B are all essential for mammalian development. DNA methylation, catalyzed by the DNMTs, plays an important role in maintaining genome stability. Aberrant expression of DNMTs and disruption of DNA methylation patterns are closely associated with many forms of cancer, although the exact mechanisms underlying this link remain elusive. DNA damage repair systems have evolved to act as a genome-wide surveillance mechanism to maintain chromosome integrity by recognizing and repairing both exogenous and endogenous DNA insults. Impairment of these systems gives rise to mutations and directly contributes to tumorigenesis. Evidence is mounting for a direct link between DNMTs, DNA methylation, and DNA damage repair systems, which provide new insight into the development of cancer. Like tumor suppressor genes, an array of DNA repair genes frequently sustain promoter hypermethylation in a variety of tumors. In addition, DNMT1, but not the DNMT3s, appear to function coordinately with DNA damage repair pathways to protect cells from sustaining mutagenic events, which is very likely through a DNA methylation-independent mechanism. This chapter is focused on reviewing the links between DNA methylation and the DNA damage response.

1.1 Introduction

DNA methyltransferases (DNMTs), responsible for the transfer of a methyl group from the universal methyl donor, *S*-adenosyl-L-methionine (SAM), to the 5-position of cytosine residues in DNA, are essential for mammalian development [1].

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There are four members of the DNMT family, including DNMT1, DNMT3A, DNMT3B, and DNMT3L. DNMT3L, unlike the other DNMTs, does not possess any inherent enzymatic activity [2]. The other three family members are active on DNA. *DNMT1* encodes the maintenance methyltransferase and *DNMT3A/DNMT3B* encode the de novo methyltransferases [3, 4], required to establish and maintain genomic methylation. While this maintenance vs. de novo division has been convenient, there is clear evidence for functional overlap between the maintenance and the de novo methyltransferases [5, 6]. Gene knockout analysis in mice has shown that *Dnmt1* and *Dnmt3a/Dnmt3b* genes are all essential for viability. *Dnmt1* inactivation leads to very early lethality at embryonic day (E) 9.5, shortly after gastrulation [7–9], whereas *Dnmt3b* knockout induces embryo death at E14.5–18.5, due to multiple developmental defects including growth impairment and rostral neural tube defects [3, 8, 9]. *Dnmt3a*$^{-/-}$ mice become runted and die at about 4 weeks of age, although they appear to be relatively normal at birth [3].

DNMTs play an important role in genomic integrity, disruption of which may result in chromosome instability and tumor progression. It is well established that DNMTs are required for transcriptional silencing of a number of sequence classes, including imprinted genes, genes on the inactive X chromosome and transposable elements [1, 10], and silencing of these sequences is essential for maintaining chromosome stability. Much compelling evidence has come from targeted deletion experiments showing that all three DNMTs are involved in stabilization of the genome, particularly repetitive sequences [3, 11, 12]. For example, either single knockout of *Dnmt1* or double knockout of *Dnmt3a* and *Dnmt3b* enhances telomere recombination [11]. DNMT3B is specifically required for stabilization of pericentromeric satellite repeats. DNMT3B deficiency results in expansion and rearrangements of pericentromeric repeats [3, 12]. Immunodeficiency, centromere instability, and facial anomalies (ICF) syndrome is the only human genetic disorder known to involve biallelic mutations in *DNMT3B*. It is characterized by chromosomal instability arising due to destabilization of pericentromeric repeats, particularly those at juxtacentromeric regions of chromosomes 1, 9, and 16 [3, 12]. Of note, cells null for *DNMT1* or with hypomorphic mutations in *DNMT1* that partially reduce its levels to 30% of WT DNMT1 display significantly greater microsatellite instability (MSI) [13–17], a greater frequency of chromosomal translocations [18] and much higher sensitivity to genotoxic agents [17], which may promote the development of cancer.

The DNA damage response (DDR) is a genome-wide surveillance system that protects cells from potentially mutagenic DNA insults derived from either endogenous or exogenous sources. The DDR usually functions through the coordinated actions of DNA repair and checkpoint systems to promote DNA damage repair before replication or to activate cell death pathways if excessive damage exists [19]. Like the cellular DNA methylation machinery, an intact DDR is crucial for preventing cancer. Evidence is mounting to support a link between the DNA methylation and DNA damage repair systems, as first suggested by promoter hypermethylation and silencing of DNA repair genes in multiple types of cancer [20]. More importantly, DNMT1 may be directly involved in DNA damage repair in a DNA methylation-independent manner [14, 17, 21–23]. Strong support for this latter notion

comes from recent observations that DNMT1 is rapidly and transiently recruited to regions of DNA double-strand breaks (DSBs) via its interaction with proliferating cell nuclear antigen (PCNA) [21, 24], as well the PCNA-like DNA damage sliding clamp component RAD9 (of the 9-1-1 complex) [21]. In this chapter, we examine and outline the links between DNMTs and DNA repair systems and discuss the possible mechanisms of how they are orchestrated, with a focus on cancer.

1.2 Epigenetic Silencing of DNA Repair Pathways Through Aberrant Promoter Hypermethylation

DNA repair systems have evolved to maintain genomic integrity by countering threats posed by DNA lesions [19]. Deficiency in the DNA repair pathways may leave these lesions unrepaired or cause them to be repaired incorrectly, eventually leading to genome instability or mutations that contribute directly to a large array of human diseases including cancer. Carcinogenesis is believed to originate from and be driven by the acquisition of abnormal genetic and/or epigenetic changes. Aberrant DNA hypermethylation, when it occurs at promoter CpG islands (CGIs), leads to potent and heritable transcriptional silencing that inactivates key cellular pathways much like genetic changes (e.g., mutation/deletion) do. In addition to genetic mutations, promoter hypermethylation in DNA repair genes is closely linked to a variety of human tumor types including colorectal, breast, lung cancers, and glioma [20] (Table 1.1), suggesting that epigenetic silencing of DNA repair pathways is an important contributor to the development of cancer.

1.2.1 Epigenetic Inactivation of the DNA Mismatch Repair Pathway

Mismatch repair (MMR) is a genome surveillance system to maintain genomic integrity through recognizing and correcting mismatched nucleotides arising during DNA replication, homologous recombination (HR), or other forms of DNA damage. Impairment of this system gives rise to MSI [25, 26], which has now been recognized as a hallmark of MMR gene-deficient cancers. Microsatellite loci, widely dispersed in the genome, are repetitive sequences consisting of short runs of nucleotides, typically one to four bases in length. Repetitive regions may give rise to the formation of secondary structures, which are subject to expansion or contraction. The secondary structures, if incorrectly resolved, lead to slippage of DNA polymerases along repetitive sequences during replication. Microsatellites are particularly susceptible to length change mutations during replication and transcription, resulting in frameshift mutations if they are located within a gene [25, 26]. MMR deals with these changes to maintain microsatellite stability. MMR comprises

Table 1.1 Genes in DNA damage repair pathways that are hypermethylated in cancer

Repair pathway	Methylated gene	Cancer type	Samples studied	Samples methylated	Methylation frequency (%)	References
MMR	MLH1	Sporadic CRC (MSI+)	110	67	61	[41–44]
		Sporadic CRC (MSI−)	128	38	30	[42, 43]
		Sporadic early-onset CRC	110	55	50	[45]
		NSCLC	77	43	56	[32]
		Acute myeloid leukemia	177	11	6	[34–36]
		Ovarian cancer	672	72	11	[33]
		Oral squamous cell carcinoma	99	8	8	[29]
		HNPCC	179	2	1	[39, 40]
		Gastric cancer	306	58	19	[30, 31]
		HNSCC	49	14	29	[37]
	MSH2	NSCLC	14	4	29	[32]
		Gastric cancer	200	27	14	[30]
		Ovarian cancer	56	29	52	[46]
		Sporadic CRC	36	1	3	[47]
		HNPCC	46	11	24	[48]
	MSH3	Gastric cancer	200	25	13	[30]
	MSH6	Breast cancer	33		92–95[a]/20©	[50]
BER	TDG	Multiple myeloma	KAS-6/1 cell line			[52]
	MBD4	CRC	39		24[a]/14©	[53]
	OGG1	Thyroid cancer	38	2	5	[54]
NER	XPC	Bladder cancer	37	12	32	[56]
	ERCC1	Glioma	32	Unknown		[57]
	XRCC1	Gastric cancer	25	Unknown		[60]
	RAD23B	Multiple myeloma	KAS-6/1 cell line			[61]

HR	BRCA1	NSCLC	98	29	30	[69]
		Sporadic ovarian cancer	81	12	15	[66]
		Sporadic breast cancer	190	24	13	[64, 70]
		Hereditary breast cancer	162	18	11	[70]
		Early onset gastric cancer	104	0.6	1	[67]
		Bladder cancer	96	0.71	1	[68]
	BRCA2	Breast cancer	33		59–64[a]/10©	[50]
		NSCLC	98	41	42	[69]
	FANCC	Acute lymphoblastic leukemia	97	3	3	[74]
		Acute myeloid leukemia	143	1	1	[74]
	FANCF	HNSCC	89	13	15	[75]
		NSCLC	158	22	14	[75]
		Cervical cancer	91	27	30	[76]
		Ovarian cancer	53	7	13	[77]
	FANCL	Acute lymphoblastic leukemia	97	1	1	[74]
NHEJ	XRCC5	NSCLC	98	19	19	[69]
ATM/ATR	ATM	HNSCC	100	25	25	[101]
		Breast cancer	23	18	78	[100]
		CRC	HCT116 cell line			[99]
	CHK2	NSCLC	139	39	28	[106]
		Glioma	5	5	100	[107]

(continued)

Table 1.1 (continued)

Repair pathway	Methylated gene	Cancer type	Samples studied	Samples methylated	Methylation frequency (%)	References
Others	MGMT	Oral squamous cell carcinoma	99	40	40	[29]
		Gastric cancer	200	50	25	[30]
		CRC	36	14	39	[80]
		HNSCC	21	6	29	[80]
		NSCLC	34	10	29	[80]
		Lymphomas	61	15	25	[80]
		Glioma	140	54	39	[80]
	WRN	Gastric cancer	38	10	26	[91]
		CRC	182	69	38	[91]
		NSCLC	56	21	38	[91]
		Prostate cancer	20	4	20	[91]
		Breast cancer	58	10	17	[91]
		Thyroid cancer	32	4	13	[91]
		Non-Hodgkin lymphoma	118	28	24	[91]
		Acute lymphoblastic leukemia	21	2	10	[91]
		Acute myeloblastic leukemia	36	3	8	[91]
		Chondrosarcomas	15	5	33	[91]
		Osteosarcomas	27	3	11	[91]

CRC colorectal cancer; *NSCLC* non-small cell lung cancer; *HNPCC* hereditary non-polyposis colorectal cancer; *HNSCC* head and neck squamous cell carcinoma

[a]Mean methylation level (%) in cancer vs. © mean methylation level (%) in control

©Indicates references from which methylation data derived from similar samples was pooled for this summary

the MutS complex and the MutL complex. MutS recognizes the mismatched base, while MutL recruits repair enzymes to damage sites via its binding with MutS [27]. There are two main MutS complexes in humans, MutSα and MutSβ. MutSα, consisting of the MutS homologue 2 (MSH2) protein bound to MSH6, recognizes single-base mismatches or small insertion/deletion loops (indels), whereas MutSβ, consisting of MSH2 and MSH3, repairs only indels [28]. The main complex for MutL in humans is MutLα, consisting of a heterodimer of MLH1 and PMS2 [26]. Mutations in or epigenetic silencing of MMR genes like *MLH1* and *MSH2* is closely associated with a variety of human cancers such as hereditary non-polyposis colon cancer (HNPCC), sporadic colon cancer, and ovarian cancer [29].

MLH1 plays a central role in coordinating various steps in MMR via interacting with other MMR proteins and modulating their activities. Hypermethylation of the *MLH1* promoter is observed in a variety of cancers including oral squamous cell carcinoma [30], gastric cancer [31, 32], non-small cell lung cancer (NSCLC) [33], ovarian cancer [34], acute myeloid leukemia [35–37], head and neck squamous cell carcinoma (HNSCC) [38], HNPCC [39–41], and particularly in colorectal cancer (CRC) [42–45] (Table 1.1). The reduced MLH1 protein expression is correlated with high-level methylation detected in human CRC samples, whereas samples with low-level methylation display expression levels similar to those observed in methylation-negative samples [46], strongly suggesting that the *MLH1* gene is inactivated via promoter hypermethylation in a dose-dependent manner. Nonetheless, it is not clear whether a moderate degree of methylation affects MLH1 gene expression or not. On the basis of observations made in germ line cells, it has long been believed that *MLH1* promoter methylation involves only one allele of maternal origin. However, more recent findings demonstrate that there is biallelic involvement of *MLH1* promoter hypermethylation in many cancers [46]. The causal link between MSI and epigenetic inactivation of *MLH1* is further highlighted by the observation that 90% of MSI+ HNPCC have *MLH1* hypermethylation, while 95% of MSI samples do not [20].

MSH2 is also hypermethylated in multiple tumor types, including gastric cancer [31], NSCLC [33], ovarian cancer [47], sporadic CRC [48], and HNPCC [49] (Table 1.1). Interestingly, promoter methylation of *MSH2* in HNPCC occurs primarily in patients with germ line mutations in *MSH2* rather than in germ line mutation-negative cases [49]. Seventy percent of patients with *MSH2* methylation also present germ line mutations in this gene, clearly indicating that methylation is the second inactivating hit in these tumors [49]. DNA hypermethylation can be caused by transcription across a CGI within a promoter region. Recent studies have revealed that deletions of the last exons of the *EpCAM* gene, located immediately upstream of *MSH2*, give rise to somatic hypermethylation of the *MSH2* promoter [50]. Deletions at the most 3′-end of the *EpCAM* gene result in loss of its polyadenylation signal, which abolishes transcription termination. Transcription of *EpCAM* then continues downstream into the *MSH2* promoter and induces promoter hypermethylation of *MSH2*. DNA methylation triggered by transcriptional read-through of a neighboring gene, in either sense or antisense, direction may represent a general mutational mechanism that promotes aberrant epigenetic changes. Like *MLH2*, other MutS

homologues, including *MSH3* and *MSH6*, are also inactivated by hypermethylation in tumors such as breast [51] and gastric cancers [31] (Table 1.1).

1.2.2 Epigenetic Inactivation of the Base Excision Repair and Nucleotide Excision Repair Pathways

The specific pairing of DNA bases in the genome is constantly challenged by endogenous metabolic by-products and environmental insults. Base excision repair (BER) is responsible for the removal of damaged DNA bases and their backbones to prevent mutations that could give rise to cancer [19, 52]. In BER, abnormal DNA bases are recognized and removed by specific glycosylases, followed by recruitment of other enzymes including nuclease, polymerase, and ligase proteins, to complete the repair process via excising the remaining sugar fragments and reinstalling an intact correctly based-paired nucleotide [19].

Either thymine DNA glycosylase (TDG) or methyl-CpG-binding domain 4 (MBD4) mediate a specific BER pathway for the correction of G/T mismatches arising due to 5-methylcytosine deamination leading to C to T transitions. DNA hypermethylation-mediated silencing of *TDG* and *MBD4* may contribute to the frequent genomic instability that occurs in cancer cells [53] (Table 1.1). *TDG* promoter hypermethylation negatively correlates with its expression. TDG down-regulation leads to less efficient DNA repair activity in response to hydrogen peroxide-induced DNA damage. Ectopic expression of TDG, however, functionally compensates for lower repair activities of damaged DNA in the KAS-6/1 myeloma cell line with extensive endogenous *TDG* gene hypermethylation [53]. *MBD4*, like *TDG*, is also subject to promoter hypermethylation and gene silencing in tumors like sporadic CRC and ovarian cancer [54]. Another DNA glycosylase, OGG1, which mediates removal of 8-oxoguanine induced by oxidative damage, is also subject to inactivation via promoter methylation in cancer cells [55] (Table 1.1).

Of all the repair systems, nucleotide excision repair (NER) recognizes the most varied types of DNA lesions, contending with the diverse class of helix-distorting damage that interferes with base pairing and obstructs replication and transcription. In NER, there exist two sub-pathways that differ in the mechanism of lesion recognition: global genome-NER (GG-NER) that surveys the entire genome for distortions, and transcription-coupled repair (TCR), which targets damage that blocks elongating RNA polymerases [19, 56]. NER, therefore, plays a particularly important role in preventing mutations. Thus far, three syndromes, xeroderma pigmentosum, Cockayne syndrome, and trichothiodystrophy (TTD), are closely associated with NER defects [56]. Of these, patients with xeroderma pigmentosum, attributable to mutations in one of the seven xeroderma pigmentosum (XP) group genes (*XPA*–*XPG*), show a dramatically increased incidence of UV light-induced skin cancer [19, 56].

It was reported recently that the *XPC* promoter is epigenetically inactivated in bladder cancer [57] (Table 1.1). *XPC* promoter methylation is significantly elevated

in cancerous bladder compared to normal tissue, leading to reduced mRNA levels in the tumor [57]. Epigenetic defects in the *XPC* gene may also influence malignant behavior and prognosis. ERCC1 is a crucial protein in the NER pathway primarily involved in the repair of platinum-DNA adducts. Aberrant CGI methylation in the *ERCC1* promoter region has been observed in human glioma cell lines and primary tumors, which is associated with cisplatin chemosensitivity [58]. In a rat lung cancer model, however, *ERCC1* methylation is detected in only a very small proportion of samples [59]. Deficiency in XRCC1, a scaffolding protein for BER and single-strand break repair (SSBR), is associated with enhanced risk of lung cancer [60]. *XRCC1* is subject to aberrant promoter methylation in human gastric cancer tissues [61]. In lung cancer, infiltrating carcinomas exhibit statistically higher levels of methylation at the *XRCC1* promoter compared to normal, hyperplastic, and squamous metaplastic tissues [59]. RAD23B, a key component for damage recognition in NER, is also hypermethylated in multiple myeloma [62].

1.2.3 Epigenetic Inactivation of HR and Nonhomologous End-Joining DNA Repair Pathway Components

HR not only provides an important mechanism to repair several types of DNA lesions that pose a threat to genome integrity, including DNA DSBs, DNA damage encountered during DNA replication, and DNA interstrand cross-links (ICLs), but is also required to restart stalled replication forks during the late S and G2 phases of the cell cycle [63, 64]. HR promotes precise repair of DNA damage using the intact sister chromatid as a template. Deficiency of HR leads to more error-prone repair, which is associated with mutagenesis and predisposition to cancer [63].

The *BRCA1* and *BRCA2* genes are both essential for HR-mediated DNA repair. BRCA1 appears to act as a signal integrator that links DNA damage sensors with response mechanisms. BRCA2, however, is more directly involved in homology-directed DSB repair, as it mediates formation of a RAD51-DNA nucleoprotein filament that catalyzes strand invasion during HR. *BRCA1* and *BRCA2* are frequently mutated in hereditary breast and ovarian cancers, but seldom in sporadic cases of these tumor types. Epigenetic inactivation of *BRCA1* via promoter hypermethylation, however, plays an important role in tumorigenesis in a wide array of cancers including breast [65, 66], ovarian [67], gastric [68], bladder [69], and NSCLCs [70], both hereditary [71] and sporadic forms [20, 39] (Table 1.1). It is believed that epigenetic silencing of BRCA1 creates a new mutator pathway that generates mutations and gross chromosomal rearrangements via p53 signaling. This idea is supported by several observations including one demonstrating that p53 inactivation rescues the impact of BRCA1 deficiency on cell survival [20, 72]. Although much less frequently than *BRCA1*, *BRCA2* also acquires promoter region hypermethylation that is closely associated with its reduced expression in breast cancer [51] and NSCLC [70] (Table 1.1).

The primary function of the Fanconi anemia (FA) pathway is to repair interstrand DNA cross-links, which promotes HR via coordinating other DNA damage-responsive events to stabilize stalled replication forks, to convey signals to DNA checkpoint pathways, and to facilitate recovery of replication forks [73]. FA is a genomic instability syndrome characterized by bone marrow failure, developmental abnormalities, and increased cancer incidence, which is caused by mutations in one of thirteen distinct genes (*FANCA*, *FANCB*, *FANCC*, *FANCD1*, *FANCD2*, *FANCE*, *FANCF*, *FANCG*, *FANCI*, *FANCJ*, *FANCL*, *FANCM*, and *FANCN*) [73]. Eight of them (FANCA, B, C, E, F, G, L, and M) form the FA core complex. This group of genes contains a high GC content and CGIs at their promoter regions, making them potential targets for aberrant hypermethylation-mediated silencing [74]. This idea has received support from observations that *FANCC*, *FANCF,* and *FANCL* acquire promoter methylation during human carcinogenesis [39, 75]. Of these, *FANCF* displays hypermethylation the most frequently, occurring in 14–28% of different cancers including NSCLC [76], HNSCC [76], cervical [77], and ovarian [39, 78] (Table 1.1).

Unlike HR, which performs error-free repair, nonhomologous end-joining (NHEJ) simply restores DNA integrity by joining the two DNA ends. This type of repair is error-prone and frequently results in the loss or addition of several nucleotides at the break site. Despite its mutagenic consequences, NHEJ is the major DSB repair pathway in mammalian cells. Defects in NHEJ lead to chromosomal translocations and genomic instability. In NHEJ, DSBs are detected by the KU70/KU80 heterodimer; the KU complex then activates the protein kinase DNA-PKcs (DNA-dependent protein kinase catalytic subunit), leading to recruitment and activation of end-processing enzymes, polymerases, and finally ligation of the breaks by the XRCC4/DNA ligase IV complex. In the NHEJ pathway, only the *XRCC5* gene, encoding the KU80 protein, has been reported to be inactivated via epigenetic mechanisms [70] (Table 1.1). Low expression of XRCC5 in squamous cell carcinoma and NSCLC is significantly associated with promoter region hypermethylation. Treatment of NSCLC cells with the DNA demethylating agent 5-aza-2′-deoxycytidine (5-aza-CdR), however, does not result in increased KU80 expression [70]. Thus, the underlying mechanisms promoting and maintaining XRCC5 silencing await further investigation, particularly in more samples and more types of cancer.

1.2.4 Epigenetic Silencing of O⁶-Methylguanine-DNA Methyltransferase

O^6-methylguanine, which arises due to alkylation reactions, pairs with thymine rather than cytosine, resulting in G:C to A:T mutations during DNA replication. O^6-methylguanine-DNA methyltransferase (MGMT), also known as O^6-alkylguanine-DNA alkyltransferase (AGT), repairs DNA damage by transferring the methyl groups on the O^6 position of guanine to an active site cysteine residue to protect cells from sustaining mutagenic events, which has been demonstrated by gain- or loss-of-function experiments in vitro and in vivo [79]. The MGMT protein is unique among DNA-repair components because it acts alone to remove DNA adducts.

Although MGMT is ubiquitously expressed in normal human tissues, mean enzymatic activity in malignant tissues is usually higher than in their normal counterparts. However, there is a variety of tumors such as glioma, CRC, NSCLC, and HNSCC that lack MGMT expression [20, 39] (Table 1.1). It has been well documented that MGMT deficiency often arises due to abnormal promoter methylation [20, 39, 80]. For example, 29% of NSCLCs and 38% of CRCs display aberrant *MGMT* methylation, in which the presence of hypermethylation is highly associated with loss of MGMT protein [81]. *MGMT* is the most frequently methylated gene in central nervous system tumors. Epigenetic silencing of *MGMT* via promoter hypermethylation occurs in approximately 40% of primary glioblastomas and over 70% of secondary glioblastomas. It is also detected in 50% of the diffuse and anaplastic astrocytomas and approximately two-thirds of oligodendroglial and mixed tumors [82]. These results, together with a causal relationship between DNA methylation of the *MGMT* CGI and decreased transcription of the gene in cell culture-based studies, demonstrate that DNA methylation is an important mechanism for silencing the *MGMT* gene in human cancers.

Epigenetic silencing of *MGMT* may initiate an important mutator signaling cascade in human cancers since MGMT loss causes G:C to A:T transitions, which lead to downstream gene mutations. This proposal is strongly supported by an analysis of point mutations in *KRAS* and *p53*. KRAS, the most commonly altered oncogene in cancer, is an early key player in multiple signal pathways. Loss of MGMT is associated with increased *KRAS* mutations possessing G:C to A:T transitions in colon [83] and gastric cancer [84]. *p53* is the most frequently mutated tumor suppressor gene (TSG) in human cancer, and the majority of known *p53* mutations are G:C to A:T transitions [66, 85]. Epigenetic inactivation of *MGMT* may lead to G:C to A:T transition mutations in *p53*, which has been observed in several types of cancer including colorectal [66], liver [86], lung [87], esophageal squamous cell carcinomas [88], and glioma [89]. Interestingly, *MGMT* promoter methylation is associated with improved disease chemosensitivity and prolonged survival time in patients treated with alkylating agent-based therapies [90]. However, it is unclear whether the improved survival is specifically due to loss of MGMT expression or accompanying drug sensitivity.

1.2.5 Epigenetic Silencing of WRN

Werner syndrome (WS) is a rare autosomal recessive disease, characterized by premature onset of aging, genomic instability, and increased cancer incidence. WS is caused by null mutations at the *WRN* locus at 8p11.2–p12, which codes for a DNA helicase belonging to the RecQ family. Deficiency in WRN function causes defects in DNA replication and recombination, as well as DNA repair.

WRN is a 180-kd nuclear protein that has a unique interaction with its DNA substrates through its C terminal RQC domain during base separation [91]. In addition to two C-terminal ATPase domains encoding for helicase activity, the WRN protein contains an N-terminal domain coding for exonuclease activity. Its helicase

and exonuclease activities function in a coordinated manner, suggestive of roles in DNA repair, recombination, and replication. Recently, the WRN protein was also shown to be involved in telomere maintenance based on the discovery that its deficiency leads to accelerated telomere shortening in WS cells [92]. These multiple roles of the WRN protein highlight its importance in aging and cancer.

The evidence suggesting that WRN acts as a TSG is derived primarily from WS, which is characterized by the early onset development of a variety of cancers due to germ line *WRN* mutation; somatic mutations in the *WRN* gene have not been reported. Epigenetic inactivation of *WRN* provides additional support for its TSG role in sporadic cancer. The *WRN* promoter undergoes hypermethylation in a wide array of tumors including colorectal, gastric, prostate, non-small cell lung, and breast cancers [93, 94] (Table 1.1). Epigenetic silencing of *WRN* via methylation not only leads to the loss of protein and enzyme activity, but also to chromosomal instability. Furthermore, the above phenotype is reversed by DNA-demethylating agents. Most importantly, restoration of WRN expression induces its tumor-suppressor effects, such as inhibition of colony formation and tumor growth [93]. Taken together, aberrant epigenetic silencing of *WRN*, a candidate TSG, may play an important role in human cancers. Interestingly, WRN was recently shown to be associated with promoter methylation of the *OCT4* gene [95], which encodes a crucial transcription factor for the maintenance of cell pluripotency. During differentiation of human pluripotent NCCIT embryonic carcinoma cells, WRN localizes to the *OCT4* promoter region with *de novo* DNA methyltransferase DNMT3B and promotes differentiation-dependent *OCT4* silencing and promoter methylation [95]. Deficiency in WRN blocks DNMT3B recruitment to the promoter and leads to decreased promoter methylation of *OCT4* [95]. Therefore, WRN may also contribute to the control of stem cell differentiation via epigenetic silencing of the key pluripotency transcription factor OCT4.

1.2.6 Epigenetic Inactivation of ATM/ATR Signaling

DNA damage signaling requires the coordinated action of a large array of molecules that can be categorized as DNA damage sensors, transducers, mediators, and effectors according to their functions. Upon damage of DNA, the MRE11–RAD50–NBS1 (MRN) sensor complex recognizes DSBs and the replication protein A (RPA) complex processes accumulated single-stranded DNA (ssDNA). The transducer ataxia-telangiectasia mutated (ATM) and ATR kinases are recruited to and activated by DSBs and RPA-coated ssDNA, respectively. With the help of mediators (including 53BP1, MDC1, BRCA1, MCPH1, and PTIP in ATM signaling, and TopBP1 and Claspin in ATR signaling), ATM and ATR activate the effector kinases CHK2 and CHK1, respectively, which then spread the signal throughout the nucleus [96–98]. CHK1 and CHK2 decrease cyclin-dependent kinase (CDK) activity, which slows down or arrests cell cycle progression. Meanwhile, ATM/ATR

signaling promotes DNA repair through various mechanisms. Through ATM/ATR signaling, DNA repair and cell cycle progression are closely coordinated. The coordinated action of DNA repair and cell cycle controls either promotes the resumption of normal cell functioning before replication or triggers apoptosis/cell death when normal cell functioning cannot be restored; both mechanisms act as barriers to tumorigenesis [19].

Ataxia-telangiectasia (AT) is a rare autosomal recessive disorder, characterized by progressive cerebellar ataxia, oculocutaneous telangiectasia, susceptibility to bronchopulmonary disease, and lymphoid tumors. AT is caused by deficiency in the *ATM* gene, localized on chromosome 11q22–23. ATM is an Ser/Thr protein kinase of the phosphoinositide 3-kinase (PI3K)-related protein kinase (PIKK) family, which also includes ATR, DNA-PKcs, and SMG1. ATM may have as many as 700 substrates [99, 100], highlighting its multiple functions in various biological processes including cancer. Loss of heterozygosity in *ATM* results in reduced protein expression; however, this mechanism explains only a small proportion of cancers where ATM down-regulation is observed. In sporadic cancer, which accounts for 90–95% of tumors, the probability of *ATM* gene mutations is low, whereas altered expression of ATM is frequently observed. It is therefore likely that epigenetic modifications have an impact on ATM expression in these cases (Table 1.1). Initial proof for this idea came from studies using the human colon cancer cell line HCT116 [101]. In this cell line, *ATM* displays aberrant promoter methylation, which inversely correlates with its low expression and low radiosensitivity. The significance of this finding is underscored by further observations that treatment of HCT116 cells with 5-azacytidine (a DNA demethylating agent) restores expression of ATM and radiosensitivity [101]. *ATM* is also epigenetically silenced in primary cancers. For example, 78% of surgically removed breast tumors [102] and 25% of HNSCC [103] display aberrant methylation in the *ATM* promoter region accompanied by reduced ATM.

CHK2, the mammalian homologue of the yeast Rad53 and Cds1, is located at chromosome 22q12.1, spans approximately 50 kb, and consists of 14 exons [104]. CHK2, activated by ATM, responds primarily to DSBs. Its fundamental role is to coordinate cell cycle progression with DNA repair and cell survival or death. Germ line mutations in the *CHK2* gene predispose to Li–Fraumeni syndrome (LFS), characterized by multiple tumors at early age with a predominance of breast cancer and sarcomas [105]. Somatic mutations in *CHK2* exist also, although they occur in only a small subset of sporadic human malignancies, including carcinomas of the breast, lung, colon, and ovary, osteosarcomas, and lymphomas [106]. The finding of both germ line and somatic mutations suggests that *CHK2* acts as a TSG. This is further supported by the observation that down-regulation of CHK2 is associated with promoter methylation in sporadic cancers including lung cancer, glioma, and Hodgkin's lymphoma [107–109]. For example, DNA hypermethylation of the distal *CHK2* CGIs occurs in 28.1% of NSCLCs and 40.0% of squamous cell carcinomas, which inversely correlates with *CHK2* mRNA levels. It should be noted, however, that observations in breast, colon, and

ovarian cancers do not support a causative link between DNA methylation and gene expression of CHK2 [110, 111].

1.3 DNA Methyltransferase 1 and Mismatch Repair

The function of the MMR pathway is to correct base substitution mismatches and insertion–deletion mismatches generated in newly replicated DNA [112]. Deficiencies in or inactivation of this pathway has profound biological consequences. Loss of MMR activity is attributed to the initiation and promotion of multistage carcinogenesis [113]. A growing number of reports have demonstrated that loss of DNMT1 function has a significant impact on MSI—a hallmark of MMR efficiency, suggesting it has a role in the MMR pathway (Fig. 1.1). Using genetic screens in *Blm*-deficient embryonic stem (ES) cells, *Dnmt1* was identified as an MMR modifier gene. *Dnmt1* deficiency in murine ES cells results in a fourfold increase in the MSI rate [13]. Further support for this finding comes from several other laboratories [14–17, 114]. DNMT1 deficiency enhances microsatellite mutations for both integrated reporter genes [13, 14, 16, 17] and endogenous repeats [15]. This finding holds true for both ES cells and somatic cells. In a murine ES cell line with homologous deletion of *Dnmt1*, the stability of five endogenous microsatellite repeats (two mononucleotides and three dinucleotides), exhibiting instabilities in MMR-deficient cells was analyzed. A significantly higher frequency of instability was detected at three of the five markers in *Dnmt1*$^{-/-}$ ES cells compared to the wild-type ES cells [15]. The slippage rate of a stable reporter gene was also monitored. Dnmt1 deficiency led to a sevenfold higher rate of microsatellite slippage in *Dnmt1*$^{-/-}$ ES cells compared to wild-type cells [14]. Notably, no DNA methylation in the region flanking the reporter gene was discovered, regardless of Dnmt1 status, suggesting that the effect of Dnmt1 on MMR was not at the level of DNA methylation [14]. Enhanced MSI is associated with higher levels of histone H3 acetylation and lower MeCP2 binding at regions near the assayed microsatellite, suggesting that Dnmt1 loss decreases MMR efficiency by modifying chromatin structure. CAG repeat expansions are closely associated with human age-related diseases including 12 neurodegenerative disorders. Repeat instability induced by CAG repeat expansion requires the MMR components [16, 115]. DNMT1 deficiency induces destabilization and intergenerational expansion of CAG triplet repeats [16]. Double knockdown of MLH1 and DNMT1, however, additively increases the frequency of CAG contraction [114]. Specific targeting of DNMT1 in hTERT-immortalized normal human fibroblasts by siRNA induces both resistance to MSI and the drug 6-thioguanine (which induces cytotoxic DNA damage due to its misincorporation opposite thymine [116]) at a CA17 reporter gene; two hallmarks of MMR deficiency. Mutation rates correspond well with DNMT1 levels, ranging from 4.1-fold in cells with 31% of the normal DNMT1 protein level to tenfold in cells with 12% of the normal DNMT1 protein level [17]. This suggests that DNMT1 regulates microsatellite stability in a dose-dependent manner. The exact underlying mechanism of how

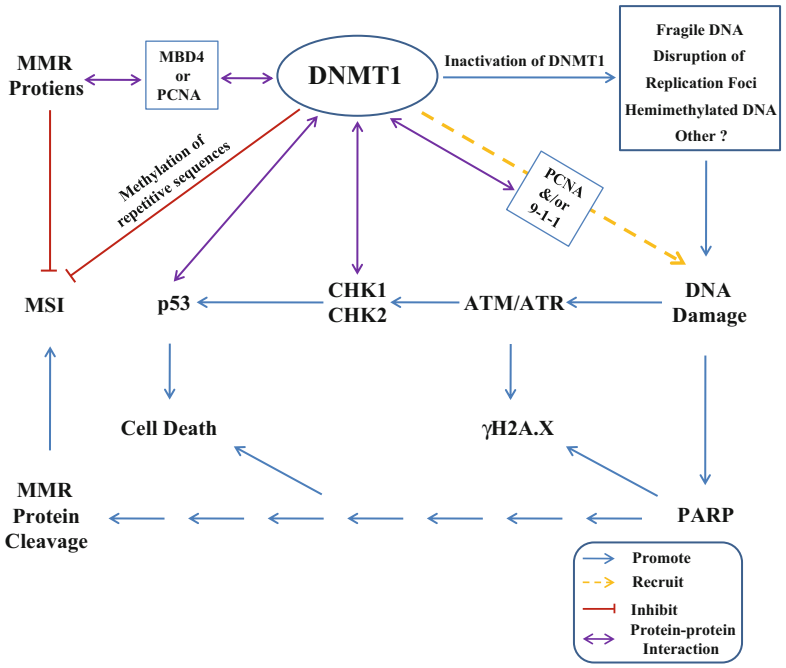

Fig. 1.1 Impact of DNMT1 on MMR and DDR. DNMT1 may promote stabilization of microsatellites via methylation of CpG repeats and it also interacts with DNA repair proteins via third-party mediators (e.g., MBD4 and PCNA). Moreover, deficiency in DNMT1 leads to activation of PARP signaling, eventually resulting in MMR protein cleavage. DNMT1 is also closely associated with DDR. Inactivation of DNMT1 may induce several changes to DNA and/or chromatin including increased DNA fragility, disruption of replication foci, and accumulation of hemimethylated DNA, which may be recognized as "damage" and activate the DDR. Strong support for a direct link between DNMT1 and DDR comes from the identification of several protein-protein interactions involving DNMT1 and DDR proteins. DNMT1 is recruited to sites of DNA damage via its interaction with PCNA and 9-1-1. DNMT1 is also capable of binding CHK1 and p53, which promote cell cycle arrest and apoptosis, respectively

DNMT1 is involved in MSI appears complex and remains elusive. Microsatellite methylation probably provides a mechanism for length stabilization by subsequent transcriptional repression of genes containing or proximal to microsatellites with methylated CpG repeats. However, increased mutations usually occur at microsatellite repeats that do not contain any CpG sites in the repeat itself [13, 15, 16, 114] or nearby [14], indicating that DNA methylation changes around microsatellite repeats, at least in some cases, are not the primary cause of the instability. Alternatively, DNMT1 might influence transcriptional repression and MSI through chromatin remodeling [14].

The impact of DNMT1 on the MMR pathway is further highlighted by the observation that DNMT1 and the MMR proteins probably interact with each other through a third-party mediator (Fig. 1.1). The methyl CpG-binding protein MBD4/MED1 may provide a functional link between MMR and DNMT1 through protein–protein

interaction. MBD4, which possesses glycosylase repair activity for G:T mismatches, is involved in NER as well as MMR. MBD4 binds MLH1 via its C-terminal glycosylase domain [117, 118]. Deletion of *Mbd4* in MEFs induced destabilization of MMR proteins and conferred resistance to antitumor drugs including 5-FU and platinum [119]. MBD4 and TDG have functional overlap and they interact with the de novo methyltransferases DNMT3A and DNMT3B [120, 121]. MBD4 also interacts with maintenance methyltransferase DNMT1 via its N-terminal MBD domain [118]. Based on a combination of immunoprecipitation and GST-pull down experiments in mouse, rat, and *Xenopus*, a minimal domain of approximately 70 amino acids in the N-terminal targeting sequence region of DNMT1 was shown to be required for MBD4 to bind to DNMT1 [118], which overlaps with a region in rat DNMT1 that interacts with MECP2 [122]. Through interacting directly with both DNMT1 and MLH1, MBD4 recruits MLH1 to heterochromatic sites that are coincident with DNMT1 localization [118]. Similarly, MBD4/MLH1 accumulates at DNA damage sites where DNMT1 is recruited after laser microirradiation [118]. Loss of DNMT1 induces p53-dependent apoptosis, which can be rescued by inactivation of p53 [123]. The MBD4/MLH1 complex also mediates the apoptotic response to DNMT1 depletion [118]. Colocalization of these proteins at damaged regions implies that they function coordinately in the cellular decision to repair the lesion or activate apoptosis. Like MBD4, PCNA may act as a mediator between MMR and DNMT1 because of its direct interaction with both systems. PCNA interacts with multiple components of the MMR pathway including MSH6, MSH3, and MLH1. Disruption of this interaction confers an MMR defect in vivo and in vitro [124–126]. Both MSH6 and MSH3 colocalize with PCNA at replication foci during S-phase [127]. MLH1 is recruited to damage sites where PCNA and DNMT1 also accumulate, although with slower kinetics than DNMT1 [118, 128]. The recruitment of DNMT1 to both the replication fork and DNA damage sites is through a direct interaction with PCNA and possibly CHK1 and the 9-1-1 complex as well [21, 24]. However, there is no report showing that PCNA, MLH1, and DNMT1 colocalize together, implying that PCNA might interact with each protein at a different time. Nonetheless, the functional mechanisms of whether and how these factors are orchestrated in response to DNA damage requires further investigation.

Most recently, DNMT1 deficiency has been shown to induce the depletion of multiple repair factors at the protein level (Fig. 1.1) [17], highlighting its importance not only in MMR efficiency, but also in DDR signaling. In normal human fibroblasts and CRC cell lines, DNMT1 knockdown leads to a matching decrease in MLH1 at the protein, but not the mRNA level [17]. Loss of MLH1, however, does not lead to expression changes in DNMT1 [17]. Promoter hypermethylation of *MLH1*, although frequently observed in sporadic colon cancers [39], does not appear to be the cause leading to gene inactivation in the context of DNMT1 deficiency. *MLH1* hypermethylation in DNMT1-deficient cells was further ruled out using a bisulfite pyrosequencing assay [17]. Further observations suggest that DNMT1 deficiency affects the steady-state levels of a number of repair proteins, including MSH2, MSH6, and PMS2, as well as MBD4 [17]. Loss of multiple MMR components in DNMT1 hypomorphic cells indicates that DNMT1 might play an

indirect role in the stabilization or proteolytic cleavage of these proteins, rather than directly interacting with each of them. It is documented that DNMT1 deficiency activates the DDR, which leads to cell cycle arrest [21, 123] and the triggering of cell death pathways [123] that may result in cleavage of proteins including MLH1 [129], which might account for MMR protein depletion after DNMT1 knockdown. Loss of DNMT1 activates ATM/ATR, which normally phosphorylate H2A.X leading to focal accumulation of γH2A.X, a hallmark of DDR [21]. If excessive damage exists, p53-dependent [123] and other cell death pathways are activated to maintain genomic integrity. Elevated γH2A.X levels in DNMT1 hypomorphic cells can be partially reduced through inhibition of ATM/ATR signaling [17]. However, the PAR polymerase (PARP) inhibitor DPQ also reduces the level of γH2A.X, to an extent exceeding that observed with the ATM/ATR inhibitor caffeine. In keeping with these observations, the viability of DNMT1-depleted cells treated with DPQ is enhanced to a greater extent than treatment of cells with agents that inhibit caspases or p53 [17]. These findings, together with the observation that PARylation increases after DNMT1 loss, clearly demonstrate that PARP is involved in the DDR and cell death process in cells deficient in DNMT1 (Fig. 1.1). PARP catalyzes the polymerization of ADP-ribose (PAR) units on target proteins using nicotinamide adenine dinucleotide (NAD^+) molecules as a donor [130]. NAD^+ depletion, induced by severe DNA damage, gives rise to mitochondrial membrane depolarization and apoptosis initiation factor (AIF) translocation. It eventually results in an activation of caspases that lead to protein cleavage and cell death. DNA repair protein MLH1 [129], along with BLM1 [131] and ATM [132], are preferred targets of caspases. Treatment with the PARP inhibitor DPQ, as expected, leads to an increase in full-length MLH1 protein levels in DNMT1-depleted cells [17]. Taken together, DDR signaling, particularly the cell death pathway mediated by PARP, may play a substantial role in regulating cleavage of MMR repair proteins in cells deficient for DNMT1 (Fig. 1.1).

1.4 DNMT1 and the DNA Damage Response

Reduction of DNMT1 levels activates a DDR usually initiated by the most lethal form of DNA damage-DSBs (Fig. 1.1). DNMT1 deficiency also inhibits DNA replication [22, 23, 133]. It was reported that DNMT1 knockdown triggers an intra-S-phase arrest of DNA replication, independent of DNA demethylation [22]. Similar to the observations for DNA damage checkpoints [134], the intra-S-phase arrest is transient, disappearing after 10 days of treatment with *DNMT1* siRNA. The S-phase cells induced by DNMT1 knockdown exist in two distinct populations: 70% incorporate BrdUr, while 30% do not, consistent with the presence of an intra-S-phase checkpoint triggering cell cycle arrest [134]. Cells are arrested at different positions throughout S-phase, suggesting that this response is not specific to distinct classes of origins of DNA replication. 5-aza-CdR, a nucleoside analogue, is a well-characterized and widely used inhibitor of DNA methylation, which inhibits

DNA methylation by trapping DNMT1 at the replication fork after being incorporated into DNA. 5-aza-CdR does not inhibit the de novo synthesis of DNMT1 protein or its presence in the nucleus. S-phase cells treated with 5-aza-CdR, which causes genome-wide demethylation, do not exhibit two distinct population distributions as observed in cells deficient in DNMT1. These results suggest that the intra-S-phase arrest is not correlated with the degree of DNA methylation, consistent with observations that DNA replication arrest following DNMT1 inhibition is probably due to a reduction in the physical presence of DNMT1 at the replication fork, rather than DNA demethylation [133]. As discussed above, the cell cycle distribution in DNMT1 knockdown cells resembles the transient intra-S-phase arrest in DNA replication that is evoked by genotoxic insults [135–137]. In addition, DNMT1 inhibition also leads to the induction of a set of genes that are implicated in the genotoxic stress response including *p21* [133], *p53* [123], and the growth arrest DNA damage inducible 45β gene (GADD45*β*) [22]. These results imply that DNMT1 is linked to DNA damage repair machineries to maintain chromosome integrity via blocking DNA replication, a notion further strengthened by observations that DNMT1 knockdown activates the checkpoint pathways in an ATR-dependent manner [23]. Upon DNMT1 depletion, CHK1 and CHK2, key proteins in ATM/ATR signaling, are phosphorylated, which in turn induce phosphorylation and degradation of cell division control protein 25 A (CDC25A) as well as CDC25B [23]. As a consequence, the capacity for loading CDC45, an essential factor for DNA replication [138], onto replication forks is decreased, resulting in replication arrest. DNMT1 knockdown also induces the formation of histone γH2A.X foci, a hallmark of the DNA DSB response. The response elicited by DNMT1 knockdown is blocked by siRNA-mediated depletion of ATR, suggestive of its ATR dependency. Further support for the importance of ATR came from the finding that the cellular response to DNMT1 depletion is markedly attenuated in cells derived from a patient with Seckel syndrome, a disorder due to ATR deficiency [23]. However, it is not clear whether ATM, another key transducer like ATR in the checkpoint pathway, is involved in the process or not. DNA demethylating agents do not trigger the stress response like genetic DNMT1 depletion does [23]. Moreover, this response is abolished by ectopic expression of either wild-type DNMT1 or a mutant form of DNMT1 lacking the catalytic domain [23], suggesting that loss of catalytic activity of DNMT1 is not driving this response. Also of importance, DNMT1 knockdown leads to very limited genomic demethylation [22, 23], consistent with observations made in cells containing hypomorphic mutations in *DNMT1* [139, 140]. One explanation for this limited demethylation is that de novo DNMTs compensate for the reduction of DNMT1 activity [139]. Another possibility is that DNMT1 loss triggers a checkpoint pathway (Fig. 1.1) to block DNA replication, preventing loss of DNA methylation in an attempt to maintain genome stability. Double knockdown of DNMT1 and ATR does indeed induce global DNA demethylation, whereas single knockdowns of either DNMT1 or ATR do not, implying that the arrest of DNA replication activated by ATR signaling following DNMT1 depletion prevents loss of DNA methylation and that blocking this

response results in global loss of DNA methylation [23]. Taken together, it appears that reduction of DNMT1 levels activates ATR signaling to block DNA replication in a DNA methylation-independent manner (Fig. 1.1). How this response to DNMT1 reduction is initiated, however, is still uncertain. It is possible that removal of DNMT1 from replication forks disrupts fork progression and eventually results in DSBs that elicit checkpoint signaling (Fig. 1.1). Alternatively, the presence of low levels of hemimethylated DNA due to the absence of DNMT1 may trigger this response (Fig. 1.1).

Complete inactivation of DNMT1 via genetic mechanisms also activates the DDR and causes genomic demethylation. The degree of demethylation, however, varies greatly depending on cellular context, ranging from 20% loss in human cancer cells [141] to 90% loss of genomic methylation in murine ES cells [7, 8]. As the principal enzyme responsible for maintaining DNA methylation, DNMT1 is essential for embryonic development and cell survival. Disruption of *Dnmt1* in mice results in loss of 90% of genomic methylation and embryonic lethality [7, 8]. Murine ES cells deficient for *Dnmt1* die when introduced to differentiate [7], mouse fibroblasts die within 2–4 cell divisions after conditional deletion in *Dnmt1* [123], and the human colon cancer cell line HCT116 undergoes marked apoptosis and cell death within one cell division if *DNMT1* is completely inactivated by cre-mediated conditional knockout [141, 142]. Notably, complete inactivation of DNMT1 triggers the DDR before cells die [141]. Deletion of *DNMT1* activates p53 [123, 141], a target of ATM whose phosphorylation correlates with accumulation of p53 in response to DNA damage [143]. Disruption of both alleles of *DNMT1* leads to activation of the G2/M checkpoint and G2 arrest, as verified by the presence of phosphorylated ATM and γH2A.X at discrete nuclear DNA damage foci [141]. Further support for checkpoint activation comes from the finding that treatment of cells with an ATM/ATR inhibitor, caffeine, facilitates mitotic entry and cell death in *DNMT1* null cells [141]. Most of these cells, however, eventually escape G2 arrest and reenter interphase with their unrepaired DNA, resulting in severe chromosomal and mitotic abnormalities (mitotic catastrophe) [141]. Thus far, the mechanisms by which DNMT1 inactivation leads to activation of DNA damage repair remains elusive. In the complete absence of DNMT1, DNA may become more fragile owing to reduced methylation and/or defective chromatin structure in critical regions of the genome, leading to activation of DNA damage signaling (Fig. 1.1) [142]. Alternatively, the accumulation of hemimethylated DNA in *DNMT1* mutant cells may be recognized as damage and trigger the damage response (Fig. 1.1). Both of these possibilities are consistent with the observation that agents that affect overall chromatin structure without damaging DNA also activate ATM [144]. Nonetheless, it cannot be excluded that oncogene activation or gene mutations initiate the DDR, as Dnmt1-deficient ES cells exhibit significantly increased mutation rates, particularly in the form of deletions and mutations [145].

Recruitment of DNMT1 to sites of DNA damage has been observed by our laboratory [21, 146] and others [24], providing compelling evidence to support the notion that DNMT1 is directly involved in DNA damage repair (Fig. 1.1).

Immediately after laser microirradiation-induced DSBs, an accumulation of DNMT1 and PCNA occurs at the damage sites in S and non-S phase cells, colocalizing with γH2A.X—a marker of DSBs. Recruitment of DNMT1 to damage sites is dependent on its interaction with PCNA through its PCNA-binding domain (PBD) [21, 24], but is independent of its catalytic activity [21]. In addition to PCNA, DNMT1 also interacts with other components of the DNA damage machinery including CHK1 [21, 146] and the 9-1-1 complex [21]. PCNA, along with CHK1 and 9-1-1, is essential for DNMT1's recruitment to DNA damage sites. After recruitment to damaged regions, DNMT1 modulates the rate of ATR signaling and is essential for suppressing abnormal activation of the DDR in the absence of exogenous damage [21]. Taken together, these data have revealed a direct link between DNMT1 and the DNA damage repair process.

PCNA mediates recruitment of DNMT1, not only to DNA replication sites, but also to DNA damage sites. The DNMT1–PCNA interaction implies that the role of DNMT might be to restore epigenetic information after damage repair. However, recent studies demonstrate that this interaction is not essential for maintaining DNA methylation [5, 147]. Furthermore, the observation [21] that DNMT1 is very rapidly recruited and retained only transiently, likely before resynthesis is completed, suggest that genomic methylation is not the main function of DNMT1 at these sites, at least in the early part of the DDR. The recruitment kinetics of WT *DNMT1* and *DNMT1* with a point mutation in the catalytic domain are almost identical [21]. CHK1/CHK2 activation and γH2A.X foci formation induced by DNMT1 deficiency are rescued by expression of a catalytically inactive form of DNMT1 [23]. Therefore, although the possibility that DNMT1 participates in the restoration of DNA methylation patterns during damage repair cannot be excluded, it seems more likely that DNMT1 functions in sensing and/or mobilizing the response to certain forms of DNA damage (Fig. 1.1).

In summary, both DNMTs and DNA damage repair systems have evolved to maintain genomic integrity and disruption of these pathways contributes to the development of cancer [19]. Therefore, we have examined and outlined the interaction of DNMTs and DNA methylation with DNA damage repair systems and have discussed possible mechanisms for how the two systems may function coordinately to deal with DNA damage. Promoter methylation, catalyzed by DNMTs, plays an established role in silencing key genes in multiple DNA damage repair pathways; inactivation of these pathways may predispose to a large array of tumors [20]. These findings are consistent with observations that TSGs are frequently silenced via epigenetic mechanisms in cancer cells. Unexpectedly perhaps, more recent observations strongly suggest that DNMTs, particular DNMT1, are directly involved in DNA damage repair systems via what is likely to be a DNA-methylation-independent mechanism [17, 21–23, 141]. The exact nature of the links between the DNMTs, DNA methylation, and DNA damage repair systems is complex and remains to be further investigated. A more thorough understanding of these links will not only help dissect the mechanisms of tumor development, but also identify new antitumor targets and therapeutic strategies.

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References

1. Robertson KD (2005) DNA methylation and human disease. Nat Rev Genet 6(8):597–610
2. Kareta MS, Botello ZM, Ennis JJ, Chou C, Chedin F (2006) Reconstitution and mechanism of the stimulation of de novo methylation by human DNMT3L. J Biol Chem 281(36): 25893–25902
3. Okano M, Bell DW, Haber DA, Li E (1999) DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell 99(3):247–257
4. Okano M, Xie S, Li E (1998) Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases. Nat Genet 19(3):219–220
5. Egger G, Jeong S, Escobar SG et al (2006) Identification of DNMT1 (DNA methyltransferase 1) hypomorphs in somatic knockouts suggests an essential role for DNMT1 in cell survival. Proc Natl Acad Sci USA 103(38):14080–14085
6. Riggs AD, Xiong Z (2004) Methylation and epigenetic fidelity. Proc Natl Acad Sci USA 101(1):4–5
7. Li E, Bestor TH, Jaenisch R (1992) Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. Cell 69(6):915–926
8. Lei H, Oh SP, Okano M et al (1996) De novo DNA cytosine methyltransferase activities in mouse embryonic stem cells. Development 122(10):3195–3205
9. Li E (2002) Chromatin modification and epigenetic reprogramming in mammalian development. Nat Rev Genet 3(9):662–673
10. Yoder JA, Walsh CP, Bestor TH (1997) Cytosine methylation and the ecology of intragenomic parasites. Trends Genet 13(8):335–340
11. Gonzalo S, Jaco I, Fraga MF et al (2006) DNA methyltransferases control telomere length and telomere recombination in mammalian cells. Nat Cell Biol 8(4):416–424
12. Xu GL, Bestor TH, Bourc'his D et al (1999) Chromosome instability and immunodeficiency syndrome caused by mutations in a DNA methyltransferase gene. Nature 402(6758): 187–191
13. Guo G, Wang W, Bradley A (2004) Mismatch repair genes identified using genetic screens in Blm-deficient embryonic stem cells. Nature 429(6994):891–895
14. Kim M, Trinh BN, Long TI, Oghamian S, Laird PW (2004) Dnmt1 deficiency leads to enhanced microsatellite instability in mouse embryonic stem cells. Nucleic Acids Res 32(19): 5742–5749
15. Wang KY, James Shen CK (2004) DNA methyltransferase Dnmt1 and mismatch repair. Oncogene 23(47):7898–7902
16. Dion V, Lin Y, Hubert L Jr, Waterland RA, Wilson JH (2008) Dnmt1 deficiency promotes CAG repeat expansion in the mouse germline. Hum Mol Genet 17(9):1306–1317
17. Loughery JE, Dunne PD, O'Neill KM, Meehan RR, McDaid JR, Walsh CP (2011) DNMT1 deficiency triggers mismatch repair defects in human cells through depletion of repair protein levels in a process involving the DNA damage response. Hum Mol Genet 20(16): 3241–3255
18. Karpf AR, Matsui S (2005) Genetic disruption of cytosine DNA methyltransferase enzymes induces chromosomal instability in human cancer cells. Cancer Res 65(19):8635–8639
19. Jackson SP, Bartek J (2009) The DNA-damage response in human biology and disease. Nature 461(7267):1071–1078

20. Jacinto FV, Esteller M (2007) Mutator pathways unleashed by epigenetic silencing in human cancer. Mutagenesis 22(4):247–253
21. Ha K, Lee GE, Palii SS et al (2011) Rapid and transient recruitment of DNMT1 to DNA double-strand breaks is mediated by its interaction with multiple components of the DNA damage response machinery. Hum Mol Genet 20(1):126–140
22. Milutinovic S, Zhuang Q, Niveleau A, Szyf M (2003) Epigenomic stress response. Knockdown of DNA methyltransferase 1 triggers an intra-S-phase arrest of DNA replication and induction of stress response genes. J Biol Chem 278(17):14985–14995
23. Unterberger A, Andrews SD, Weaver IC, Szyf M (2006) DNA methyltransferase 1 knockdown activates a replication stress checkpoint. Mol Cell Biol 26(20):7575–7586
24. Mortusewicz O, Schermelleh L, Walter J, Cardoso MC, Leonhardt H (2005) Recruitment of DNA methyltransferase I to DNA repair sites. Proc Natl Acad Sci USA 102(25):8905–8909
25. Laghi L, Bianchi P, Malesci A (2008) Differences and evolution of the methods for the assessment of microsatellite instability. Oncogene 27(49):6313–6321
26. Kunkel TA, Erie DA (2005) DNA mismatch repair. Annu Rev Biochem 74:681–710
27. Raschle M, Dufner P, Marra G, Jiricny J (2002) Mutations within the hMLH1 and hPMS2 subunits of the human MutLalpha mismatch repair factor affect its ATPase activity, but not its ability to interact with hMutSalpha. J Biol Chem 277(24):21810–21820
28. Kantelinen J, Kansikas M, Korhonen MK et al (2010) MutSbeta exceeds MutSalpha in dinucleotide loop repair. Br J Cancer 102(6):1068–1073
29. Thibodeau SN, French AJ, Cunningham JM et al (1998) Microsatellite instability in colorectal cancer: different mutator phenotypes and the principal involvement of hMLH1. Cancer Res 58(8):1713–1718
30. Viswanathan M, Tsuchida N, Shanmugam G (2003) Promoter hypermethylation profile of tumor-associated genes p16, p15, hMLH1, MGMT and E-cadherin in oral squamous cell carcinoma. Int J Cancer 105(1):41–46
31. Kim HG, Lee S, Kim DY et al (2010) Aberrant methylation of DNA mismatch repair genes in elderly patients with sporadic gastric carcinoma: A comparison with younger patients. J Surg Oncol 101(1):28–35
32. Brucher BL, Geddert H, Langner C et al (2006) Hypermethylation of hMLH1, HPP1, p14(ARF), p16(INK4A) and APC in primary adenocarcinomas of the small bowel. Int J Cancer 119(6):1298–1302
33. Wang YC, Lu YP, Tseng RC et al (2003) Inactivation of hMLH1 and hMSH2 by promoter methylation in primary non-small cell lung tumors and matched sputum samples. J Clin Invest 111(6):887–895
34. Murphy MA, Wentzensen N (2011) Frequency of mismatch repair deficiency in ovarian cancer: a systematic review. Int J Cancer 129:1914–1922
35. Seedhouse CH, Das-Gupta EP, Russell NH (2003) Methylation of the hMLH1 promoter and its association with microsatellite instability in acute myeloid leukemia. Leukemia 17(1):83–88
36. Lenz G, Hutter G, Hiddemann W, Dreyling M (2004) Promoter methylation and expression of DNA repair genes hMLH1 and MGMT in acute myeloid leukemia. Ann Hematol 83(10):628–633
37. Nomdedeu JF, Perea G, Estivill C et al (2005) Microsatellite instability is not an uncommon finding in adult de novo acute myeloid leukemia. Ann Hematol 84(6):368–375
38. Tawfik HM, El-Maqsoud NM, Hak BH, El-Sherbiny YM (2011) Head and neck squamous cell carcinoma: mismatch repair immunohistochemistry and promoter hypermethylation of hMLH1 gene. Am J Otolaryngol 32(6):528–36
39. Lahtz C, Pfeifer GP (2011) Epigenetic changes of DNA repair genes in cancer. J Mol Cell Biol 3(1):51–58
40. Valle L, Carbonell P, Fernandez V et al (2007) MLH1 germline epimutations in selected patients with early-onset non-polyposis colorectal cancer. Clin Genet 71(3):232–237
41. Gazzoli I, Loda M, Garber J, Syngal S, Kolodner RD (2002) A hereditary nonpolyposis colorectal carcinoma case associated with hypermethylation of the MLH1 gene in normal tissue

and loss of heterozygosity of the unmethylated allele in the resulting microsatellite instability-high tumor. Cancer Res 62(14):3925–3928
42. Herman JG, Umar A, Polyak K et al (1998) Incidence and functional consequences of hMLH1 promoter hypermethylation in colorectal carcinoma. Proc Natl Acad Sci USA 95(12):6870–6875
43. Nakagawa H, Nuovo GJ, Zervos EE et al (2001) Age-related hypermethylation of the 5′ region of MLH1 in normal colonic mucosa is associated with microsatellite-unstable colorectal cancer development. Cancer Res 61(19):6991–6995
44. Kuismanen SA, Holmberg MT, Salovaara R et al (1999) Epigenetic phenotypes distinguish microsatellite-stable and -unstable colorectal cancers. Proc Natl Acad Sci USA 96(22): 12661–12666
45. Wheeler JM, Beck NE, Kim HC, Tomlinson IP, Mortensen NJ, Bodmer WF (1999) Mechanisms of inactivation of mismatch repair genes in human colorectal cancer cell lines: the predominant role of hMLH1. Proc Natl Acad Sci USA 96(18):10296–10301
46. Auclair J, Vaissiere T, Desseigne F et al (2011) Intensity-dependent constitutional MLH1 promoter methylation leads to early onset of colorectal cancer by affecting both alleles. Genes Chromosomes Cancer 50(3):178–185
47. Zhang H, Zhang S, Cui J, Zhang A, Shen L, Yu H (2008) Expression and promoter methylation status of mismatch repair gene hMLH1 and hMSH2 in epithelial ovarian cancer. Aust N Z J Obstet Gynaecol 48(5):505–509
48. Vlaykova T, Mitkova A, Stancheva G et al (2011) Microsatellite instability and promoter hypermethylation of MLH1 and MSH2 in patients with sporadic colorectal cancer. J BUON 16(2):265–273
49. Nagasaka T, Rhees J, Kloor M et al (2010) Somatic hypermethylation of MSH2 is a frequent event in Lynch Syndrome colorectal cancers. Cancer Res 70(8):3098–3108
50. Ligtenberg MJ, Kuiper RP, Chan TL et al (2009) Heritable somatic methylation and inactivation of MSH2 in families with Lynch syndrome due to deletion of the 3′ exons of TACSTD1. Nat Genet 41(1):112–117
51. Moelans CB, Verschuur-Maes AH, van Diest PJ (2011) Frequent promoter hypermethylation of BRCA2, CDH13, MSH6, PAX5, PAX6 and WT1 in ductal carcinoma in situ and invasive breast cancer. J Pathol 225(2):222–231
52. David SS, O'Shea VL, Kundu S (2007) Base-excision repair of oxidative DNA damage. Nature 447(7147):941–950
53. Peng B, Hurt EM, Hodge DR, Thomas SB, Farrar WL (2006) DNA hypermethylation and partial gene silencing of human thymine- DNA glycosylase in multiple myeloma cell lines. Epigenetics 1(3):138–145
54. Howard JH, Frolov A, Tzeng CW et al (2009) Epigenetic downregulation of the DNA repair gene MED1/MBD4 in colorectal and ovarian cancer. Cancer Biol Ther 8(1):94–100
55. Guan H, Ji M, Hou P et al (2008) Hypermethylation of the DNA mismatch repair gene hMLH1 and its association with lymph node metastasis and T1799A BRAF mutation in patients with papillary thyroid cancer. Cancer 113(2):247–255
56. Hoeijmakers JH (2001) Genome maintenance mechanisms for preventing cancer. Nature 411(6835):366–374
57. Yang J, Xu Z, Li J et al (2010) XPC epigenetic silence coupled with p53 alteration has a significant impact on bladder cancer outcome. J Urol 184(1):336–343
58. Chen HY, Shao CJ, Chen FR, Kwan AL, Chen ZP (2010) Role of ERCC1 promoter hypermethylation in drug resistance to cisplatin in human gliomas. Int J Cancer 126(8):1944–1954
59. Liu WB, Ao L, Cui ZH et al (2011) Molecular analysis of DNA repair gene methylation and protein expression during chemical-induced rat lung carcinogenesis. Biochem Biophys Res Commun 408(4):595–601
60. Jiang J, Liang X, Zhou X et al (2010) DNA repair gene X-ray repair cross complementing group 1 Arg194Trp polymorphism on the risk of lung cancer: a meta-analysis on 22 studies. J Thorac Oncol 5(11):1741–1747

61. Wang P, Tang JT, Peng YS, Chen XY, Zhang YJ, Fang JY (2010) XRCC1 downregulated through promoter hypermethylation is involved in human gastric carcinogenesis. J Dig Dis 11(6):343–351
62. Peng B, Hodge DR, Thomas SB et al (2005) Epigenetic silencing of the human nucleotide excision repair gene, hHR23B, in interleukin-6-responsive multiple myeloma KAS-6/1 cells. J Biol Chem 280(6):4182–4187
63. Moynahan ME, Jasin M (2010) Mitotic homologous recombination maintains genomic stability and suppresses tumorigenesis. Nat Rev Mol Cell Biol 11(3):196–207
64. Mazon G, Mimitou EP, Symington LS. SnapShot: homologous recombination in DNA double-strand break repair. Cell. 2010;142(4):646, 646.e1.
65. Esteller M, Silva JM, Dominguez G et al (2000) Promoter hypermethylation and BRCA1 inactivation in sporadic breast and ovarian tumors. J Natl Cancer Inst 92(7):564–569
66. Esteller M, Risques RA, Toyota M et al (2001) Promoter hypermethylation of the DNA repair gene O(6)-methylguanine-DNA methyltransferase is associated with the presence of G:C to A:T transition mutations in p53 in human colorectal tumorigenesis. Cancer Res 61(12): 4689–4692
67. Baldwin RL, Nemeth E, Tran H et al (2000) BRCA1 promoter region hypermethylation in ovarian carcinoma: a population-based study. Cancer Res 60(19):5329–5333
68. Bernal C, Vargas M, Ossandon F et al (2008) DNA methylation profile in diffuse type gastric cancer: evidence for hypermethylation of the BRCA1 promoter region in early-onset gastric carcinogenesis. Biol Res 41(3):303–315
69. Cabello MJ, Grau L, Franco N et al (2011) Multiplexed methylation profiles of tumor suppressor genes in bladder cancer. J Mol Diagn 13(1):29–40
70. Lee MN, Tseng RC, Hsu HS et al (2007) Epigenetic inactivation of the chromosomal stability control genes BRCA1, BRCA2, and XRCC5 in non-small cell lung cancer. Clin Cancer Res 13(3):832–838
71. Esteller M, Fraga MF, Guo M et al (2001) DNA methylation patterns in hereditary human cancers mimic sporadic tumorigenesis. Hum Mol Genet 10(26):3001–3007
72. Ludwig T, Chapman DL, Papaioannou VE, Efstratiadis A (1997) Targeted mutations of breast cancer susceptibility gene homologs in mice: lethal phenotypes of Brca1, Brca2, Brca1/Brca2, Brca1/p53, and Brca2/p53 nullizygous embryos. Genes Dev 11(10): 1226–1241
73. Kee Y, D'Andrea AD (2010) Expanded roles of the Fanconi anemia pathway in preserving genomic stability. Genes Dev 24(16):1680–1694
74. Meier D, Schindler D (2011) Fanconi anemia core complex gene promoters harbor conserved transcription regulatory elements. PLoS One 6(8):e22911
75. Hess CJ, Ameziane N, Schuurhuis GJ et al (2008) Hypermethylation of the FANCC and FANCL promoter regions in sporadic acute leukaemia. Cell Oncol 30(4):299–306
76. Marsit CJ, Liu M, Nelson HH, Posner M, Suzuki M, Kelsey KT (2004) Inactivation of the Fanconi anemia/BRCA pathway in lung and oral cancers: implications for treatment and survival. Oncogene 23(4):1000–1004
77. Narayan G, Arias-Pulido H, Nandula SV et al (2004) Promoter hypermethylation of FANCF: disruption of Fanconi anemia-BRCA pathway in cervical cancer. Cancer Res 64(9): 2994–2997
78. Lim SL, Smith P, Syed N et al (2008) Promoter hypermethylation of FANCF and outcome in advanced ovarian cancer. Br J Cancer 98(8):1452–1456
79. Pegg AE, Dolan ME, Moschel RC (1995) Structure, function, and inhibition of O6-alkylguanine-DNA alkyltransferase. Prog Nucleic Acid Res Mol Biol 51:167–223
80. Gerson SL (2004) MGMT: its role in cancer aetiology and cancer therapeutics. Nat Rev Cancer 4(4):296–307
81. Esteller M, Hamilton SR, Burger PC, Baylin SB, Herman JG (1999) Inactivation of the DNA repair gene O6-methylguanine-DNA methyltransferase by promoter hypermethylation is a common event in primary human neoplasia. Cancer Res 59(4):793–797

82. Weller M, Stupp R, Reifenberger G et al (2010) MGMT promoter methylation in malignant gliomas: ready for personalized medicine? Nat Rev Neurol 6(1):39–51
83. Whitehall VL, Walsh MD, Young J, Leggett BA, Jass JR (2001) Methylation of O-6-methylguanine DNA methyltransferase characterizes a subset of colorectal cancer with low-level DNA microsatellite instability. Cancer Res 61(3):827–830
84. Park TJ, Han SU, Cho YK, Paik WK, Kim YB, Lim IK (2001) Methylation of O(6)-methylguanine-DNA methyltransferase gene is associated significantly with K-ras mutation, lymph node invasion, tumor staging, and disease free survival in patients with gastric carcinoma. Cancer 92(11):2760–2768
85. Greenblatt MS, Bennett WP, Hollstein M, Harris CC (1994) Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. Cancer Res 54(18): 4855–4878
86. Zhang YJ, Chen Y, Ahsan H et al (2003) Inactivation of the DNA repair gene O6-methylguanine-DNA methyltransferase by promoter hypermethylation and its relationship to aflatoxin B1-DNA adducts and p53 mutation in hepatocellular carcinoma. Int J Cancer 103(4): 440–444
87. Wolf P, Hu YC, Doffek K, Sidransky D, Ahrendt SA (2001) O(6)-Methylguanine-DNA methyltransferase promoter hypermethylation shifts the p53 mutational spectrum in non-small cell lung cancer. Cancer Res 61(22):8113–8117
88. Zhang L, Lu W, Miao X, Xing D, Tan W, Lin D (2003) Inactivation of DNA repair gene O6-methylguanine-DNA methyltransferase by promoter hypermethylation and its relation to p53 mutations in esophageal squamous cell carcinoma. Carcinogenesis 24(6):1039–1044
89. Nakamura M, Watanabe T, Yonekawa Y, Kleihues P, Ohgaki H (2001) Promoter methylation of the DNA repair gene MGMT in astrocytomas is frequently associated with G:C –> A:T mutations of the TP53 tumor suppressor gene. Carcinogenesis 22(10):1715–1719
90. Sabharwal A, Middleton MR (2006) Exploiting the role of O6-methylguanine-DNA-methyltransferase (MGMT) in cancer therapy. Curr Opin Pharmacol 6(4):355–363
91. Kitano K, Kim SY, Hakoshima T (2010) Structural basis for DNA strand separation by the unconventional winged-helix domain of RecQ helicase WRN. Structure 18(2):177–187
92. Opresko PL (2008) Telomere ResQue and preservation—roles for the Werner syndrome protein and other RecQ helicases. Mech Ageing Dev 129(1–2):79–90
93. Agrelo R, Cheng WH, Setien F et al (2006) Epigenetic inactivation of the premature aging Werner syndrome gene in human cancer. Proc Natl Acad Sci USA 103(23):8822–8827
94. Kawasaki T, Ohnishi M, Suemoto Y et al (2008) WRN promoter methylation possibly connects mucinous differentiation, microsatellite instability and CpG island methylator phenotype in colorectal cancer. Mod Pathol 21(2):150–158
95. Smith JA, Ndoye AM, Geary K, Lisanti MP, Igoucheva O, Daniel R (2010) A role for the Werner syndrome protein in epigenetic inactivation of the pluripotency factor Oct4. Aging Cell 9(4):580–591
96. Polo SE, Jackson SP (2011) Dynamics of DNA damage response proteins at DNA breaks: a focus on protein modifications. Genes Dev 25(5):409–433
97. Harrison JC, Haber JE (2006) Surviving the breakup: the DNA damage checkpoint. Annu Rev Genet 40:209–235
98. Lazzaro F, Giannattasio M, Puddu F et al (2009) Checkpoint mechanisms at the intersection between DNA damage and repair. DNA Repair (Amst) 8(9):1055–1067
99. Linding R, Jensen LJ, Ostheimer GJ et al (2007) Systematic discovery of in vivo phosphorylation networks. Cell 129(7):1415–1426
100. Matsuoka S, Ballif BA, Smogorzewska A et al (2007) ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage. Science 316(5828): 1160–1166
101. Kim WJ, Vo QN, Shrivastav M, Lataxes TA, Brown KD (2002) Aberrant methylation of the ATM promoter correlates with increased radiosensitivity in a human colorectal tumor cell line. Oncogene 21(24):3864–3871

102. Vo QN, Kim WJ, Cvitanovic L, Boudreau DA, Ginzinger DG, Brown KD (2004) The ATM gene is a target for epigenetic silencing in locally advanced breast cancer. Oncogene 23(58): 9432–9437
103. Ai L, Vo QN, Zuo C et al (2004) Ataxia-telangiectasia-mutated (ATM) gene in head and neck squamous cell carcinoma: promoter hypermethylation with clinical correlation in 100 cases. Cancer Epidemiol Biomarkers Prev 13(1):150–156
104. Bartek J, Falck J, Lukas J (2001) CHK2 kinase—a busy messenger. Nat Rev Mol Cell Biol 2(12):877–886
105. Bell DW, Varley JM, Szydlo TE et al (1999) Heterozygous germ line hCHK2 mutations in Li-Fraumeni syndrome. Science 286(5449):2528–2531
106. Bartek J, Lukas J (2003) Chk1 and Chk2 kinases in checkpoint control and cancer. Cancer Cell 3(5):421–429
107. Kato N, Fujimoto H, Yoda A et al (2004) Regulation of Chk2 gene expression in lymphoid malignancies: involvement of epigenetic mechanisms in Hodgkin's lymphoma cell lines. Cell Death Differ 11(Suppl 2):S153–161
108. Kim DS, Kim MJ, Lee JY et al (2009) Epigenetic inactivation of checkpoint kinase 2 gene in non-small cell lung cancer and its relationship with clinicopathological features. Lung Cancer 65(2):247–250
109. Wang H, Wang S, Shen L et al (2010) Chk2 down-regulation by promoter hypermethylation in human bulk gliomas. Life Sci 86(5–6):185–191
110. Sullivan A, Yuille M, Repellin C et al (2002) Concomitant inactivation of p53 and Chk2 in breast cancer. Oncogene 21(9):1316–1324
111. Williams LH, Choong D, Johnson SA, Campbell IG (2006) Genetic and epigenetic analysis of CHEK2 in sporadic breast, colon, and ovarian cancers. Clin Cancer Res 12(23):6967–6972
112. Jascur T, Boland CR (2006) Structure and function of the components of the human DNA mismatch repair system. Int J Cancer 119(9):2030–2035
113. Loeb LA, Loeb KR, Anderson JP (2003) Multiple mutations and cancer. Proc Natl Acad Sci USA 100(3):776–781
114. Lin Y, Wilson JH (2009) Diverse effects of individual mismatch repair components on transcription-induced CAG repeat instability in human cells. DNA Repair (Amst) 8(8):878–885
115. Lin Y, Dion V, Wilson JH (2006) Transcription promotes contraction of CAG repeat tracts in human cells. Nat Struct Mol Biol 13(2):179–180
116. Karran P (2006) Thiopurines, DNA damage, DNA repair and therapy-related cancer. Br Med Bull 79–80:153–170
117. Bellacosa A, Cicchillitti L, Schepis F et al (1999) MED1, a novel human methyl-CpG-binding endonuclease, interacts with DNA mismatch repair protein MLH1. Proc Natl Acad Sci USA 96(7):3969–3974
118. Ruzov A, Shorning B, Mortusewicz O, Dunican DS, Leonhardt H, Meehan RR (2009) MBD4 and MLH1 are required for apoptotic induction in xDNMT1-depleted embryos. Development 136(13):2277–2286
119. Cortellino S, Turner D, Masciullo V et al (2003) The base excision repair enzyme MED1 mediates DNA damage response to antitumor drugs and is associated with mismatch repair system integrity. Proc Natl Acad Sci USA 100(25):15071–15076
120. Boland MJ, Christman JK (2008) Characterization of Dnmt3b:thymine-DNA glycosylase interaction and stimulation of thymine glycosylase-mediated repair by DNA methyltransferase(s) and RNA. J Mol Biol 379(3):492–504
121. Li YQ, Zhou PZ, Zheng XD, Walsh CP, Xu GL (2007) Association of Dnmt3a and thymine DNA glycosylase links DNA methylation with base-excision repair. Nucleic Acids Res 35(2): 390–400
122. Kimura H, Shiota K (2003) Methyl-CpG-binding protein, MeCP2, is a target molecule for maintenance DNA methyltransferase, Dnmt1. J Biol Chem 278(7):4806–4812
123. Jackson-Grusby L, Beard C, Possemato R et al (2001) Loss of genomic methylation causes p53-dependent apoptosis and epigenetic deregulation. Nat Genet 27(1):31–39

124. Flores-Rozas H, Clark D, Kolodner RD (2000) Proliferating cell nuclear antigen and Msh2p-Msh6p interact to form an active mispair recognition complex. Nat Genet 26(3):375–378
125. Iyer RR, Pohlhaus TJ, Chen S et al (2008) The MutSalpha-proliferating cell nuclear antigen interaction in human DNA mismatch repair. J Biol Chem 283(19):13310–13319
126. Plotz G, Welsch C, Giron-Monzon L et al (2006) Mutations in the MutSalpha interaction interface of MLH1 can abolish DNA mismatch repair. Nucleic Acids Res 34(22):6574–6586
127. Kleczkowska HE, Marra G, Lettieri T, Jiricny J (2001) hMSH3 and hMSH6 interact with PCNA and colocalize with it to replication foci. Genes Dev 15(6):724–736
128. Umar A, Buermeyer AB, Simon JA et al (1996) Requirement for PCNA in DNA mismatch repair at a step preceding DNA resynthesis. Cell 87(1):65–73
129. Chen F, Arseven OK, Cryns VL (2004) Proteolysis of the mismatch repair protein MLH1 by caspase-3 promotes DNA damage-induced apoptosis. J Biol Chem 279(26):27542–27548
130. Kim MY, Zhang T, Kraus WL (2005) Poly(ADP-ribosyl)ation by PARP-1: 'PAR-laying' NAD+ into a nuclear signal. Genes Dev 19(17):1951–1967
131. Bischof O, Galande S, Farzaneh F, Kohwi-Shigematsu T, Campisi J (2001) Selective cleavage of BLM, the bloom syndrome protein, during apoptotic cell death. J Biol Chem 276(15): 12068–12075
132. Wang J, Pabla N, Wang CY, Wang W, Schoenlein PV, Dong Z (2006) Caspase-mediated cleavage of ATM during cisplatin-induced tubular cell apoptosis: inactivation of its kinase activity toward p53. Am J Physiol Renal Physiol 291(6):F1300–1307
133. Knox JD, Araujo FD, Bigey P et al (2000) Inhibition of DNA methyltransferase inhibits DNA replication. J Biol Chem 275(24):17986–17990
134. Bartek J, Lukas J (2001) Mammalian G1- and S-phase checkpoints in response to DNA damage. Curr Opin Cell Biol 13(6):738–747
135. Kastan MB, Lim DS (2000) The many substrates and functions of ATM. Nat Rev Mol Cell Biol 1(3):179–186
136. Falck J, Mailand N, Syljuasen RG, Bartek J, Lukas J (2001) The ATM-Chk2-Cdc25A checkpoint pathway guards against radioresistant DNA synthesis. Nature 410(6830):842–847
137. Maser RS, Mirzoeva OK, Wells J et al (2001) Mre11 complex and DNA replication: linkage to E2F and sites of DNA synthesis. Mol Cell Biol 21(17):6006–6016
138. Hardy CF (1997) Identification of Cdc45p, an essential factor required for DNA replication. Gene 187(2):239–246
139. Rhee I, Bachman KE, Park BH et al (2002) DNMT1 and DNMT3b cooperate to silence genes in human cancer cells. Nature 416(6880):552–556
140. Ting AH, Jair KW, Suzuki H, Yen RW, Baylin SB, Schuebel KE (2004) CpG island hypermethylation is maintained in human colorectal cancer cells after RNAi-mediated depletion of DNMT1. Nat Genet 36(6):582–584
141. Chen T, Hevi S, Gay F et al (2007) Complete inactivation of DNMT1 leads to mitotic catastrophe in human cancer cells. Nat Genet 39(3):391–396
142. Brown KD, Robertson KD (2007) DNMT1 knockout delivers a strong blow to genome stability and cell viability. Nat Genet 39(3):289–290
143. Canman CE, Lim DS, Cimprich KA et al (1998) Activation of the ATM kinase by ionizing radiation and phosphorylation of p53. Science 281(5383):1677–1679
144. Bakkenist CJ, Kastan MB (2003) DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. Nature 421(6922):499–506
145. Chen RZ, Pettersson U, Beard C, Jackson-Grusby L, Jaenisch R (1998) DNA hypomethylation leads to elevated mutation rates. Nature 395(6697):89–93
146. Palii SS, Van Emburgh BO, Sankpal UT, Brown KD, Robertson KD (2008) DNA methylation inhibitor 5-Aza-2′-deoxycytidine induces reversible genome-wide DNA damage that is distinctly influenced by DNA methyltransferases 1 and 3B. Mol Cell Biol 28(2):752–771
147. Spada F, Haemmer A, Kuch D et al (2007) DNMT1 but not its interaction with the replication machinery is required for maintenance of DNA methylation in human cells. J Cell Biol 176(5):565–571

Chapter 2
DNA Hypomethylation and Hemimethylation in Cancer

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Abstract In contrast to earlier views that there was much compartmentalization of the types of sequences subject to cancer-linked changes in DNA epigenetics, it is now clear that both cancer-associated DNA hypomethylation and hypermethylation are found throughout the genome. The hypermethylation includes promoters of tumor suppressor genes whose expression becomes repressed, thereby facilitating cancer formation. How hypomethylation contributes to carcinogenesis has been less clear. Recent insights into tissue-specific intra- and intergenic methylation and into cancer methylomes suggest that some of the DNA hypomethylation associated with cancers is likely to aid in tumor formation and progression by many different pathways, including effects on transcription in *cis*. Cancer-associated loss of DNA methylation from intergenic enhancers, promoter regions, silencers, and chromatin boundary elements may alter transcription rates. In addition, cancer-associated intragenic DNA hypomethylation might modulate alternative promoter usage,

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production of intragenic noncoding RNA transcripts, cotranscriptional splicing, and transcription initiation or elongation. Initial studies of hemimethylation of DNA in cancer and many new studies of DNA demethylation in normal tissues suggest that active demethylation with spreading of hypomethylation can explain much of the cancer-associated DNA hypomethylation. The new discoveries that genomic 5-hydroxymethylcytosine is an intermediate in DNA demethylation, a base with its own functionality, and a modified base that, like 5-methylcytosine, exhibits cancer-associated losses, suggest that both decreased hydroxymethylation and decreased methylation of DNA play important roles in carcinogenesis.

2.1 Introduction

Altered methylation of DNA in human cancers was first described as overall genomic hypomethylation in various cancers vs. a wide variety of normal tissues [1] and as hypomethylation of a few gene regions in colon adenocarcinomas vs. normal colonic epithelium [2]. Almost all types of cancers exhibit both hypermethylation of some DNA sequences and hypomethylation of others relative to appropriate controls that account for the tissue specificity of DNA methylation [3]. The cancer-associated hypermethylation and hypomethylation of the genome are generally independent of each other [4, 5]. Until recently, it appeared that cancer-specific changes in DNA methylation were usually hypermethylation of unique gene regions and hypomethylation of DNA repeats, albeit with many notable exceptions [6–11]. Deep sequencing of the genome has revealed far greater size and complexity to the transcriptome than previously appreciated [12]. Similarly, recent whole-genome analysis of the cancer methylome demonstrates that there is much more cancer-linked hypomethylation of unique gene sequences and hypermethylation of repeated sequences than previously found, although there are differences in the frequency with which subsets of sequences undergo hypo- or hypermethylation [13–18].

This chapter reviews new insights into genome-wide DNA and chromatin epigenetics in normal cell populations as well as in cancers [19–29]. Recent studies are drawing attention to previously unsuspected roles of epigenetic marks in the body of genes as well as at promoters and intergenic transcription control regions. These findings are likely to be relevant to the biological impact of cancer-associated DNA hypomethylation. In addition to effects on normal gene expression, cancer-associated DNA hypomethylation probably favors oncogenesis by enhancing recombination [30–33]; occasionally activating a small number of endogenous retroviral elements [34, 35]; altering the intranuclear positioning of chromatin; and modulating the sequestration transcription factors at tandem DNA repeats, as reviewed previously [3, 6]. In addition, the little-studied area of DNA hemimethylation in cancer is discussed in this chapter in the context of our growing understanding of pathways for the conversion of genomic 5-methylcytosine (5mC) residues to C residues.

2.2 Genomic Hypomethylation Profiles in Cancer and Their Relevance at Promoters and Enhancers

Until recent high-resolution genome-wide analyses of DNA methylation, cancer-specific portions of methylomes were considered to consist predominantly of hypomethylated DNA repeats and hypermethylated gene regions [3, 7, 36]. DNA repeats are often used as a surrogate for average genomic methylation changes (usually losses of 5mC), with DNA epigenetic changes in some classes of repeats more closely associated with certain tumor types [6, 18, 35, 37–39]. In our 1983 analysis of global DNA hypomethylation in human cancers by high performance liquid chromatography analysis of enzymatic DNA digests [1], we fractionated one adenocarcinoma DNA into highly repetitive, moderately repetitive, and unique sequence classes. Because we found that each of these cancer DNA fractions had similar ratios of mol% 5mC to those from normal human tissues, we concluded that cancer-linked hypomethylation was not confined to repeated DNA. Indeed, cancer-linked DNA hypomethylation often occurs in unique sequences in and around genes, including metastasis-associated genes, as originally revealed in studies using CpG methylation-sensitive restriction endonucleases or sodium sulfite-based methods to study individual gene regions [2, 6, 40].

Recent genome-wide studies of DNA methylation in various normal and cancer cell populations indicate much tissue specificity throughout the genome in normal samples and pervasive cancer-linked DNA hypomethylation and hypermethylation [13, 15, 16, 41–45]. Regions of cancer-associated changes in DNA methylation are found in short interspersed or clustered regions as well as in long blocks [7, 42, 44, 46, 47]. There is increasing evidence for cause-and-effect relationships between normal tissue-specific DNA hypomethylation and increased transcription as well as many associations between cancer-linked hypomethylation and cancer-linked increases in gene expression [16, 17, 19, 21, 24, 48–55]. The inverse relationships between expression and DNA methylation include imprinted genes implicated in carcinogenesis [56].

A small percentage of annotated gene promoters overlap tissue-specific (T-DMR) or cancer-specific (C-DMR) differentially methylated DNA regions [49, 57]. However, most of the non-imprinted, autosomal T-DMR promoters are not the main type of vertebrate DNA promoters, which are part of CpG islands (CGIs, a class of CpG-rich regions surrounded by CpG-poor DNA). Among the genes with T-DMR promoters are some that become activated upon experimentally induced demethylation with a low dose of 5-deoxyazacytidine but not upon treatment with a histone deacetylase inhibitor, trichostatin A [49].

Enhancers too sometimes show a correlation between upregulation of expression of the associated gene and DNA demethylation in normal cells. For example, the binding of FoxA1/FOXA1 to enhancers is inhibited by site-specific DNA methylation at the corresponding binding site [58]. This differentiation-associated transcription regulatory factor can open up DNA compacted in chromatin of inactive enhancers (as a “pioneer” factor) and then recruit effector transcription factors to

make the enhancer active [59–61]. A window of DNA demethylation provided by previous binding of FoxD3, another pioneer factor, allows recruitment of FoxA1 and conversion of the enhancer to a state that is poised for activity. Moreover, in embryonal stem cells, local DNA demethylation per se, rather than any changes in histone H3K27 or H3K9 methylation, is associated with the binding of pioneer factors to certain tissue-specific non-CGI promoters [58]. Pioneer factors, including FOXA1, are implicated in various types of carcinogenesis [62]. Given the extensive hypomethylation of DNA in cancers, many known and yet more unknown enhancer regions are likely to become demethylated specifically in tumors. However, specific losses of DNA methylation from transcription regulatory regions might facilitate, but not independently cause, changes in expression [63].

Broad DNA regions enriched in hypomethylation are sometimes also associated with increases in copy number of DNA regions and can, thereby, synergistically increase expression of some of the affected genes [13, 33, 42]. Such broad regional hypomethylation (which can encompass occasional sites of persistent methylation) might reflect higher order chromatin structure. The latter is influenced, in turn, by the type, frequency, and spacing of DNA repeats; the G+C and CpG contents of subregions; the gene density; the nucleosome density; broad regions of distinct histone composition modification; and the presence of clusters of co-regulated genes. Nonetheless, a long region of cancer-linked DNA hypermethylation can be adjacent to a region of cancer-linked DNA hypomethylation with a sharp border between them, as demonstrated for a tandem repeat array (D4Z4) and its border sequences [9]. Despite evidence for functionality, DNA demethylation in cancer probably involves frequent overshooting of targeted sequences. These are referred to as *passenger* DNA methylation changes [64]. The hypomethylation in cancers of many more sites than are biologically relevant is probably due to a relaxed specificity of the demethylation apparatus during carcinogenesis and tumor progression and to the spreading of DNA demethylation patterns.

2.3 Genomic Hypomethylation in Cancer Within Gene Bodies

Recent findings implicate intragenic epigenetic marks in the regulation of normal gene expression. T-DMRs have been found inside many genes, and increased methylation in the central gene body or downstream promoter-flanking region of certain subsets of genes is associated with increased transcription [23, 65–68]. Moreover, there are nonrandom associations between positions of CpG methylation within genes and exon–intron boundaries, distance from the transcription start site, and distance from the 3′ end of the gene [66, 69]. Besides first exons, T-DMRs are present in various exonic and intronic sequences, including internal CGIs, sequences adjacent to internal CGIs (“CGI shores”), insulators, intragenic ncRNA genes, and 3′ terminal regions [17, 19, 28, 59, 70, 71]. They are present in both repeated and unique sequences. These findings are consistent with the many interrelationships between DNA and chromatin epigenetics and tissue-specific chromatin epigenetic marks inside genes [65, 68, 72, 73].

Differentiation-related DNA and/or chromatin epigenetic marks within genes may help determine alternative promoter usage, modulate the rate of transcription initiation or elongation, and possibly help direct the choice of alternative splice sites [19, 21, 24, 26, 27, 29, 34, 52, 74, 75]. The average DNA methylation level in the central portion of moderately expressed genes is associated with higher average transcription levels, possibly by being related to nucleosome positioning [76]. For example, immediately downstream of proximal CpG-poor promoters, it was unexpectedly found that methylation of sequences antagonizes binding of Polycomb repressor complexes [68]. Methylation of gene-body CGIs appears to be associated with repression of intragenic promoters [28]. However, for some sets of genes under certain conditions, lower expression was correlated with increases in gene-body methylation [69].

With respect to alternative splicing, evidence implicates certain histone modifications in helping to regulate the choice of splice junctions by altering rates of transcription, nucleosome positioning, or direct interactions with proteins that mark exon–intron junctions of pre-mRNA [77, 78]. Changes in physiological conditions can alter the chromatin modifications at these junctions and concomitantly modulate exon skipping [78]. DNA methylation may also be involved in regulating alternative splicing because of the many DNA methylation/chromatin epigenetic interrelationships and the finding that intron–exon junctions are enriched in sharp transitions in DNA methylation levels [66]. A recent report that malignant prostate cancer cells have enrichment of DNA hypermethylation at exon–intron junctions [45] is consistent with the cancer-linked involvement of DNA methylation levels in determining alternative splicing.

Programmed changes in DNA methylation in intra- and intergenic regions are not restricted to differentiation-related events. For example, electroconvulsive stimulation of mouse neuronal cells in vivo was recently demonstrated to cause rapid decreases and increases in DNA methylation in a substantial minority of CpG sites, especially at CpG-poor regions [69]. The physiologically linked DNA demethylation included rapid demethylation of exons and introns in various positions of the genes. Importantly, there was enrichment in these DNA epigenetic changes in the vicinity of brain-related genes. Thus, there is ample precedent from studies of normal cell functioning to suggest that cancer-associated DNA hypomethylation in intronic and exonic sequences can modulate the amount and type of gene products and thereby contribute to tumor formation or progression.

Cancer-linked DNA hypomethylation in the gene body is illustrated in Fig. 2.1 for three genes whose expression has been reported to be altered in certain cancers [79–81]. *TGFB2* has an intronic Alu repeat that was hypomethylated in some cancer cell lines relative to a wide variety of normal tissues (Fig. 2.1a) and untransformed cell cultures. The only exceptions to this intronic region being highly methylated in normal tissues and cell strains were found in skeletal muscle (Fig. 2.1a), myoblasts, and myotubes (data not shown). Their hypomethylation at this site might be related to the significant upregulation of *TGFB2* in myoblasts and myotubes vs. 19 types of non-muscle cell cultures [82] and is an example of the frequent relationship between targets for cancer-associated hypo- or hypermethylation and targets for differentiation-associated epigenetic changes [17, 83]. Like *TGFB2, PRDM16* (Fig. 2.1b) exhibited gene-body hypomethylation in

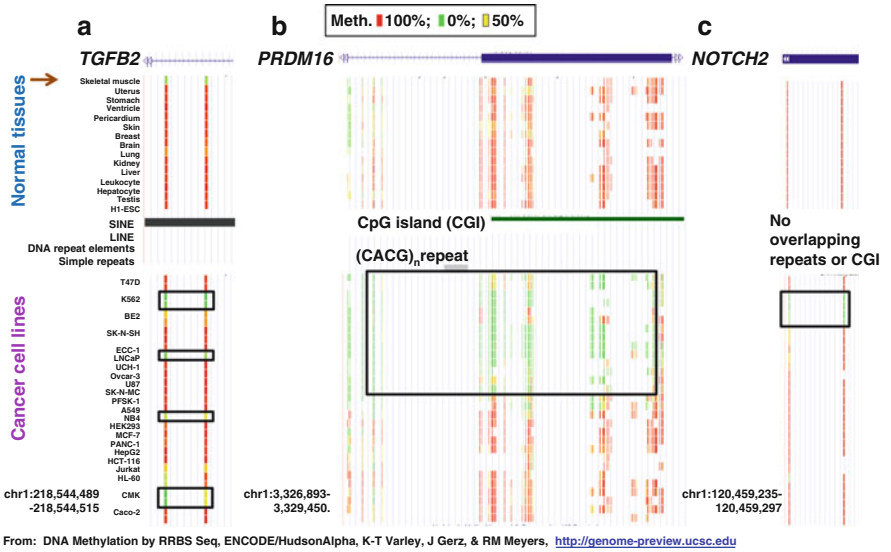

Fig. 2.1 Examples of cancer cell-associated hypomethylation (*boxed*) within gene bodies and overlapping a DNA repeat (**a**), a CGI (**b**), or neither (**c**) as determined by whole-genome analysis using reduced representation bisulfite sequencing (RRBS). (**a**), *TGFB2* , intron 1; the cancer hypomethylation overlaps an Alu repeat that is also hypomethylated in skeletal muscle (see *arrow*). (**b**), *PRDM16,* exon 9 and intron 8; the cancer hypomethylation overlaps a CGI and CGI shore. (**c**), *NOTCH2*, exon 34; no overlapping repeats or CGI. In contrast to the cancer-derived cell lines, non-immortalized cell strains (not shown) showed the same hypermethylation seen in normal tissues with the exception of myoblasts and myotubes for *TGFB2.* Myoblasts and myotubes overexpress *TGFB2* relative to 19 other types of cultured cell popula*tions*. All analyses were done in duplicate, and representative duplicates are shown

some of the cancer cell lines; however, this hypomethylation was in a region largely overlapping a CGI in an exon. *NOTCH2* (Fig. 2.1c) also showed gene-body hypomethylation in several cancer cell lines, but this hypomethylation was neither in a subregion with a CGI nor a DNA repeat. We note that some of the cancer cell lines with *TGFB2* or *PRDM16* gene hypomethylation also displayed cancer cell-linked promoter hypermethylation (data not shown).

Recently, the presence of 5-hydroxymethylcytosine (5hmC) as the sixth naturally programmed base in vertebrate DNA has been established [84]. It is generated from 5mC by hydroxylation via the enzymes TET1, TET2, or TET3 and is even more highly tissue specific in its relative levels in DNA than is 5mC [84–86]. It is implicated in stem cell renewal and distinct types of differentiation [87–89], as described further in an accompanying chapter by Pradhan and Kinney. Like 5mC, 5hmC is enriched in certain intragenic regions and exhibits major decreases in its genomic levels in cancer [84–86]. However, unlike 5mC, exons, intragenic CGIs, and enhancers have significantly elevated 5hmC levels relative to other portions of the genome [87, 90, 91]. These findings further highlight the need for studies of the functional significance of decreases in intragenic DNA epigenetic marks in cancer. In addition, they introduce a complication into almost all studies to date of 5mC that use either bisulfite or conventional CpG methylation-sensitive

restriction analysis to distinguish 5mC from unmethylated C, as these methods cannot resolve 5hmC and 5mC [69, 92, 93]. Therefore, a caveat to conclusions about 5mC distribution is that 5hmC might have been monitored instead, especially in exonic or enhancer regions in more 5hmC-rich tissues like brain [84, 85]. However, in some other cell types, like breast, heart, cell lines, and cancers, 5hmC is very much lower [84–86, 93], and 5hmC levels are also low in intronic and intergenic regions [90, 94].

2.4 Hypomethylation of DNA Repeats in Cancer

Global losses of DNA methylation with less numerous increases in methylation in other portions of the genome are typical of cancer [5, 6] although there are exceptions [18]. A major contributor toward the overall DNA hypomethylation is hypomethylation of tandem and interspersed DNA repeats, which is observed in most examined cancers [6, 95–97]. Most hypomethylation of DNA repeats in cancers is apparently the result of demethylation and not preexisting hypomethylation in a cancer stem cell [3], with the exception of seminomas as discussed below. Besides the effects on transcription and possible effects on alternative splicing described in the previous section, hypomethylation of a minor portion of interspersed DNA repeats may occasionally cause induction of retroviral element transcription [35]. In addition, hypomethylation of certain promoter-containing interspersed DNA repeats may affect chromatin boundaries resulting in effects on transcription of nearby genes [98, 99].

In a study of mononuclear cells from a few patients with chronic lymphocytic leukemia vs. the analogous cells from controls, Dante et al. described hypomethylation of LINE-1, a highly repeated interspersed repeat [100]. Hypomethylation of LINE-1 and Alu repeats was subsequently observed in many other types of cancers [38, 101–104]. Similarly, we found that tandem repeats in centromeric and juxtacentromeric satellite DNA are frequently hypomethylated in breast adenocarcinomas, ovarian epithelial cancers, and Wilms tumors [30, 105, 106], as confirmed for many other types of cancers [3, 107]. Additional classes of tandem repeats (including macrosatellite DNAs) and segmental duplications are also susceptible to DNA hypomethylation in malignancies [9, 18, 39, 43, 83, 108–110], although different subclasses of DNA repeat families can vary in their susceptibility to loss of DNA methylation in cancer [38, 39, 102, 111–113]. In some cancers, satellite DNA repeats showed the strongest DNA hypomethylation of all types of sequences analyzed [18, 33].

The frequency of cancer-associated hypomethylation of DNA repeats depends on the grade, the stage, and the individual tumor specimen [46, 114]. This hypomethylation is seen sometimes in non-tumor tissue adjacent to the cancer and in benign neoplasms and tissue lesions such as breast fibroadenomas and ovarian cystadenomas, although often to a lesser extent than in cancers [13, 51, 95, 105, 106, 112, 115]. In a mouse model of prostate tumor progression, repeat DNA hypomethylation was observed at the stage of prostatic intraepithelial neoplasia and prior to promoter hypermethylation [116]. However, depending on the tumor type

or specimen, repeat DNA hypomethylation may increase with tumor progression, a relationship inferred since the 1980s [1, 117]. In many types of cancer, repeat DNA hypomethylation is a highly informative prognostic marker and/or predictor of survival [46, 107, 118–122].

2.5 DNA Hypomethylation and Germ Cells: Comparison to Cancer Hypomethylation

Differential methylation of testes-specific genes has some similarities to cancer-associated DNA hypomethylation. Most genes that are specifically expressed in testis (like the cancer-testis genes) have little or no methylation in their promoter regions in testis and sperm although they are highly methylated, and transcriptionally repressed, in somatic tissues [123]. In sperm, as well as in many cancers, tandem DNA repeats and certain subclasses of interspersed DNA repeats display low methylation levels compared with normal postnatal somatic tissues [38, 112, 124–126]. Reminiscent of the tendency (with many exceptions, as described above) towards DNA repeats and unique sequences having opposite methylation changes in cancer, single-copy genes become demethylated but tandem and interspersed repeats retain their methylation in murine primordial germ cells at 12.5–13.5 dpc [123].

Another interface between the germ line epigenome and cancer is seen in the exceptionally strong global DNA hypomethylation in seminomatous testicular germ cell tumors. In our 1982 study of 62 tumors representing 23 different types, we found that a testicular seminoma had only 1.4% of its genomic C present as 5mC, while the next lowest 5mC level for a cancer was 2.4% [1]. The range of genomic 5mC levels among the normal tissues that we studied was 3.5–4.1% of C residues methylated. Smiraglia et al. confirmed the extraordinary depletion of 5mC in the genomes of many seminomas [127]. This finding has been ascribed to the origin of seminomas from primordial germ cells that had undergone massive demethylation before oncogenic transformation without subsequent de novo methylation thereafter [127, 128]. Importantly, seminomas generally show none of the CGI hypermethylation so prevalent in other types of cancer, but rather display extreme overall DNA hypomethylation [127]. Therefore, cancers can develop without gene region hypermethylation but with extreme overall genomic hypomethylation.

2.6 Opposite Cancer-Linked Changes in DNA Methylation in DNA Repeats: Hypo- and Hypermethylation

Opposite types of cancer-linked DNA methylation changes can occur in the same DNA sequence, as we found in a Southern blot study of methylation of NBL2, a 1.4-kb sequence repeated in tandem mostly near the centromeres of acrocentric chromosomes [39]. NBL2 was hypomethylated at HhaI sites (5′-CGCG-3′ sites) in

17% of ovarian carcinomas and hypermethylated in >70% of ovarian carcinomas and Wilms tumors at the same sites [39]. Various normal postnatal somatic tissues exhibited partial methylation at HhaI sites in NBL2 and were similar to each other in their methylation patterns at this tandem repeat. Using NotI (5′-GCGGCCGC-3′) for Southern blotting, only the cancer-linked hypomethylation of NBL2 was previously observed [108, 110] because NotI cleaves control somatic DNA too infrequently to reveal hypermethylation in cancers. This is an example of the importance of considering the technique used in evaluating results on DNA methylation [92] as well as the appropriate control DNA for comparison to the cancer. A few cancer DNAs digested with HhaI displayed two distinct fractions of NBL2 sequences, one with overall hypermethylation and the other with overall hypomethylation relative to all the somatic controls, which suggests that the repeats at one chromosomal location underwent de novo methylation and at another underwent demethylation during carcinogenesis. Hairpin genomic sequencing [129] (see below) at two ~0.3-kb subregions of the 1.4-kb *NBL2* ([8] and Nishiyama and Ehrlich, unpublished data) confirmed that hypomethylation at NBL2 predominated in some cancers and hypermethylation in others in comparison to normal somatic tissues, which displayed much site specificity in the methylation status of individual CpG sites. Therefore, a small region of DNA can be made unstable epigenetically during carcinogenesis so that CpG sites that are very near to each other undergo opposite changes in DNA methylation. The plasticity of the directionality of methylation changes at DNA repeats in cancers has also been seen in recent genome-wide studies [15, 18].

D4Z4, a heterologous tandem array (macrosatellite) located at subtelomeric 4q and 10q, also exhibited strong hypomethylation in the bulk of the array in some cancers and hypermethylation in others of the same type [9]. Several of the cancers had extremely high levels of methylation in more than three consecutive 3.3-kb repeat units of D4Z4, indicative of the spreading of de novo methylation. This methylation spreading seems to have limits to its processivity and to be prone to stop at certain subregions of the repeat unit.

2.7 Tagging Classes of DNA Sequences for Demethylation

Because NBL2 and D4Z4 tandem repeats displayed overall hypomethylation in some cancers and hypermethylation in others, it was highly informative to compare their methylation changes in a given cancer. Among 17 ovarian carcinomas and 44 Wilms tumors, there was a significant correlation ($p<0.001$) between the direction (either hypo- or hypermethylation) and degree of methylation change (strong, moderate, or weak) at D4Z4 and the dissimilar NBL2 [9]. This suggests that diverse sequences on different chromosomes may be similarly tagged for demethylation or de novo methylation (methylation of symmetrically unmethylated CpG dyads) during carcinogenesis. However, many cancers with extensive hypermethylation of D4Z4 and NBL2 repeats displayed hypomethylation of another, heterologous tandem repeat, juxtacentromeric satellite 2 on chromosome 1 (Sat2) [39].

NBL2 (mostly in the short arm of the acrocentric chromosomes) and D4Z4 (in the subtelomeric region of chromosomes 4 and 10) are both rich in G+C and look like very long CGIs. However, they differ appreciably in their G+C composition (61% and 73%, respectively) and their CpG content (5.7% and 9.9%, respectively). Analysis of histone modification and DNaseI sensitivity has been done for D4Z4 and indicates that its chromatin has properties midway between constitutive heterochromatin and unexpressed euchromatin [130, 131]. In contrast, Sat2, which is in the pericentromeric region, is constitutively heterochromatic and highly condensed in interphase. It has only 38% G+C but, nonetheless, it has 5.1% CpG. Therefore, the CpG suppression seen in the overall genome is not evident in Sat2. Sometimes even Sat2, with its rather CpG-rich character, becomes hypermethylated in cancers at a CpG dyad that exhibits a low methylation level in normal somatic tissues [132].

That the G+C content and chromatin structure is important for recruiting machinery for either demethylation or de novo methylation is consistent with our findings on the HpaII site immediately proximal to the D4Z4 array. It is located in a 0.2-kb D4Z4-proximal subregion that has 43% G+C, while D4Z4 has 73% G+C in all of its essentially identical, tandem 3.3-kb repeats. This 0.2-kb sequence immediately adjacent to the array is prone to tumor-linked hypomethylation even in cancers displaying strong hypermethylation within the array [9]. Surprisingly, even the adjacent D4Z4 repeat unit at the proximal end of the array became hypomethylated in cancers with hypermethylation of the bulk of the array. Probably, the array-adjacent sequence with its much lower G+C content helps confer a different chromatin structure on the neighboring D4Z4 repeat unit, which, in turn, affects the directionality of cancer-linked methylation change. Interestingly, a study of tandem transgenic repeats in mice revealed that, in some animals, all of the (G+C)-rich transgene units became methylated except for one copy adjacent to cellular DNA [133]. Despite the regional properties of DNA and chromatin that may recruit cancer-associated DNA methylation or demethylation apparati, there are, as mentioned above, very local sequence-specific effects which allow individual CpG dyads to circumvent regional demethylation or de novo methylation [8, 9].

DNA demethylation both influences and is strongly influenced by histone modifications. For example, histone H3 trimethylation at lysine 4 (H3K4me3) correlates best with the lack of DNA methylation around the transcription start site [66]. This was found for both CGI promoters [134] and promoters that do not contain a CGI, and for CpG methylation as well as the appreciable amount of CpA methylation in embryonal stem cells [66]. A histone H3 unmethylated at lysine 4 has been implicated as necessary for de novo methylation by DNMT3A in conjunction with its interacting partner DNMT3L [135]. Increased activity of the histone lysine demethylase LSD1 (KDM1A), which, depending on its interacting partners, demethylates K4- or K9-methylated histone H3, has been found to correlate with an adverse outcome and a less differentiated phenotype in neuroblastomas [136]. Conversely, mutation of the *Lsd1* gene blocks murine gastrulation [137] and results in global DNA hypomethylation. This may be partially due to the need for Lsd1/LSD1 to demethylate the DNMT1 enzyme itself and thereby increase its stability

[137] but also could reflect the role of this enzyme in the demethylation of H3K9me3. There are many other players that could influence DNA methylation during carcinogenesis by their effects on chromatin structure, e.g., poly(ADP-ribosyl)ation, other types of histone modifications, histone variants, nonhistone chromatin proteins, specific interactions with DNMT proteins, and modulation of the set of DNA methyltransferase isoforms produced at the RNA or protein levels [138–143]. Nonetheless, multi-functionality of LSD1 in its ability to demethylate proteins and both activating and repressive histone methylation marks may serve as a paradigm for how, paradoxically, there can be both increases and decreases in DNA methylation in a given cancer cell.

2.8 Active Versus Passive DNA Demethylation

There are two broad classes of mechanisms by which 5mC residues can be replaced by C residues (DNA demethylation). During replicative or repair DNA synthesis there may be a failure to methylate the newly synthesized DNA strand at a symmetrically methylated CpG dyad (passive demethylation), which will initially result in a hemimethylated dyad (Fig. 2.2). If this failure occurs again at the same CpG dyad in the next round of replication, then a symmetrically unmethylated CpG dyad will be the result. Active demethylation involves 5mC residues being physically replaced with C residues (at the base or mononucleotide level) or, less likely, the methyl group being removed enzymatically. Accumulating evidence favors mainly active demethylation contributing to the naturally occurring DNA demethylation by the replacement of C residues [144, 145]. Active demethylation is consistent with

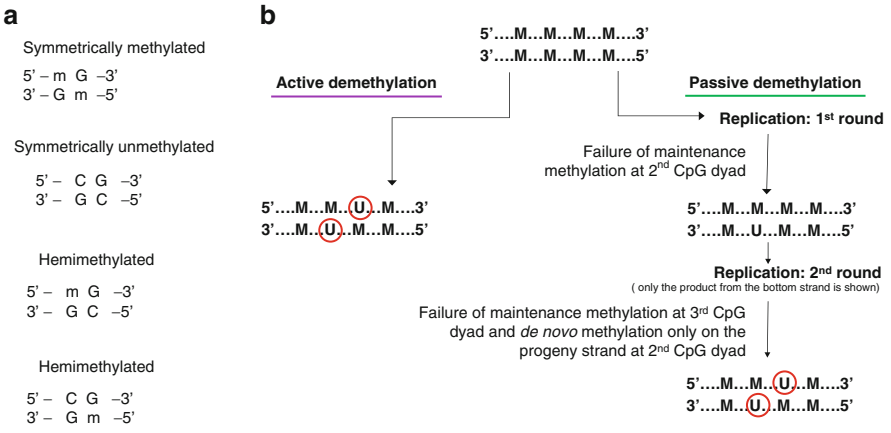

Fig. 2.2 Findings of consecutive hemimethylated dyads of opposite orientation in normal and cancer cells are best explained by active demethylation. (**a**) m, 5mC; C, unmethylated cytosine. (**b**) M, 5′-5mCpG-3′; U, 5′-CpG-3′. The generation of hemimethylated dyads of opposite orientation by passive demethylation would involve improbable changes in the second round of replication

the rapid and distributive loss of 5mC and the replication independence that has been demonstrated for many examples of naturally programmed demethylation of mammalian genomes [146, 147]. However, passive demethylation or a combination of active and passive demethylation due to inadequate maintenance methylation [148] is likely to also play a role in normal and pathological decreases in DNA methylation. Hemimethylated dyads (Fig. 2.2) can be intermediates in both active and passive demethylation of DNA as well as being intermediates in maintenance methylation.

2.9 Maintenance of DNA Methylation Patterns Through Hemimethylated Intermediates

The processes by which DNA methylation patterns are maintained are highly relevant to understanding how DNA demethylation occurs. Over 30 years ago, mechanisms for the inheritance of DNA methylation were initially proposed [149, 150]. In the traditional view, methylation at each site is assumed to be governed by the processes of de novo methylation and maintenance methylation, and these processes are independent of one another. The maintenance of methylation patterns has been attributed to the methyltransferase Dnmt1. As summarized in a 2009 review by Jones and Liang, "The basis of this model is that DNA methylation patterns are established in germ cells and in developing embryos by the activity of the de novo DNA methyltransferases Dnmt3a and Dnmt3B. Subsequently, methylation patterns are inherited after DNA replication primarily owing to the activity of Dnmt1, which has a preference for hemimethylated sites that are generated through DNA synthesis" [151]. The premise of independently acting mechanisms for de novo and maintenance methylation has led to the construction of stochastic models for methylation inheritance [152–157].

2.10 Alternative Mechanisms for Maintenance Methylation

The accepted dogma of de novo methylation catalyzed by DNMT3A/Dnmt3a, DNMT3B/Dnmt3b, and maintenance methylation through obligatory hemimethylated intermediates via DNMT1/Dnmt1 has recently been called into question. According to the original model for maintenance methylation, hemimethylated CpG dyads (Fig. 2.2) should be short-lived and difficult to detect. However, as early as 1986, demethylation with long-lived hemimethylated CpG dyads was observed at individual CpG sites in the avian vitellogenin II gene following treatment with estradiol, which suggested an active pathway through excision repair and/or enzymatic demethylation [158]. A later study of the rat alpha-actin gene promoter provided evidence for hemimethylated intermediates persisting more than 48 hours prior to becoming fully demethylated and suggested active demethylation involving

cis-acting DNA elements [159]. Subsequently, Liang et al. [160] developed an assay that allowed determination of hemimethylation at *Hpa*II sites (CCGG). In mouse embryonic stem cells, levels of hemimethylation in some repetitive sequence regions were significantly higher than the traditional model of maintenance methylation by Dnmt1 would predict. By looking at gene knockouts for *Dnmt1* and *Dnmt3a* and *Dnmt3b*, they deduced that ongoing de novo methylation by Dnmt3a or Dnmt3b in a highly cooperative manner with Dnmt1 in embryonal stem cells compensated for inefficient maintenance methylation by Dnmt1 in these regions. These results suggest a constant, rather than sporadic or only differentiation-associated, role for de novo methylation in vivo. They concluded that sequences would gradually become demethylated without this constant role for de novo methylation to compensate for inefficient replication-coupled maintenance methylation. Furthermore, in a study by Chen et al. [161], loss of Dnmt1 gave only a 10% decrease in methylation overall following one cell cycle of replication in human colorectal carcinoma cells. This conditional knockout resulted in hemimethylation of 18% of sites analyzed by hairpin genomic sequencing in the CGI of an L1 transposable element. The overall level of methylation at CpG dyads in these sequences in cells with normal Dnmt1 was around 85% with no detectable hemimethylation.

In the alternative model for maintaining DNA methylation patterns that was proposed by Jones and Liang [151], DNMT1, the most abundant DNA methyltransferase is still considered to be primarily a maintenance methylase and is responsible for most of the replication-associated DNA methylation. However, they propose that DNMT3A and DNMT3B enzymes remain bound to nucleosomes that contain high levels of DNA methylation. Following replication, CpG dyads whose methylation fails to be correctly maintained by DNMT1 would then be "corrected" by DNMT3A and DNMT3B, so that these enzymes would preserve highly methylated regions without strictly "reading" the patterns on the parental strand. In this way, the methylation state of a region is maintained rather than a site-specific methylation pattern. In addition, DNMT1 might participate in some of this correction of lingering hemimethylated sites that have left the vicinity of the replication fork, perhaps recruited by proteins such as UHRF1 which recognizes hemimethylated sites (see below). This concept of repair methylation is consistent with findings that methylation patterns in highly methylated regions tend to vary among molecules and higher rates of de novo methylation are observed in highly methylated sequences [129]. Moreover, non-CpG methylation at asymmetrical sites, which is found mostly in embryonal stem cells [70], should rely on de novo methyltransferase activity for perpetuating the DNA methylation patterns, as described below.

In cancers, the frequent presence of long blocks of hypomethylated DNA [7, 16, 42, 47, 105] and the usual predominance of overall decreases rather than increases in 5mC content of the genome suggest that passive demethylation contributes to cancer-associated genomic hypomethylation. Passive demethylation might involve either a lack of methylation of hemimethylated sites by DNMT1 or a failure of DNMT3A or DNMT3B to retain dense methylation of a normally highly methylated region. However, the current, more layered view of the maintenance of DNA methylation patterns suggests that while some of the demethyla-

tion of DNA in cancer occurs by a failure of maintenance methylation, most is due to an active mechanism. Recent studies of normal differentiation- or physiology-associated DNA demethylation support an active type of DNA methylation involving enzymatically catalyzed modification of 5mC residues to 5hmC residues (and subsequent oxidation products) or thymine residues followed by DNA repair [162–164]. Three-step processes for active DNA demethylation have been proposed in which 5mC is first enzymatically modified; then demethylated on one strand, most likely by excision repair; and later fully demethylated by a mechanism that avoids inducing double-strand breaks during removal of both 5mCs of a 5mCpG dyad [165]. The last step could involve a repair mechanism that preferentially acts on hemimethylated substrates [165] or passive demethylation of a hemimethylated or hemihydroxymethylated dyad. The latter could be due to the 5hmC residues on one strand of a hemihydroxymethylated dyad not being recognized for maintenance methylation [148].

UHRF1 (also known as NP95) is a cofactor that interacts specifically with hemimethylated DNA and may participate in demethylation as well as de novo methylation of cancer epigenomes. UHRF1 also interacts with DNMT1, and even more strongly with DNMT3A and DNMT3B [166], and thereby, may be involved in the recruitment of DNMT3A/3B to unmethylated regions during tumorigenesis leading to de novo methylation [167]. However, recent work on gliomas has identified the disruption of DNMT1, PCNA, and UHRF1 interactions as a crucial oncogenic event promoting DNA hypomethylation-induced tumorigenesis in the absence of DNMT1 deficiencies [168]. Thus, while upregulation of UHRF1 may contribute to the silencing of tumor suppressors through de novo methylation, the disruption of DNMT1/PCNA/UHRF1 interactions might result in cancer-associated DNA hypomethylation affecting transcription.

2.11 Insights into Cancer-Associated DNA Demethylation from Studies of DNA Hemimethylation

The introduction of hairpin-bisulfite PCR (hairpin genomic sequencing) by Laird et al. in 2004 [129] has enabled the observation of the methylation status on both strands of individual DNA molecules on a site-by-site basis. In bisulfite-based genomic sequencing, bisulfite causes deamination of unmethylated C residues, but not methylated C residues [169]. Hairpin genomic sequencing allows analysis of methylation at every CG dinucleotide pair in a given region on covalently linked DNA strands of a restriction fragment. A caveat about these studies of DNA hemimethylation is that bisulfite-based DNA methylation analysis cannot distinguish between 5hmC and 5mC, as described above, and 5hmC on one strand at a CpG dyad is not recognized for maintenance methylation [170]. Therefore, it is possible that the detected hemimethylation is actually a CpG dyad with one unmethylated C residue and one 5hmC residue. However, in the studies of tandem DNA repeats in cancers described below, this is unlikely because 5hmC is predominantly in gene

regions and all studied cancers and cancer cell lines have extremely low levels of 5hmC [84–86].

By sodium bisulfite-based whole-methylome analysis using next-generation sequencing, Lister et al. analyzed more than 90% of the cytosines in human H1 embryonic stem cells (H1 ES) and IMR90 fetal lung fibroblasts [70]. While nearly all of the methylcytosines detected in the IMR90 fibroblasts were in the CG dinucleotide context, considerable methylation in non-CG contexts (mCHG and mCHH, where H = A, C or T) was observed in the H1 stem cells, comprising almost 25% of the total methylation, in agreement with a recent study by Laurent et al. [66]. Methylation at mCHG sites in H1 ES was also highly asymmetrical, with 98% of such sites observed to be methylated on only one strand. Non-CpG methylation was also found to be significantly higher on the antisense strand of gene bodies, suggesting a nonrandom bias in the observed asymmetry. Non-CpG methylation disappeared upon differentiation of the H1 stem cells, but was restored in differentiated cells induced to form pluripotent stem cells. These findings suggest that asymmetrical methylation at non-CG dinucleotide sites may contribute to maintenance of the pluripotent state. They are reminiscent of the less frequent, hemimethylated CG dinucleotide sites that we and Laird et al. have seen in various DNA repeats [8, 132, 171] or single-copy sequences [129] in normal or cancer tissues.

2.12 Hemimethylated CpG Dyads in Cancer

Although reports of DNA hemimethylation in cancer are few, our studies of hemimethylated DNA in cancers support the involvement of active demethylation in generating cancer-linked genomic hypomethylation. We analyzed DNA methylation changes in depth at the above-mentioned tandem repeats NBL2 and at Sat2 in ovarian epithelial tumors and Wilms tumors by hairpin genomic sequencing [8, 132]. In a study of 13 CpGs in a 0.2-kb subregion of Sat2 in ovarian carcinomas and somatic control tissues, hairpin genomic sequencing not only revealed significantly greater clonal variability in methylation patterns in the cancers than in diverse control tissues but also provided statistically significant evidence of clustering among both hemimethylated and fully demethylated sites [132]. Runs of hemimethylated sites with identical orientation were seen at higher than expected rates in the cancers. Similarly, an analysis of 14 CpGs in the NBL2 repeat unit identified both hypomethylation and hypermethylation in ovarian carcinomas and Wilms tumors, again with a high degree of clonal variation in methylation patterns within each sample [8].

Diverse control and cancer samples contained some DNA clones derived from unusual, consecutive hemimethylated CpG dyads of opposite polarity. Figure 2.2b illustrates how an M/U (5′-5mCpG-3′/3′-GpC-5′) dyad near a U/M dyad (5′-CpG-3′/3′-Gp5mC-5′) could be generated by active vs. passive demethylation. Passive demethylation would require inhibition of maintenance methylation (by DNMT1 alone or in conjunction with DNMT3A and DNMT3B, as discussed above) at a single CpG dyad in one round of replicative DNA synthesis. The next round of replica-

tion would then have to involve both asymmetrical de novo methylation of only the opposite strand of this dyad and inhibition, once again, of maintenance methylation at a neighboring CpG dyad. In contrast to this highly unlikely sequence of events, active demethylation can easily explain the generation of various patterns of hemimethylation in contiguous CpG dyads with either identical or opposite orientation.

In a simulation study jointly analyzing the Sat2 and NBL2 regions, we found that the observed methylation patterns in the carcinomas were best explained by a mechanism that accounted for site-to-site correlation [157]. Prior studies have produced evidence of spreading of methylation in cancer [172–176]. Our analysis suggests that demethylation may progress by spreading as well.

We propose that during carcinogenesis a highly methylated DNA sequence becomes partially demethylated by active demethylation. The sequence may then attain a density of 5mC residues in an atypical intermediate range. This intermediate level of methylation might confer less stability during successive cell divisions for maintenance of the methylation pattern or methylation density. The stability of a given partially methylated sequence could be determined, in part, by the efficiency with which DNMT3A and DNMT3B recognize unmethylated CpG sites in the sequence for repair methylation. Abnormally low methylation levels may favor the generation of yet lower levels, with some site-specific effects superimposed on the regional ones. Thus, active demethylation might start cancer-associated demethylation and a failure of maintenance methylation (including repair methylation) might continue it. The result could explain the observation that tumor progression is frequently linked to a progressive decrease in methylation.

2.13 Conclusions

Recently, there has been a burst of studies increasing our understanding of the importance of changes in DNA methylation in intragenic, promoter, and intergenic regions during differentiation and in response to some types of physiological change. These findings suggest that much more of the cancer-associated DNA hypomethylation contributes to tumor formation and progression than previously recognized. Similarly, high-resolution analysis of cancer methylomes in comparison to appropriate controls indicates that the extent of cancer-linked hypomethylation is larger than previously appreciated and affects a greater variety of DNA sequences. We propose that the pathways for normal DNA demethylation that operate during differentiation or induction of certain physiological changes become hijacked during carcinogenesis and tumor progression, leading to the initiation of cancer-associated DNA demethylation. This demethylation then may spread in *cis* by both additional rounds of active demethylation and by passive demethylation involving failures in classical maintenance methylation and replication-associated repair methylation. The net result of some of this cancer-associated DNA demethylation could be abnormal modulation of transcription and even some aberrant posttranscriptional processing of transcripts as well as increases in DNA recombination, thereby contributing to tumor formation and progression.

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References

1. Gama-Sosa MA, Slagel VA, Trewyn RW, Oxenhandler R, Kuo KC, Gehrke CW, Ehrlich M (1983) The 5-methylcytosine content of DNA from human tumors. Nucleic Acids Res 11:6883–6894
2. Feinberg AP, Vogelstein B (1983) Hypomethylation distinguishes genes of some human cancers from their normal counterparts. Nature 301(5895):89–92
3. Ehrlich M (2009) DNA hypomethylation in cancer cells. Epigenomics 1(2):239–259
4. Ehrlich M, Jiang G, Fiala ES, Dome JS, Yu MS, Long TI, Youn B, Sohn O-S, Widschwendter M, Tomlinson GE, Chintagumpala M, Champagne M, Parham DM, Liang G, Malik K, Laird PW (2002) Hypomethylation and hypermethylation of DNA in Wilms tumors. Oncogene 21(43):6694–6702
5. Ehrlich M (2006) Cancer-linked DNA hypomethylation and its relationship to hypermethylation. Curr Top Microbiol Immunol 310:251–274
6. Ehrlich M (2002) DNA methylation in cancer: too much, but also too little. Oncogene 21(35):5400–5413
7. Pfeifer GP, Rauch TA (2009) DNA methylation patterns in lung carcinomas. Semin Cancer Biol 19(3):181–187
8. Nishiyama R, Qi L, Lacey M, Ehrlich M (2005) Both hypomethylation and hypermethylation in a 0.2-kb region of a DNA repeat in cancer. Molec Cancer Res 3:617–626
9. Tsumagari K, Qi L, Jackson K, Shao C, Lacey M, Sowden J, Tawil R, Vedanarayanan V, Ehrlich M (2008) Epigenetics of a tandem DNA repeat: chromatin DNaseI sensitivity and opposite methylation changes in cancers. Nucleic Acids Res 36:2196–2207
10. Lindsey JC, Lusher ME, Anderton JA, Gilbertson RJ, Ellison DW, Clifford SC (2007) Epigenetic deregulation of multiple S100 gene family members by differential hypomethylation and hypermethylation events in medulloblastoma. Br J Cancer 97(2):267–274
11. Grunau C, Brun ME, Rivals I, Selves J, Hindermann W, Favre-Mercuret M, Granier G, De Sario A (2008) BAGE hypomethylation, a new epigenetic biomarker for colon cancer detection. Cancer Epidemiol Biomarkers Prev 17(6):1374–1379
12. Sultan M, Schulz MH, Richard H, Magen A, Klingenhoff A, Scherf M, Seifert M, Borodina T, Soldatov A, Parkhomchuk D, Schmidt D, O'Keeffe S, Haas S, Vingron M, Lehrach H, Yaspo ML (2008) A global view of gene activity and alternative splicing by deep sequencing of the human transcriptome. Science 321(5891):956–960
13. Alvarez H, Opalinska J, Zhou L, Sohal D, Fazzari MJ, Yu Y, Montagna C, Montgomery EA, Canto M, Dunbar KB, Wang J, Roa JC, Mo Y, Bhagat T, Ramesh KH, Cannizzaro L, Mollenhauer J, Thompson RF, Suzuki M, Meltzer SJ, Melnick A, Greally JM, Maitra A, Verma A (2011) Widespread hypomethylation occurs early and synergizes with gene amplification during esophageal carcinogenesis. PLoS Genet 7(3):e1001356
14. Sandoval J, Heyn HA, Moran S, Serra-Musach J, Pujana MA, Bibikova M, Esteller M (2011) Validation of a DNA methylation microarray for 450,000 CpG sites in the human genome. Epigenetics 6(6):692–702
15. Ruike Y, Imanaka Y, Sato F, Shimizu K, Tsujimoto G (2010) Genome-wide analysis of aberrant methylation in human breast cancer cells using methyl-DNA immunoprecipitation combined with high-throughput sequencing. BMC Genomics 11:137
16. Hansen KD, Timp W, Bravo HC, Sabunciyan S, Langmead B, McDonald OG, Wen B, Wu H, Liu Y, Diep D, Briem E, Zhang K, Irizarry RA, Feinberg AP (2011) Increased methylation variation in epigenetic domains across cancer types. Nat Genet 43(8):768–775

17. Irizarry RA, Ladd-Acosta C, Wen B, Wu Z, Montano C, Onyango P, Cui H, Gabo K, Rongione M, Webster M, Ji H, Potash JB, Sabunciyan S, Feinberg AP (2009) The human colon cancer methylome shows similar hypo- and hypermethylation at conserved tissue-specific CpG island shores. Nat Genet 41(2):178–186
18. Feber A, Wilson GA, Zhang L, Presneau N, Idowu B, Down TA, Rakyan VK, Noon LA, Lloyd AC, Stupka E, Schiza V, Teschendorff AE, Schroth GP, Flanagan A, Beck S (2011) Comparative methylome analysis of benign and malignant peripheral nerve sheath tumors. Genome Res 21(4):515–524
19. Brenet F, Moh M, Funk P, Feierstein E, Viale AJ, Socci ND, Scandura JM (2011) DNA methylation of the first exon is tightly linked to transcriptional silencing. PLoS One 6(1):e14524
20. Song L, Zhang Z, Grasfeder LL, Boyle AP, Giresi PG, Lee BK, Sheffield NC, Graf S, Huss M, Keefe D, Liu Z, London D, McDaniell RM, Shibata Y, Showers KA, Simon JM, Vales T, Wang T, Winter D, Zhang Z, Clarke ND, Birney E, Iyer VR, Crawford GE, Lieb JD, Furey TS (2011) Open chromatin defined by DNaseI and FAIRE identifies regulatory elements that shape cell-type identity. Genome Res 21(10):1757–67
21. Tao Y, Xi S, Briones V, Muegge K (2010) Lsh mediated RNA polymerase II stalling at HoxC6 and HoxC8 involves DNA methylation. PLoS One 5(2):e9163
22. Bauer AP, Leikam D, Krinner S, Notka F, Ludwig C, Langst G, Wagner R (2010) The impact of intragenic CpG content on gene expression. Nucleic Acids Res 38(12):3891–3908
23. Schwartz S, Ast G (2010) Chromatin density and splicing destiny: on the cross-talk between chromatin structure and splicing. Embo J 29(10):1629–1636
24. Okitsu CY, Hsieh CL (2007) DNA methylation dictates histone H3K4 methylation. Mol Cell Biol 27(7):2746–2757
25. Okitsu CY, Hsieh JC, Hsieh CL (2010) Transcriptional activity affects the H3K4me3 level and distribution in the coding region. Mol Cell Biol 30(12):2933–2946
26. Lorincz MC, Dickerson DR, Schmitt M, Groudine M (2004) Intragenic DNA methylation alters chromatin structure and elongation efficiency in mammalian cells. Nat Struct Mol Biol 11(11):1068–1075
27. Deaton AM, Webb S, Kerr AR, Illingworth RS, Guy J, Andrews R, Bird A (2011) Cell type-specific DNA methylation at intragenic CpG islands in the immune system. Genome Res 21(7):1074–1086
28. Maunakea AK, Nagarajan RP, Bilenky M, Ballinger TJ, D'Souza C, Fouse SD, Johnson BE, Hong C, Nielsen C, Zhao Y, Turecki G, Delaney A, Varhol R, Thiessen N, Shchors K, Heine VM, Rowitch DH, Xing X, Fiore C, Schillebeeckx M, Jones SJ, Haussler D, Marra MA, Hirst M, Wang T, Costello JF (2010) Conserved role of intragenic DNA methylation in regulating alternative promoters. Nature 466(7303):253–257
29. Aporntewan C, Phokaew C, Piriyapongsa J, Ngamphiw C, Ittiwut C, Tongsima S, Mutirangura A (2011) Hypomethylation of intragenic LINE-1 represses transcription in cancer cells through AGO2. PLoS One 6(3):e17934
30. Qu G, Grundy PE, Narayan A, Ehrlich M (1999) Frequent hypomethylation in Wilms tumors of pericentromeric DNA in chromosomes 1 and 16. Cancer Genet Cytogenet 109:34–39
31. Eden A, Gaudet F, Waghmare A, Jaenisch R (2003) Chromosomal instability and tumors promoted by DNA hypomethylation. Science 300(5618):455
32. Yamada Y, Jackson-Grusby L, Linhart H, Meissner A, Eden A, Lin H, Jaenisch R (2005) Opposing effects of DNA hypomethylation on intestinal and liver carcinogenesis. Proc Natl Acad Sci USA 102(38):13580–13585
33. Cadieux B, Ching TT, Vandenberg SR, Costello JF (2006) Genome-wide hypomethylation in human glioblastomas associated with specific copy number alteration, methylenetetrahydrofolate reductase allele status, and increased proliferation. Cancer Res 66(17):8469–8476
34. Trejbalova K, Blazkova J, Matouskova M, Kucerova D, Pecnova L, Vernerova Z, Heracek J, Hirsch I, Hejnar J (2011) Epigenetic regulation of transcription and splicing of syncytins, fusogenic glycoproteins of retroviral origin. Nucleic Acids Res 39(20):8728–39

35. Goering W, Ribarska T, Schulz WA (2011) Selective changes of retroelement expression in human prostate cancer. Carcinogenesis 32(10):1484–92
36. Park SY, Yoo EJ, Cho NY, Kim N, Kang GH (2009) Comparison of CpG island hypermethylation and repetitive DNA hypomethylation in premalignant stages of gastric cancer, stratified for Helicobacter pylori infection. J Pathol 219(4):410–6
37. Yang AS, Estecio MR, Doshi K, Kondo Y, Tajara EH, Issa JP (2004) A simple method for estimating global DNA methylation using bisulfite PCR of repetitive DNA elements. Nucleic Acids Res 32(3):e38
38. Weisenberger DJ, Campan M, Long TI, Kim M, Woods C, Fiala E, Ehrlich M, Laird PW (2005) Analysis of repetitive element DNA methylation by MethyLight. Nucleic Acids Res 33(21):6823–6836
39. Nishiyama R, Qi L, Tsumagari K, Dubeau L, Weissbecker K, Champagne M, Sikka S, Nagai H, Ehrlich M (2005) A DNA repeat, NBL2, is hypermethylated in some cancers but hypomethylated in others. Cancer Biol Ther 4(4):440–448
40. Pulukuri SM, Estes N, Patel J, Rao JS (2007) Demethylation-linked activation of urokinase plasminogen activator is involved in progression of prostate cancer. Cancer Res 67(3): 930–939
41. Clark SJ (2007) Action at a distance: epigenetic silencing of large chromosomal regions in carcinogenesis. Hum Mol Genet 16 Spec No 1:R88–95
42. Andrews J, Kennette W, Pilon J, Hodgson A, Tuck AB, Chambers AF, Rodenhiser DI (2010) Multi-platform whole-genome microarray analyses refine the epigenetic signature of breast cancer metastasis with gene expression and copy number. PLoS One 5(1):e8665
43. Novak P, Jensen T, Oshiro MM, Watts GS, Kim CJ, Futscher BW (2008) Agglomerative epigenetic aberrations are a common event in human breast cancer. Cancer Res 68(20): 8616–8625
44. Coolen MW, Stirzaker C, Song JZ, Statham AL, Kassir Z, Moreno CS, Young AN, Varma V, Speed TP, Cowley M, Lacaze P, Kaplan W, Robinson MD, Clark SJ (2010) Consolidation of the cancer genome into domains of repressive chromatin by long-range epigenetic silencing (LRES) reduces transcriptional plasticity. Nat Cell Biol 12(3):235–246
45. Yegnasubramanian S, Wu Z, Haffner MC, Esopi D, Aryee MJ, Badrinath R, He TL, Morgan JD, Carvalho B, Zheng Q, De Marzo AM, Irizarry RA, Nelson WG (2011) Chromosome-wide mapping of DNA methylation patterns in normal and malignant prostate cells reveals pervasive methylation of gene-associated and conserved intergenic sequences. BMC Genomics 12:313
46. Widschwendter M, Jiang G, Woods C, Muller HM, Fiegl H, Goebel G, Marth C, Holzner EM, Zeimet AG, Laird PW, Ehrlich M (2004) DNA hypomethylation and ovarian cancer biology. Cancer Res 64(13):4472–4480
47. Weber M, Davies JJ, Wittig D, Oakeley EJ, Haase M, Lam WL, Schubeler D (2005) Chromosome-wide and promoter-specific analyses identify sites of differential DNA methylation in normal and transformed human cells. Nat Genet 37(8):853–862
48. Ehrlich M (2003) Expression of various genes is controlled by DNA methylation during mammalian development. J Cell Biochem 88:899–910
49. Shen L, Kondo Y, Guo Y, Zhang J, Zhang L, Ahmed S, Shu J, Chen X, Waterland RA, Issa JP (2007) Genome-wide profiling of DNA methylation reveals a class of normally methylated CpG island promoters. PLoS Genet 3(10):2023–2036
50. Ortmann CA, Eisele L, Nuckel H, Klein-Hitpass L, Fuhrer A, Duhrsen U, Zeschnigk M (2008) Aberrant hypomethylation of the cancer-testis antigen PRAME correlates with PRAME expression in acute myeloid leukemia. Ann Hematol 87(10):809–818
51. Milicic A, Harrison LA, Goodlad RA, Hardy RG, Nicholson AM, Presz M, Sieber O, Santander S, Pringle JH, Mandir N, East P, Obszynska J, Sanders S, Piazuelo E, Shaw J, Harrison R, Tomlinson IP, McDonald SA, Wright NA, Jankowski JA (2008) Ectopic expression of P-cadherin correlates with promoter hypomethylation early in colorectal carcinogenesis and enhanced intestinal crypt fission in vivo. Cancer Res 68(19):7760–7768

52. Cheung HH, Davis AJ, Lee TL, Pang AL, Nagrani S, Rennert OM, Chan WY (2011) Methylation of an intronic region regulates miR-199a in testicular tumor malignancy. Oncogene 30(31):3404–3415
53. Colaneri A, Staffa N, Fargo DC, Gao Y, Wang T, Peddada SD, Birnbaumer L (2011) Expanded methyl-sensitive cut counting reveals hypomethylation as an epigenetic state that highlights functional sequences of the genome. Proc Natl Acad Sci USA 108(23):9715–9720
54. Kwon MJ, Shin YK (2011) Epigenetic regulation of cancer-associated genes in ovarian cancer. Int J Mol Sci 12(2):983–1008
55. Laursen KB, Wong PM, Gudas LJ (2011) Epigenetic regulation by RAR{alpha} maintains ligand-independent transcriptional activity. Nucleic Acids Res 40(1):102–15
56. Baba Y, Nosho K, Shima K, Huttenhower C, Tanaka N, Hazra A, Giovannucci EL, Fuchs CS, Ogino S (2010) Hypomethylation of the IGF2 DMR in colorectal tumors, detected by bisulfite pyrosequencing, is associated with poor prognosis. Gastroenterology 139(6):1855–1864
57. Eckhardt F, Lewin J, Cortese R, Rakyan VK, Attwood J, Burger M, Burton J, Cox TV, Davies R, Down TA, Haefliger C, Horton R, Howe K, Jackson DK, Kunde J, Koenig C, Liddle J, Niblett D, Otto T, Pettett R, Seemann S, Thompson C, West T, Rogers J, Olek A, Berlin K, Beck S (2006) DNA methylation profiling of human chromosomes 6, 20 and 22. Nat Genet 38(12):1378–1385
58. Smale ST (2010) Pioneer factors in embryonic stem cells and differentiation. Curr Opin Genet Dev 20(5):519–526
59. Serandour AA, Avner S, Percevault F, Demay F, Bizot M, Lucchetti-Miganeh C, Barloy-Hubler F, Brown M, Lupien M, Metivier R, Salbert G, Eeckhoute J (2011) Epigenetic switch involved in activation of pioneer factor FOXA1-dependent enhancers. Genome Res 21(4): 555–565
60. Xu J, Pope SD, Jazirehi AR, Attema JL, Papathanasiou P, Watts JA, Zaret KS, Weissman IL, Smale ST (2007) Pioneer factor interactions and unmethylated CpG dinucleotides mark silent tissue-specific enhancers in embryonic stem cells. Proc Natl Acad Sci USA 104(30): 12377–12382
61. Taube JH, Allton K, Duncan SA, Shen L, Barton MC (2010) Foxa1 functions as a pioneer transcription factor at transposable elements to activate Afp during differentiation of embryonic stem cells. J Biol Chem 285(21):16135–16144
62. Magnani L, Eeckhoute J, Lupien M (2011) Pioneer factors: directing transcriptional regulators within the chromatin environment. Trends Genet 27(11):465–74
63. Hatada I, Namihira M, Morita S, Kimura M, Horii T, Nakashima K (2008) Astrocyte-specific genes are generally demethylated in neural precursor cells prior to astrocytic differentiation. PLoS One 3(9):e3189
64. Kalari S, Pfeifer GP (2010) Identification of driver and passenger DNA methylation in cancer by epigenomic analysis. Adv Genet 70:277–308
65. Meissner A, Mikkelsen TS, Gu H, Wernig M, Hanna J, Sivachenko A, Zhang X, Bernstein BE, Nusbaum C, Jaffe DB, Gnirke A, Jaenisch R, Lander ES (2008) Genome-scale DNA methylation maps of pluripotent and differentiated cells. Nature 454(7205):766–770
66. Laurent L, Wong E, Li G, Huynh T, Tsirigos A, Ong CT, Low HM, Kin Sung KW, Rigoutsos I, Loring J, Wei CL (2010) Dynamic changes in the human methylome during differentiation. Genome Res 20(3):320–331
67. Ball MP, Li JB, Gao Y, Lee JH, LeProust EM, Park IH, Xie B, Daley GQ, Church GM (2009) Targeted and genome-scale strategies reveal gene-body methylation signatures in human cells. Nat Biotechnol 27(4):361–368
68. Wu H, Coskun V, Tao J, Xie W, Ge W, Yoshikawa K, Li E, Zhang Y, Sun YE (2010) Dnmt3a-dependent nonpromoter DNA methylation facilitates transcription of neurogenic genes. Science 329(5990):444–448
69. Guo JU, Ma DK, Mo H, Ball MP, Jang MH, Bonaguidi MA, Balazer JA, Eaves HL, Xie B, Ford E, Zhang K, Ming GL, Gao Y, Song H (2011) Neuronal activity modifies the DNA methylation landscape in the adult brain. Nat Neurosci 14(10):1345–1351
70. Lister R, Pelizzola M, Dowen RH, Hawkins RD, Hon G, Tonti-Filippini J, Nery JR, Lee L, Ye Z, Ngo QM, Edsall L, Antosiewicz-Bourget J, Stewart R, Ruotti V, Millar AH, Thomson

JA, Ren B, Ecker JR (2009) Human DNA methylomes at base resolution show widespread epigenomic differences. Nature 462(7271):315–322
71. De Bustos C, Ramos E, Young JM, Tran RK, Menzel U, Langford CF, Eichler EE, Hsu L, Henikoff S, Dumanski JP, Trask BJ (2009) Tissue-specific variation in DNA methylation levels along human chromosome 1. Epigenetics Chromatin 2(1):7
72. Ke XS, Qu Y, Cheng Y, Li WC, Rotter V, Oyan AM, Kalland KH (2010) Global profiling of histone and DNA methylation reveals epigenetic-based regulation of gene expression during epithelial to mesenchymal transition in prostate cells. BMC Genomics 11:669
73. Cheng X, Blumenthal RM (2010) Coordinated chromatin control: structural and functional linkage of DNA and histone methylation. Biochemistry 49(14):2999–3008
74. Stengel S, Fiebig U, Kurth R, Denner J (2010) Regulation of human endogenous retrovirus-K expression in melanomas by CpG methylation. Genes Chromosomes Cancer 49(5):401–411
75. Appanah R, Dickerson DR, Goyal P, Groudine M, Lorincz MC (2007) An unmethylated 3' promoter-proximal region is required for efficient transcription initiation. PLoS Genet 3(2):e27
76. Chodavarapu RK, Feng S, Bernatavichute YV, Chen PY, Stroud H, Yu Y, Hetzel JA, Kuo F, Kim J, Cokus SJ, Casero D, Bernal M, Huijser P, Clark AT, Kramer U, Merchant SS, Zhang X, Jacobsen SE, Pellegrini M (2010) Relationship between nucleosome positioning and DNA methylation. Nature 466(7304):388–392
77. Hodges E, Smith AD, Kendall J, Xuan Z, Ravi K, Rooks M, Zhang MQ, Ye K, Bhattacharjee A, Brizuela L, McCombie WR, Wigler M, Hannon GJ, Hicks JB (2009) High definition profiling of mammalian DNA methylation by array capture and single molecule bisulfite sequencing. Genome Res 19(9):1593–1605
78. Luco RF, Allo M, Schor IE, Kornblihtt AR, Misteli T (2011) Epigenetics in alternative pre-mRNA splicing. Cell 144(1):16–26
79. Shing DC, Trubia M, Marchesi F, Radaelli E, Belloni E, Tapinassi C, Scanziani E, Mecucci C, Crescenzi B, Lahortiga I, Odero MD, Zardo G, Gruszka A, Minucci S, Di Fiore PP, Pelicci PG (2007) Overexpression of sPRDM16 coupled with loss of p53 induces myeloid leukemias in mice. J Clin Invest 117(12):3696–3707
80. Chu D, Zhang Z, Zhou Y, Wang W, Li Y, Zhang H, Dong G, Zhao Q, Ji G (2011) Notch1 and Notch2 have opposite prognostic effects on patients with colorectal cancer. Ann Oncol 22(11):2440–7
81. Figueroa JD, Flanders KC, Garcia-Closas M, Anderson WF, Yang XR, Matsuno RK, Duggan MA, Pfeiffer RM, Ooshima A, Cornelison R, Gierach GL, Brinton LA, Lissowska J, Peplonska B, Wakefield LM, Sherman ME (2010) Expression of TGF-beta signaling factors in invasive breast cancers: relationships with age at diagnosis and tumor characteristics. Breast Cancer Res Treat 121(3):727–735
82. Tsumagari K, Chang S-C, Lacey M, Baribault C, Chittur SV, Sowden J, Tawil R, Crawford GE, Ehrlich M (2011) Gene expression during normal and FSHD myogenesis. BMC Medical Genomics 4:67
83. Nagai H, Kim YS, Yasuda T, Ohmachi Y, Yokouchi H, Monden M, Emi M, Konishi N, Nogami M, Okumura K, Matsubara K (1999) A novel sperm-specific hypomethylation sequence is a demethylation hotspot in human hepatocellular carcinomas. Gene 237(1): 15–20
84. Kriaucionis S, Heintz N (2009) The nuclear DNA base 5-hydroxymethylcytosine is present in Purkinje neurons and the brain. Science 324(5929):929–930
85. Li W, Liu M (2011) Distribution of 5-hydroxymethylcytosine in different human tissues. J Nucleic Acids 2011:870726
86. Haffner MC, Chaux A, Meeker AK, Esopi DM, Gerber J, Pellakuru LG, Toubaji A, Argani P, Iacobuzio-Donahue C, Nelson WG, Netto GJ, De Marzo AM, Yegnasubramanian S (2011) Global 5-hydroxymethylcytosine content is significantly reduced in tissue stem/progenitor cell compartments and in human cancers. Oncotarget 2(8):627–37
87. Stroud H, Feng S, Morey Kinney S, Pradhan S, Jacobsen SE (2011) 5-Hydroxymethylcytosine is associated with enhancers and gene bodies in human embryonic stem cells. Genome Biol 12(6):R54

88. Ficz G, Branco MR, Seisenberger S, Santos F, Krueger F, Hore TA, Marques CJ, Andrews S, Reik W (2011) Dynamic regulation of 5-hydroxymethylcytosine in mouse ES cells and during differentiation. Nature 473(7347):398–402
89. Pastor WA, Pape UJ, Huang Y, Henderson HR, Lister R, Ko M, McLoughlin EM, Brudno Y, Mahapatra S, Kapranov P, Tahiliani M, Daley GQ, Liu XS, Ecker JR, Milos PM, Agarwal S, Rao A (2011) Genome-wide mapping of 5-hydroxymethylcytosine in embryonic stem cells. Nature 473(7347):394–397
90. Szulwach KE, Li X, Li Y, Song CX, Han JW, Kim S, Namburi S, Hermetz K, Kim JJ, Rudd MK, Yoon YS, Ren B, He C, Jin P (2011) Integrating 5-hydroxymethylcytosine into the epigenomic landscape of human embryonic stem cells. PLoS Genet 7(6):e1002154
91. Song CX, Szulwach KE, Fu Y, Dai Q, Yi C, Li X, Li Y, Chen CH, Zhang W, Jian X, Wang J, Zhang L, Looney TJ, Zhang B, Godley LA, Hicks LM, Lahn BT, Jin P, He C (2011) Selective chemical labeling reveals the genome-wide distribution of 5-hydroxymethylcytosine. Nat Biotechnol 29(1):68–72
92. Robinson MD, Statham AL, Speed TP, Clark SJ (2010) Protocol matters: which methylome are you actually studying? Epigenomics 2(4):587–598
93. Kinney SM, Chin HG, Vaisvila R, Bitinaite J, Zheng Y, Esteve PO, Feng S, Stroud H, Jacobsen SE, Pradhan S (2011) Tissue-specific distribution and dynamic changes of 5-hydroxymethylcytosine in mammalian genomes. J Biol Chem 286(28):24685–24693
94. Jin SG, Wu X, Li AX, Pfeifer GP (2011) Genomic mapping of 5-hydroxymethylcytosine in the human brain. Nucleic Acids Res 39(12):5015–5024
95. Ehrlich M, Woods C, Yu M, Dubeau L, Yang F, Campan M, Weisenberger D, Long TI, Youn B, Fiala E, Laird P (2006) Quantitative analysis of association between DNA hypermethylation, hypomethylation, and DNMT RNA levels in ovarian tumors. Oncogene 25:2636–2645
96. Rodriguez J, Vives L, Jorda M, Morales C, Munoz M, Vendrell E, Peinado MA (2008) Genome-wide tracking of unmethylated DNA Alu repeats in normal and cancer cells. Nucleic Acids Res 36(3):770–784
97. Kim MJ, White-Cross JA, Shen L, Issa JP, Rashid A (2009) Hypomethylation of long interspersed nuclear element-1 in hepatocellular carcinomas. Mod Pathol 22(3):442–449
98. Roman AC, Gonzalez-Rico FJ, Molto E, Hernando H, Neto A, Vicente-Garcia C, Ballestar E, Gomez-Skarmeta JL, Vavrova-Anderson J, White RJ, Montoliu L, Fernandez-Salguero PM (2011) Dioxin receptor and SLUG transcription factors regulate the insulator activity of B1 SINE retrotransposons via an RNA polymerase switch. Genome Res 21(3):422–432
99. Wang J, Lunyak VV, Jordan IK (2011) Genome-wide prediction and analysis of human chromatin boundary elements. Nucleic Acids Res 40(2):511–29
100. Dante R, Dante-Paire J, Rigal D, Roizes G (1992) Methylation patterns of long interspersed repeated DNA and alphoid repetitive DNA from human cell lines and tumors. Anticancer Res 12(2):559–563
101. Jurgens B, Schmitz-Drager BJ, Schulz WA (1996) Hypomethylation of L1 LINE sequences prevailing in human urothelial carcinoma. Cancer Res 56(24):5698–5703
102. Florl AR, Lower R, Schmitz-Drager BJ, Schulz WA (1999) DNA methylation and expression of LINE-1 and HERV-K provirus sequences in urothelial and renal cell carcinomas. Br J Cancer 80(9):1312–1321
103. Schulz WA, Steinhoff C, Florl AR (2006) Methylation of endogenous human retroelements in health and disease. Curr Top Microbiol Immunol 310:211–250
104. Chan SW, Zilberman D, Xie Z, Johansen LK, Carrington JC, Jacobsen SE (2004) RNA silencing genes control de novo DNA methylation. Science 303(5662):1336
105. Narayan A, Ji W, Zhang X-Y, Marrogi A, Graff JR, Baylin SB, Ehrlich M (1998) Hypomethylation of pericentromeric DNA in breast adenocarcinomas. Int J Cancer 77:833–838
106. Qu G, Dubeau L, Narayan A, Yu M, Ehrlich M (1999) Satellite DNA hypomethylation vs. overall genomic hypomethylation in ovarian epithelial tumors of different malignant potential. Mut Res 423:91–101
107. Bollati V, Fabris S, Pegoraro V, Ronchetti D, Mosca L, Deliliers GL, Motta V, Bertazzi PA, Baccarelli A, Neri A (2009) Differential repetitive DNA methylation in multiple myeloma molecular subgroups. Carcinogenesis 30(8):1330–1335

108. Thoraval D, Asakawa J, Wimmer K, Kuick R, Lamb B, Richardson B, Ambros P, Glover T, Hanash S (1996) Demethylation of repetitive DNA sequences in neuroblastoma. Genes Chromosomes Cancer 17(4):234–244
109. Nagai H, Baba M, Konishi N, Kim YS, Nogami M, Okumura K, Emi M, Matsubara K (1999) Isolation of NotI clusters hypomethylated in HBV-integrated hepatocellular carcinomas by two-dimensional electrophoresis. DNA Res 6(4):219–225
110. Itano O, Ueda M, Kikuchi K, Hashimoto O, Hayatsu S, Kawaguchi M, Seki H, Aiura K, Kitajima M (2002) Correlation of postoperative recurrence in hepatocellular carcinoma with demethylation of repetitive sequences. Oncogene 21(5):789–797
111. Katargin AN, Pavlova LS, Kisseljov FL, Kisseljova NP (2009) Hypermethylation of genomic 3.3-kb repeats is frequent event in HPV-positive cervical cancer. BMC Med Genomics 2:30
112. Szpakowski S, Sun X, Lage JM, Dyer A, Rubinstein J, Kowalski D, Sasaki C, Costa J, Lizardi PM (2009) Loss of epigenetic silencing in tumors preferentially affects primate-specific retroelements. Gene 448(2):151–167
113. Choi SH, Worswick S, Byun HM, Shear T, Soussa JC, Wolff EM, Douer D, Garcia-Manero G, Liang G, Yang AS (2009) Changes in DNA methylation of tandem DNA repeats are different from interspersed repeats in cancer. Int J Cancer 125(3):723–729
114. Ehrlich M, Hopkins N, Jiang G, Dome JS, Yu MS, Woods CB, Tomlinson GE, Chintagumpala M, Champagne M, Diller L, Parham DM, Sawyer J (2003) Satellite hypomethylation in karyotyped Wilms tumors. Cancer Genet Cytogenet 141:97–105
115. Jackson K, Yu M, Arakawa K, Fiala E, Youn B, Fiegl H, Muller-Holzner E, Widschwendter M, Ehrlich M (2004) DNA hypomethylation is prevalent even in low-grade breast cancers. Cancer Biol Ther 3(12):1225–1231
116. Morey Kinney SR, Smiraglia DJ, James SR, Moser MT, Foster BA, Karpf AR (2008) Stage-specific alterations of DNA methyltransferase expression, DNA hypermethylation, and DNA hypomethylation during prostate cancer progression in the transgenic adenocarcinoma of mouse prostate model. Mol Cancer Res 6(8):1365–1374
117. Kerbel RS, Frost P, Liteplo R, Carlow DA, Elliott BE (1984) Possible epigenetic mechanisms of tumor progression: induction of high-frequency heritable but phenotypically unstable changes in the tumorigenic and metastatic properties of tumor cell populations by 5-azacytidine treatment. J Cell Physiol Suppl 3:87–97
118. Santourlidis S, Florl A, Ackermann R, Wirtz HC, Schulz WA (1999) High frequency of alterations in DNA methylation in adenocarcinoma of the prostate. Prostate 39(3):166–174
119. Itano O, Ueda M, Kikuchi K, Shimazu M, Kitagawa Y, Aiura K, Kitajima M (2000) A new predictive factor for hepatocellular carcinoma based on two- dimensional electrophoresis of genomic DNA. Oncogene 19(13):1676–1683
120. Grunau C, Sanchez C, Ehrlich M, van der Bruggen P, Hindermann W, Rodriguez C, Krieger S, De Sario A (2005) Frequent DNA hypomethylation in the human juxtacentromeric BAGE loci in cancer. Genes Chrom Cancer 43(1):11–24
121. Roman-Gomez J, Jimenez-Velasco A, Agirre X, Castillejo JA, Navarro G, San Jose-Eneriz E, Garate L, Cordeu L, Cervantes F, Prosper F, Heiniger A, Torres A (2008) Repetitive DNA hypomethylation in the advanced phase of chronic myeloid leukemia. Leuk Res 32(3): 487–490
122. Yegnasubramanian S, Haffner MC, Zhang Y, Gurel B, Cornish TC, Wu Z, Irizarry RA, Morgan J, Hicks J, DeWeese TL, Isaacs WB, Bova GS, De Marzo AM, Nelson WG (2008) DNA hypomethylation arises later in prostate cancer progression than CpG island hypermethylation and contributes to metastatic tumor heterogeneity. Cancer Res 68(21):8954–8967
123. Marchal R, Chicheportiche A, Dutrillaux B, Bernardino-Sgherri J (2004) DNA methylation in mouse gametogenesis. Cytogenet Genome Res 105(2–4):316–324
124. Zhang X-Y, Loflin PT, Gehrke CW, Andrews PA, Ehrlich M (1987) Hypermethylation of human DNA sequences in embryonal carcinoma cells and somatic tissues but not sperm. Nucleic Acids Res 15:9429–9449
125. Rubin CM, VandeVoort CA, Teplitz RL, Schmid CW (1994) Alu repeated DNAs are differentially methylated in primate germ cells. Nucleic Acids Res 22(23):5121–5127
126. Dupressoir A, Heidmann T (1997) Expression of intracisternal A-particle retrotransposons in primary tumors of oncogene-expressing transgenic mice. Oncogene 14(24):2951–2958

127. Smiraglia DJ, Szymanska J, Kraggerud SM, Lothe RA, Peltomaki P, Plass C (2002) Distinct epigenetic phenotypes in seminomatous and nonseminomatous testicular germ cell tumors. Oncogene 21(24):3909–3916
128. Netto GJ, Nakai Y, Nakayama M, Jadallah S, Toubaji A, Nonomura N, Albadine R, Hicks JL, Epstein JI, Yegnasubramanian S, Nelson WG, De Marzo AM (2008) Global DNA hypomethylation in intratubular germ cell neoplasia and seminoma, but not in nonseminomatous male germ cell tumors. Mod Pathol 21(11):1337–1344
129. Laird CD, Pleasant ND, Clark AD, Sneeden JL, Hassan KM, Manley NC, Vary JC Jr, Morgan T, Hansen RS, Stoger R (2004) Hairpin-bisulfite PCR: assessing epigenetic methylation patterns on complementary strands of individual DNA molecules. Proc Natl Acad Sci USA 101(1):204–209
130. Jiang G, Yang F, van Overveld PG, Vedanarayanan V, van der Maarel S, Ehrlich M (2003) Testing the position-effect variegation hypothesis for facioscapulohumeral muscular dystrophy by analysis of histone modification and gene expression in subtelomeric 4q. Hum Mol Genet 12:2909–2921
131. Zeng W, de Greef JC, Chen YY, Chien R, Kong X, Gregson HC, Winokur ST, Pyle A, Robertson KD, Schmiesing JA, Kimonis VE, Balog J, Frants RR, Ball AR Jr, Lock LF, Donovan PJ, van der Maarel SM, Yokomori K (2009) Specific loss of histone H3 lysine 9 trimethylation and HP1gamma/cohesin binding at D4Z4 repeats is associated with facioscapulohumeral dystrophy (FSHD). PLoS Genet 5(7):e1000559
132. Shao C, Lacey M, Dubeau L, Ehrlich M (2009) Hemimethylation footprints of DNA demethylation in cancer. Epigenetics 4(3):165–175
133. Lau S, Jardine K, McBurney MW (1999) DNA methylation pattern of a tandemly repeated LacZ transgene indicates that most copies are silent. Dev Dyn 215(2):126–138
134. Takai D, Jones PA (2002) Comprehensive analysis of CpG islands in human chromosomes 21 and 22. Proc Natl Acad Sci USA 99(6):3740–3745
135. Hu JL, Zhou BO, Zhang RR, Zhang KL, Zhou JQ, Xu GL (2009) The N-terminus of histone H3 is required for de novo DNA methylation in chromatin. Proc Natl Acad Sci USA 106(52):22187–22192
136. Schulte JH, Lim S, Schramm A, Friedrichs N, Koster J, Versteeg R, Ora I, Pajtler K, Klein-Hitpass L, Kuhfittig-Kulle S, Metzger E, Schule R, Eggert A, Buettner R, Kirfel J (2009) Lysine-specific demethylase 1 is strongly expressed in poorly differentiated neuroblastoma: implications for therapy. Cancer Res 69(5):2065–2071
137. Wang J, Hevi S, Kurash JK, Lei H, Gay F, Bajko J, Su H, Sun W, Chang H, Xu G, Gaudet F, Li E, Chen T (2009) The lysine demethylase LSD1 (KDM1) is required for maintenance of global DNA methylation. Nat Genet 41(1):125–129
138. Zampieri M, Passananti C, Calabrese R, Perilli M, Corbi N, De Cave F, Guastafierro T, Bacalini MG, Reale A, Amicosante G, Calabrese L, Zlatanova J, Caiafa P (2009) Parp1 localizes within the Dnmt1 promoter and protects its unmethylated state by its enzymatic activity. PLoS One 4(3):e4717
139. Ostler KR, Davis EM, Payne SL, Gosalia BB, Exposito-Cespedes J, Le Beau MM, Godley LA (2007) Cancer cells express aberrant DNMT3B transcripts encoding truncated proteins. Oncogene 26(38):5553–5563
140. Lopez de Silanes I, Gorospe M, Taniguchi H, Abdelmohsen K, Srikantan S, Alaminos M, Berdasco M, Urdinguio RG, Fraga MF, Jacinto FV, Esteller M (2009) The RNA-binding protein HuR regulates DNA methylation through stabilization of DNMT3b mRNA. Nucleic Acids Res 37(8):2658–2671
141. Shukla V, Coumoul X, Lahusen T, Wang RH, Xu X, Vassilopoulos A, Xiao C, Lee MH, Man YG, Ouchi M, Ouchi T, Deng CX (2010) BRCA1 affects global DNA methylation through regulation of DNMT1. Cell Res 20(11):1201–1215
142. Felle M, Joppien S, Nemeth A, Diermeier S, Thalhammer V, Dobner T, Kremmer E, Kappler R, Langst G (2011) The USP7/Dnmt1 complex stimulates the DNA methylation activity of Dnmt1 and regulates the stability of UHRF1. Nucleic Acids Res 39(19):8355–65
143. Sharma S, De Carvalho DD, Jeong S, Jones PA, Liang G (2011) Nucleosomes containing methylated DNA stabilize DNA methyltransferases 3A/3B and ensure faithful epigenetic inheritance. PLoS Genet 7(2):e1001286

144. Chen ZX, Riggs AD (2011) DNA methylation and demethylation in mammals. J Biol Chem 286(21):18347–18353
145. He YF, Li BZ, Li Z, Liu P, Wang Y, Tang Q, Ding J, Jia Y, Chen Z, Li L, Sun Y, Li X, Dai Q, Song CX, Zhang K, He C, Xu GL (2011) Tet-mediated formation of 5-carboxylcytosine and its excision by TDG in mammalian DNA. Science 333(6047):1303–1307
146. Kress C, Thomassin H, Grange T (2006) Active cytosine demethylation triggered by a nuclear receptor involves DNA strand breaks. Proc Natl Acad Sci USA 103(30):11112–11117
147. Bhutani N, Burns DM, Blau HM (2011) DNA demethylation dynamics. Cell 146(6): 866–872
148. Inoue A, Zhang Y (2011) Replication-dependent loss of 5-hydroxymethylcytosine in mouse preimplantation embryos. Science 334(6053):194
149. Holliday R, Pugh JE (1975) DNA modification mechanisms and gene activity during development. Science 187:226
150. Riggs AD (1975) X chromosome inactivation, differentiation and DNA methylation. Cytogenet Cell Genet 14:9–25
151. Jones PA, Liang G (2009) Rethinking how DNA methylation patterns are maintained. Nat Rev Genet 10(11):805–811
152. Otto SP, Walbot V (1990) DNA methylation in eukaryotes: kinetics of demethylation and de novo methylation during the life cycle. Genetics 124(2):429–437
153. Pfeifer GP, Steigerwald SD, Hansen RS, Gartler SM, Riggs AD (1990) Polymerase chain reaction-aided genomic sequencing of an X chromosome-linked CpG island: methylation patterns suggest clonal inheritance, CpG site autonomy, and an explanation of activity state stability. Proc Natl Acad Sci USA 87(21):8252–8256
154. Nicolas P, Kim KM, Shibata D, Tavare S (2007) The stem cell population of the human colon crypt: analysis via methylation patterns. PLoS Comput Biol 3(3):e28
155. Sontag LB, Lorincz MC, Georg Luebeck E (2006) Dynamics, stability and inheritance of somatic DNA methylation imprints. J Theor Biol 242(4):890–899
156. Genereux DP, Miner BE, Bergstrom CT, Laird CD (2005) A population-epigenetic model to infer site-specific methylation rates from double-stranded DNA methylation patterns. Proc Natl Acad Sci USA 102(16):5802–5807
157. Lacey M, Ehrlich M (2009) Modeling dependence in methylation patterns with application to ovarian carcinomas. Stat Appl Genet Mol Biol 8(1):40
158. Saluz HP, Jiricny J, Jost JP (1986) Genomic sequencing reveals a positive correlation between the kinetics of strand-specific DNA demethylation of the overlapping estradiol/glucocorticoid-receptor binding sites and the rate of avian vitellogenin mRNA synthesis. Proc Natl Acad Sci USA 83(19):7167–7171
159. Paroush Z, Keshet I, Yisraeli J, Cedar H (1990) Dynamics of demethylation and activation of the alpha-actin gene in myoblasts. Cell 63(6):1229–1237
160. Liang G, Chan MF, Tomigahara Y, Tsai YC, Gonzales FA, Li E, Laird PW, Jones PA (2002) Cooperativity between DNA methyltransferases in the maintenance methylation of repetitive elements. Mol Cell Biol 22(2):480–491
161. Chen T, Hevi S, Gay F, Tsujimoto N, He T, Zhang B, Ueda Y, Li E (2007) Complete inactivation of DNMT1 leads to mitotic catastrophe in human cancer cells. Nat Genet 39(3): 391–396
162. Gehring M, Reik W, Henikoff S (2009) DNA demethylation by DNA repair. Trends Genet 25(2):82–90
163. Zhu JK (2009) Active DNA demethylation mediated by DNA glycosylases. Annu Rev Genet 43:143–166
164. Bhutani N, Brady JJ, Damian M, Sacco A, Corbel SY, Blau HM (2010) Reprogramming towards pluripotency requires AID-dependent DNA demethylation. Nature 463(7284): 1042–1047
165. Kress C, Thomassin H, Grange T (2001) Local DNA demethylation in vertebrates: how could it be performed and targeted? FEBS Lett 494(3):135–140
166. Meilinger D, Fellinger K, Bultmann S, Rothbauer U, Bonapace IM, Klinkert WE, Spada F, Leonhardt H (2009) Np95 interacts with de novo DNA methyltransferases, Dnmt3a and Dnmt3b, and mediates epigenetic silencing of the viral CMV promoter in embryonic stem cells. EMBO Rep 10(11):1259–1264

167. Jeong S, Liang G, Sharma S, Lin JC, Choi SH, Han H, Yoo CB, Egger G, Yang AS, Jones PA (2009) Selective anchoring of DNA methyltransferases 3A and 3B to nucleosomes containing methylated DNA. Mol Cell Biol 29(19):5366–5376
168. Hervouet E, Lalier L, Debien E, Cheray M, Geairon A, Rogniaux H, Loussouarn D, Martin SA, Vallette FM, Cartron PF (2010) Disruption of Dnmt1/PCNA/UHRF1 interactions promotes tumorigenesis from human and mice glial cells. PLoS One 5(6):e11333
169. Frommer M, McDonald LE, Millar DS, Collis CM, Watt F, Grigg GW, Molloy PL, Paul CL (1992) A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. Proc Natl Acad Sci USA 89:1827–1831
170. Valinluck V, Sowers LC (2007) Endogenous cytosine damage products alter the site selectivity of human DNA maintenance methyltransferase DNMT1. Cancer Res 67(3):946–950
171. Burden AF, Manley NC, Clark AD, Gartler SM, Laird CD, Hansen RS (2005) Hemimethylation and non-CpG methylation levels in a promoter region of human LINE-1 (L1) repeated elements. J Biol Chem 280(15):14413–14419
172. Turker MS (2002) Gene silencing in mammalian cells and the spread of DNA methylation. Oncogene 21(35):5388–5393
173. Yan PS, Shi H, Rahmatpanah F, Hsiau TH, Hsiau AH, Leu YW, Liu JC, Huang TH (2003) Differential distribution of DNA methylation within the RASSF1A CpG island in breast cancer. Cancer Res 63(19):6178–6186
174. Nguyen C, Liang G, Nguyen TT, Tsao-Wei D, Groshen S, Lubbert M, Zhou JH, Benedict WF, Jones PA (2001) Susceptibility of nonpromoter CpG islands to de novo methylation in normal and neoplastic cells. J Natl Cancer Inst 93(19):1465–1472
175. Stirzaker C, Song JZ, Davidson B, Clark SJ (2004) Transcriptional gene silencing promotes DNA hypermethylation through a sequential change in chromatin modifications in cancer cells. Cancer Res 64(11):3871–3877
176. Homma N, Tamura G, Honda T, Matsumoto Y, Nishizuka S, Kawata S, Motoyama T (2006) Spreading of methylation within RUNX3 CpG island in gastric cancer. Cancer Sci 97(1): 51–56

Chapter 3
Ten Eleven Translocation Enzymes and 5-Hydroxymethylation in Mammalian Development and Cancer

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Abstract 5-Hydroxymethylcytosine (5hmC) is an oxidative product of 5-methylcytosine (5mC), catalyzed by the ten eleven translocation (TET) family of enzymes. Although 5hmC was discovered several decades ago, it was only after its recent identification in murine brain and stem cell DNA that it has become a major focus of epigenomic research. Part of the reason for this delay is due to the difficulty in detecting both global and locus-specific 5hmC levels. Several studies have addressed this issue with the development of novel techniques to locate and measure 5hmC, which led to multiple reports detailing 5hmC patterns in stem cells and global 5hmC levels during embryogenesis. Based on these studies of 5hmC levels and reports of tissue-specific TET expression, these enzymes are thought to play a role in mammalian development and differentiation. In addition, the TET enzymes are mutated in several types of cancer, affecting their activity and likely altering genomic 5hmC and 5mC patterns. Furthermore, oxidation of 5mC appears to be a step in several active DNA demethylation pathways, which may be important for normal processes, as well as global hypomethylation during cancer development and progression. Much has been revealed about this interesting DNA modification in recent years, but more research is needed for understanding the role of TET proteins and 5hmC in gene regulation and disease.

3.1 Discovery and History of 5-Hydroxymethylation

Methylation of cytosine residues at the 5-carbon position (5-methylcytosine, 5mC) has been studied as a stable epigenetic modification for decades [1]. However, oxidation of DNA has traditionally been considered a DNA damage event, which is readily removed by DNA repair pathways [2]. Recently, it was demonstrated that

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enzymatic oxidation of 5mC to 5hmC (5-hydroxymethylcytosine) may act as a stable modification of DNA and downstream removal of 5hmC may actually be part of a complex and intricate process of epigenetic gene regulation [3].

5-Hydroxymethylcytosine (5hmC) was first identified in T-even bacteriophages during early 1950s using paper chromatography and ultraviolet absorbance spectra [4]. This nucleotide is normally incorporated during DNA synthesis and then further glycosylated by phage encoded glucosyltransferases as a mechanism for protection of the phage DNA from bacterial restriction enzymes during infection [5, 6]. Later, during the 1970s, 5hmC was detected in genomic DNA purified from brain tissue of rats, mice, and frogs and, to a lesser extent, from liver tissue of rats [7]. The same group also observed an increase in 5hmC levels in the adult compared to newborn rat brain, as well as a decrease of 5hmC levels in brains from rats with low protein diets [8]. Unfortunately, these experiments could not be reproduced and this DNA modification was overlooked for several decades [9].

In 2009, 5hmC was rediscovered in mammalian DNA and shown to be present in substantial amounts (~10 to 20% of 5mC) in murine embryonic stem cells (ESCs) [10], Purkinje neurons, and granule cells [11]. These recent studies utilized more advanced analytical techniques, such as 2D thin layer chromatography (TLC) or high-pressure liquid chromatography (HPLC) coupled with mass spectrometry (MS), to confirm the existence of this DNA modification in mammalian DNA. In addition, 5hmC was recently identified in mammalian mitochondrial DNA [12]. As a result of these discoveries, there is a huge amount of interest in developing technologies for genome-wide mapping and site-specific quantification of 5hmC in an effort to decipher its possible role in development and disease.

3.2 TET Enzymes and Their Catalytic Activity

There are three known mammalian 5mC dioxygenases, which catalyze the conversion of 5mC to 5hmC [10]. These proteins belong to the family of ten eleven translocation (TET) enzymes, whose name is based on a common chromosomal translocation in some cancers (described in detail later in this chapter). TET1 was originally named leukemia-associated protein with a CXXC domain (LCX) when it was initially cloned in 2002 [13]. This gene was rediscovered in 2003 along with the two other members of the family and they were renamed ten eleven translocation, or TET, genes [14]. All three TET proteins share a similar catalytic domain structure to 2-oxoglutarate (2-OG) oxygenases. These types of enzymes can oxidize DNA and RNA that is methylated on either the nitrogen (N) or carbon (C) of the base by conversion of 2-OG and oxygen to carbon dioxide and succinate [15]. The TETs were identified based on their similarity to the JBP1 and JBP2 enzymes in trypanosome, which were originally named for their ability to bind to the unique nucleotide β-D-glucosylhydroxymethyl-uracil (base J) and then later were reported to hydroxylate thymine, the first step in the conversion of base J [16]. Proteins with similar

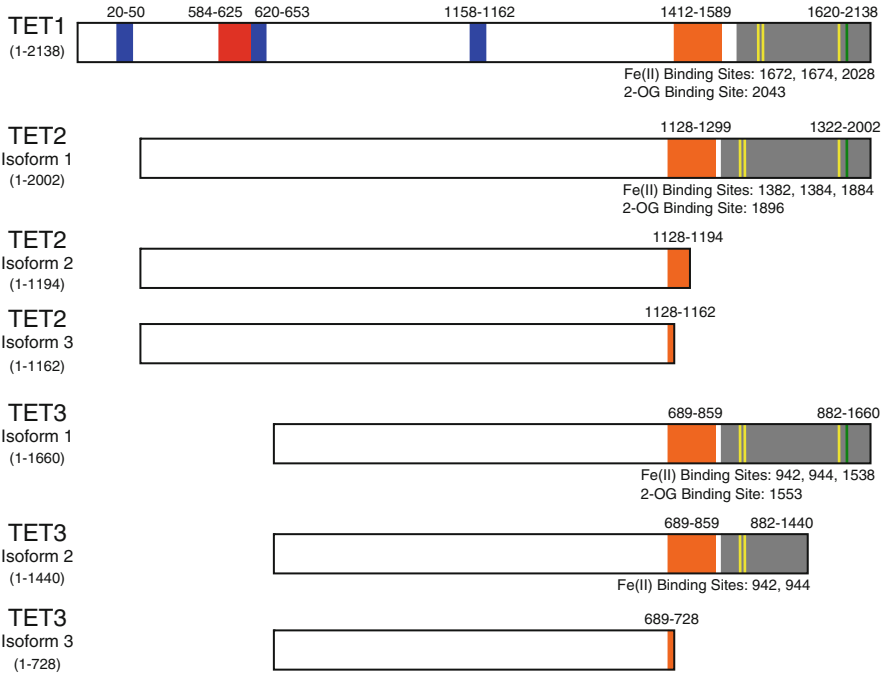

Fig. 3.1 Diagram of TET enzyme isoforms. TET1 is 2138 aa long with multiple putative Nuclear Localization Sequence (NLS), a CXXC motif, and cysteine-rich region N-terminal to the DSBH making up the core catalytic domain. There are three isoforms of TET2, the longest being 2002 aa long. TET2 does not contain any putative NLS or CXXC motif, but does have a core catalytic domain very similar to TET1. TET3 also has three isoforms, of 1660 aa or less in length. Similar to TET2, TET3 does not appear to have any other domains other than the core catalytic domain. Numbers in brackets represent length of proteins in aa or location of domains. *Blue bars* NLS; *red bar* CXXC motif; *orange* bar Cysteine-rich region; *Gray bar* DSBH; *yellow bars* Fe(II) binding sites; *green bar* 2-OG binding site

homology to the TET proteins are found in several lower eukaryote groups, including *Drosophila* [17].

The human TET1 gene is found at chromosomal location 10q21 and is approximately 134 kilobases (kb) long [18]. The resulting transcript contains 12 exons and is approximately 9.6 kb. The TET1 protein consists of ~2,136 amino acids encoding a 236 kilodalton (kDa) enzyme. TET1 is a multidomain protein containing several putative nuclear localization sequences, a binuclear Zn^{+2}-chelating CXXC domain, and a cysteine-rich region preceding the catalytic domain (Fig. 3.1). CXXC domains are frequently found in chromatin binding proteins, including DNA (cytosine-5) methyltransferase-1 (DNMT1), 5-methylcytosine binding proteins (MBDs), and mixed lineage leukemia (MLL) protein [19]. The CXXC domain of TET1 binds to CG-rich sequences of both methylated and unmethylated DNA, with some preference for unmethylated CpGs in cell free assays [19, 20].

The human TET2 gene is found on chromosome 4q24 and contains 11 exons, which can result in three known isoforms produced through alternative splicing. The longest form of TET2 is ~2,002 amino acids and similar to TET1 with approximately 70% homology in their C-terminal regions, including their catalytic domains [18]. There are two shorter isoforms of TET2 (1,164 and 1,194 amino acids long) that both lack catalytic domains due to truncation or introduction of stop codons (Fig. 3.1). The TET3 gene resides on chromosome 2p13. It is approximately 62 kb in length, with a transcript containing nine exons. Similar to TET2, the TET3 protein sequence shares approximately 70% sequence homology to TET1 in the regions surrounding the catalytic domain (Fig. 3.1). Three putative isoforms of TET3 have been identified using complementary DNA screening [18]. These include the full-length protein, as well as two shorter variants that are missing either a small portion, or most of the catalytic domain (Fig. 3.1).

TET2 and TET3 differ from TET1 in that they do not appear to contain any putative nuclear localization sequences or regions similar to a CXXC domain [18]. Interestingly, one study reported that the CXXC4 gene, at 4q22-24, is a very close neighbor to TET2 and may be the result of a chromosomal inversion of the TET2 CXXC domain followed by a translocation [17]. It has been proposed that interaction of CXXC4 and TET2 may be required for appropriate TET2 targeting and activity [17].

The catalytic domains of all 2-oxoglutarate (2-OG) oxygenases contain a double-stranded β-helix (DSBH) [10, 15]. The DSBH domain, in addition to the cysteine-rich region, of TET1 has been found to be both necessary and sufficient for catalytic activity [10]. Furthermore, the DSBH domain contains three Fe(II) binding sites and a 2-OG binding site (details in Fig. 3.1) [18]. Amino acid mutation studies have confirmed the requirement of these domains for TET catalytic activity [21].

The increased homology within the cysteine-rich region and the DSBH domain of TET1, TET2, and TET3 suggests that they have similar catalytic activity. Each protein of this family also contains unique regions indicating that they may have distinct binding affinities to chromatin and/or protein partners, resulting in the establishment of specific 5hmC patterns in various cell types and during different developmental stages. All three forms of the Tet enzymes are known to be catalytically active in cells [22] and tissue-specific expression of TET transcripts has also been reported [23, 24], supporting the above hypothesis.

Triple knockout (TKO, knockout of Dnmt1, Dnmt3a, and Dnmt3b) ESCs display decreased 5hmC levels although they have normal Tet expression. This confirms that the 5mC catalyzed by Dnmts is in fact the substrate for the Tet enzymes [22, 25]. In addition to the oxidation of 5mC to 5hmC, the TET enzymes have recently been reported to have the ability to further oxidize 5hmC to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) [26, 27]. Quantification of the three oxygenated forms of 5mC reveals unequal distribution with much more 5hmC than 5fC or 5caC in genomic DNA [27]. The function of these less frequent enzymatic products of TET enzymes is not well understood, but current knowledge

suggests they may be involved in the DNA demethylation process described later in this chapter [28].

3.3 Technologies and Advancements in 5hmC Detection

Identifying and quantifying 5hmC globally and at specific loci has been, and continues to be, quite a challenge. For example, the most accepted technique for 5mC detection and measurement, bisulfite sequencing, does not differentiate between 5mC and 5hmC or unmodified C and 5caC [28, 29]. Additionally, restriction enzymes have been used for years to specifically digest methylated or unmethylated DNA and recent data shows that many of these enzymes have different specificities or sensitivities for oxidized forms of 5mC or glucosylated 5hmC (5ghmC) [30–33]. Indeed, many of the 5mC-sensitive enzymes that have previously been used to measure DNA methylation are also sensitive to 5hmC [34]. Complicating matters further, 5mC-specific antibodies appear to have no cross reactivity with 5hmC, thus in the past oxidation of 5mC may have been mistaken for demethylation. Since the discovery of 5hmC in mammalian DNA there has been a flurry of new techniques reported to measure this elusive base, either globally or at a specific locus.

There are several techniques that have been shown to evaluate global 5hmC levels. Some are more qualitative than quantitative and each has its own range of sensitivity and accuracy. Initially, the existence of 5hmC (followed by 5fC and 5caC) in mammalian DNA was discovered using restriction enzyme-based TLC [7, 10, 11, 27, 28]. Dot blot of genomic DNA and immunofluorescence in mammalian cells using 5hmC-specific antibodies has also been used extensively to examine global 5hmC levels [22, 25, 35]. These antibodies appear to be sensitive but seem to require several proximal 5hmC sites for measurable binding to occur [36]. More recently, an antibody was developed targeting cytosine 5-methylenesulfonate (CMS), a product of sodium bisulfite treatment of hydroxymethylated DNA that can apparently detect as few as one 5hmC site on DNA [21]. Although these techniques are not truly quantitative, they offer more sensitivity as the input DNA could be as low as several nanograms. Currently, the most sensitive techniques for measuring global 5hmC, 5fC, and 5caC utilize HPLC coupled with mass spectrometry [27, 28, 37]. However, these techniques require unique expertise and complex analytical machinery. A simple, yet very accurate and sensitive, technique for measuring global 5hmC uses the T4 phage enzyme, β-glucosyltransferase (β-GT), and radioactive UDP-[^{3}H]-glucose [38, 39].

The 5hmC and CMS-specific antibodies mentioned above have also been utilized for hydroxymethylcytosine-DNA immunoprecipitation (hMe-DIP) followed by next generation sequencing, DNA array, or PCR [25, 36, 40, 41]. A second technique, (glucosylation, periodate oxidation, biotinylation, or GLIB) uses a glucosylation reaction to attach UDP-6-N3-glucose to 5hmC, which marks these sites with a reactive azide group. The azide group is further reacted with biotin using click

chemistry for subsequent pulldown with a streptavidin matrix [42, 43]. Although data derived from these techniques can be extremely useful in mapping the regions of 5hmC, it still does not offer single base resolution. Single-molecule real-time (SMRT) sequencing is a novel sequencing technique that can discriminate between unmodified cytosine, 5mC, and 5hmC due to variations in polymerase kinetics during the sequencing reaction [44]. It is also possible to differentiate 5mC from 5hmC with nanopore amperometry, as each modification causes unique breaks in current as synthetic DNA molecules are fed through nanopores [45]. Current research is focused towards optimizing the last two methods for genomic DNA samples and for high-throughput analysis, but these technologies are not yet commonly used.

Many restriction enzymes that can differentiate between 5mC and unmodified cytosine, as well as families of enzymes that target 5hmC or 5ghmC are being studied for unique properties that make them useful for measuring 5hmC [30, 33, 46]. For example, MspI and GlaI can fully digest 5mC or 5hmC in their respective target sequences, but after conversion of 5hmC to 5ghmC, digestion by both of these enzymes is blocked [32, 47]. Taq$^{\alpha}$I is a restriction enzyme that is not fully blocked by 5ghmC, but is blocked by biotin-N3-5gmC [31]. Therefore, tagging a 5hmC residue with glucose or a modified glucose may be a valuable tool for epigenetic studies. In contrast to restriction enzymes that are blocked by 5hmC or 5ghmC, but not by unmodified cytosine, another class known as PvuRts1I family show digestion preference for 5hmC or 5ghmC as compared to 5mC and cytosine [30, 33]. Using this class of enzymes for digestion followed by PCR amplification of a region of interest can reveal the level of 5hmC at a specific site. Alternatively, one could use the digested fragments for next generation sequencing for genome-wide mapping of 5hmC.

Novel and more accurate techniques for measuring 5hmC will be available in the near future as the epigenetics field progresses with reference to this modification. We must always consider how to normalize traditional techniques and any new ones that are developed to evaluate various DNA modifications when drawing conclusions about how epigenomic modification patterns relate to biological phenomenon.

3.4 Tet1 Binding and 5hmC in Embryonic Stem Cells

It is important to understand the normal function of TET enzymes and 5hmC in order to comprehend how and why they may be disrupted in disease. The study of mouse ESCs may allow us to gain some insight into these phenomena. Mouse ESCs are derived from the inner cell mass (ICM) of blastocysts and can be cultured in an undifferentiated state with use of leukemia inhibitory factor (LIF) [48]. ESCs can be differentiated into embryoid bodies (EB) with the removal of LIF or into other more specific lineages by addition or removal of cytokines and specific growth factors. As mentioned earlier, ESCs tend to have high levels of 5hmC as compared to other cell

types [10, 27]. It is thought that the TET enzymes and 5hmC may play a significant biological role in ESCs because epigenetic modifications and factors are important for both maintaining an undifferentiated state and for differentiation. *Tet1* and *Tet2* are expressed in ESCs and induced pluripotent stem (iPS) cells, while *Tet3* expression is quite low, suggesting that Tet1 and Tet2 are especially important for maintaining a pluripotent status [22, 49]. Furthermore, expression of *Tet1* and *Tet2* is repressed during differentiation and it appears that Oct4 [49], one of a few transcription factors that are required for ESC pluripotency and dedifferentiation of somatic cells, is involved in regulating Tet1 and Tet2 expression [50].

A number of reports describe Tet1 binding and/or 5hmC status throughout the genome of mouse ESCs and the relationship of these patterns to gene expression [25, 41, 42, 47, 51, 52]. Several techniques were utilized in these studies, including ChIP-seq, GLIB-Seq, hMeDIP-Seq, restriction enzyme-dependent genome-wide sequencing, and hMeDIP-Chip (with 5hmC and CMS-specific antibodies), as well as RNA-Seq and microarray analyses [25, 41, 42, 51, 52]. Even though there are some disagreements between these studies, their overall conclusions are similar. In general, Tet1 binds to CG-rich regions of the genome, which seems to be due, at least in part, to its CXXC domain. Tet1 binds to both active and inactive genes, with more binding in the gene bodies of active genes and increased binding in the promoters and transcriptional start sites (TSS) of inactive genes. Tet1 targeted genes are involved in many cellular pathways, including development, differentiation, and neural processes [22, 25, 49, 52]. Tet1 also appears to be enriched in regions containing the active H3K4me3 mark, as well as the bivalent H3K4me3 and H3K27me3 marks, and to a lesser extent at polycomb repressed genes exhibiting only H3K27me3 mark.

5hmC patterns in the genome are very similar to Tet1 binding. Both 5mC and its oxidative product 5hmC are commonly found in the gene bodies of active genes and in the promoters of inactive genes. Surprisingly, there are a number of Tet1 binding sites that do not appear to contain 5hmC. This suggests that Tet1 may have additional non-catalytic activities or that 5hmC is quickly removed specifically at these loci as part of a DNA demethylation/repair pathway. Several studies indicate that gene body 5hmC is more prevalent in exons than introns [25, 42, 51, 52]; however, results from another group indicated more enrichment in introns [41]. These ambiguities could be due to differences in the techniques utilized and will likely be sorted out in the future with base resolution mapping of the respective mammalian hydroxymethylome. Interestingly, 5hmC is enriched in and around the TSS, which is in contrast to a general reduction in 5mC at these locations [51]. Intergenic regions and repetitive elements appear to have less 5hmC than coding regions. Thus, 5hmC and 5mC coexist in some genomic regions, while also displaying unique patterns of genomic localization. Genome-wide 5hmC patterns have also been reported for human ESCs and they closely match with the description of mouse ESCs [36]. The patterning observed in both mouse and human ESCs suggests that 5hmC may have a more specific role in regulating transcription, while 5mC has additional roles in maintaining genomic integrity and transposon stability.

Upon knockdown of Tet1 expression or gene knockout, there are clear increases in both locus-specific and global 5mC with concomitant decreases in

5hmC globally and at Tet1 target sites [22, 47, 53]. In addition, loss or reduction of Tet1 consistently resulted in both increased and decreased gene expression with gene activation being associated with promoter hypo-hydroxymethylation [41, 51–53]. Tet1 enrichment occurs at almost two-thirds of all genes in mouse ESCs and thus overlaps with a number of chromatin modifying and transcriptional regulatory proteins, such as Suz12, Ezh2, Sin3a, Mbd3, and LIF activated Stat3 [41, 47, 51, 54]. Concomitantly, the binding of these proteins to the chromatin is reduced by Tet1 knockdown [41, 47, 51, 54]. It is not clear whether it is direct interaction with Tet1, possibly via other bridge proteins, or 5hmC that provides a platform for their recruitment to specific regions of the chromatin, except in the cases of Sin3a and Mbd3. These two proteins have been shown to either bind directly to Tet1 or in a complex with Tet1 by co-immunoprecipitation experiments. Mbd3 also appears to bind to 5hmC-modified DNA, which is thought to result in its recruitment to inactive genes [51, 54].

There is an overall enrichment of 5hmC at regulatory protein binding sites, such as gene promoters, enhancers, and insulators further supporting the hypothesis of 5hmC-specific binding proteins [25, 36]. In addition to transcriptional regulation by putative 5hmC binding proteins, active promoters bound by Tet1 may be maintained in an unmethylated state through constant oxidation of 5mC, allowing transcription factors and RNA polymerase to bind. Based on these observations, Tet enzymes can regulate the levels of both 5hmC and 5mC at specific gene sequences in order to direct the binding of transcriptional regulator proteins, resulting in both positive and/or negative effects on its expression.

3.5 Role of Tets and 5hmC in Early Mammalian Development and Embryonic Stem Cells

The mammalian paternal zygotic genome is thought to be actively demethylated upon fertilization of the egg and this demethylated state persists over the next several cell divisions, during which time the maternal genome undergoes passive demethylation [55]. At the blastocyst stage of development, both the maternal and paternal DNAs are remethylated by the de novo methyltransferases. The observation that the paternal genome is demethylated is based primarily on studies utilizing anti-5mC antibody staining and bisulfite sequencing of a small number of loci [56–59]. However, recent data suggests that the lack of staining of the paternal genome by the 5mC antibody is actually due to conversion of 5mC to 5hmC [35, 60]. High levels of 5hmC in the paternal genome persist for several genome replications suggesting that demethylation is not as extensive as was previously thought and may take place only at specific loci [35]. Technological advances that allow for the paternal and maternal DNA to be fully sequenced for epigenetic modifications will help in the future to resolve this important observation.

Tet3 is the most likely Tet family member that oxidizes the paternal DNA as it is expressed at high levels in oocytes and zygotes, but not at later developmental stages

[35, 60, 61]. *Tet1* seems to only be expressed at the two- and four-cell stages and in ESCs, and *Tet2* is only expressed at very low levels throughout fertilization and zygote development, except in ESCs where Tet2 expression is higher [35, 60]. Knockdown of *Tet3* by siRNA injection into the oocyte or conditional knockout of Tet3 in primordial germ cells (PGC) of mice significantly reduces oxidation of 5mC in the paternal genome [60, 61]. Furthermore, the Tet3 responsible for this process appears to be of maternal origin as wild-type (WT) females crossed with Tet3 conditionally null males did not exhibit this defect [61]. Finally, primordial germ cell gene 7 (PGC7) may be involved in protecting the maternal genome from demethylation [62] and knockout of this gene results in oxidation of the maternal genome [60]. It is not clear why only the paternal genome methylation specifically undergoes widespread oxidation during zygote development, but this process is likely involved in locus-specific 5mC erasure and epigenetic reprogramming of the chromatin.

There are several contradicting reports on whether knockdown or knockout of Tet genes alters growth and differentiation of ESCs. Two studies report that knockdown of *Tet1*, but not *Tet2* or *Tet3*, in mouse ESCs results in decreased alkaline phosphatase activity (a marker of healthy ESCs) and pluripotency associated genes, as well as an increase in differentiation markers and altered cell growth and morphology [22, 47]. It is suggested that this may be due to a decrease in *Nanog* expression as reintroduction of Nanog can rescue the phenotype. ChIP analysis shows that Tet1 binds to the Nanog promoter and depletion of Tet1 results in methylation and suppression of the *Nanog* gene. Furthermore, use of Dnmt TKO ES cells prevents the methylation and repression of *Nanog* [22].

In contrast, other studies did not report any effects on morphology or *Nanog* expression with *Tet1* knockdown or knockout in undifferentiated cells [49, 51, 53]. However, there was agreement amongst some reports that *Tet1* knockdown upregulates genes involved in trophectoderm and endoderm development and represses genes involved in neuroectoderm development [22, 49, 53]. Loss of Tet1 function in ESCs results in differentiation toward endoderm/mesoderm and trophoblast lineages. Based on this, and because *Tet1* is primarily expressed in the ICM (not the trophectoderm), it is thought that Tet1 participates in preventing the expression of trophectoderm developmental genes and maintaining proper cellular specification in embryos [22, 49]. *Tet2* knockdown did not seem to affect trophectoderm, endoderm, or mesoderm genes but did slightly increase neuroectoderm markers. In addition, knockdown of either *Tet1* or *Tet2* alters expression of unique subsets of genes suggesting that each enzyme has unique target regions in the genome [49]. *Tet3* knockdown in ESCs had minimal transcriptional effects on the differentiation genes that were examined.

Tet1 knockout ESCs are capable of producing live pups and loss of Tet1 has minimal effects on embryogenesis and mouse development, as Tet1 homozygous null mice maintain proper Mendelian ratios, appear healthy, and are fertile [53]. The only initial observations of aberrant development are that both male and female Tet1 null mice are born at lower body weight (although they are similar to WT mice as adults); they have slightly decreased neutrophil numbers, and smaller litter sizes when inter-crossed. These mice do not appear to have any myeloid or other disorders

[53]. Complete knockout of Tet2 has not yet been reported, but a mouse model has been developed that utilizes a Tet2-LacZ fusion to express an inactive Tet2 protein [63]. However, these mice maintain 20–50% of normal Tet2 transcripts, have no obvious reduction in 5hmC, are normal in overall appearance, and display expected Mendelian ratios. In spite of this, and unlike Tet1 null mice, Tet2 hypomorphs do appear to have aberrant hematopoiesis [63]. Although no changes in Tet gene expression have been reported, it is possible that the different members of the Tet family are compensating for the loss or reduction of Tet1 or Tet2 in these mouse models [53, 63].

Tet3 null mice are unique in that they exhibit neonatal lethality [61]. This lethality was overcome by creation of Tet3 conditional knockout mice [61]. As described above, the parental mice only lack Tet3 expression in PGC and thus are essentially normal, with only the females exhibiting reduced fecundity. The zygotes of these mice have decreased 5hmC levels and aberrant reprogramming of the paternal DNA, which is thought to disrupt prenatal development [61].

Tissue-specific expression of Dnmts and patterning of 5mC is known to be involved in gene regulation. It is hypothesized that Tet enzyme activity and 5hmC may be involved in specific biological functions in different tissues and organs as well. Indeed, TET enzymes display altered expression levels depending on the tissue or the stage of development [22–24, 35, 60]. A number of tissue types have been examined for TET expression, including but not limited to brain, lung, liver, heart, and kidney. *TET1* and *TET2* exhibit varied expression levels in different tissues examined [23] and isoforms 2 and 3 of *TET2* are expressed at a lower level than its isoform 1 [24]. Overall TET2 and its isoforms appear to be the most highly expressed amongst the TET enzymes in many tissues [22, 24]. *TET3* also tends to have consistently high expression across various tissues [23]. All TETs are highly expressed in hematopoietic cells, with *TET2* and *TET3* being the highest. Consistently, hypomorphic expression of Tet2 in mice has been shown to alter hematopoietic development [23, 24, 63].

Several studies have measured global 5hmC in DNA from various tissues using the techniques described above [26–28, 64]. Based on these analyses one would conclude that in addition to tissue-specific expression of TET enzymes, many tissues also display varied global 5hmC levels with some tissues having high, medium, or low levels of 5hmC. In general, tissues of the central nervous system have variable but overall high levels of global 5hmC [26, 27, 64]. Conversely, glandular tissues tend to have low 5hmC levels and the majority of key organs, such as heart, lung, and kidney tend to have midlevels of 5hmC in their genome [26–28]. This is in contrast with the stable global 5mC levels that are observed across most tissues [26]. However, it is important to note that in spite of stable global 5mC levels in various tissues there are locus-specific differences that are involved in maintaining proper tissue phenotype and function. These data suggest that high levels of 5hmC are not indicative of low 5mC levels on a genome-wide basis in somatic tissues, but that locus-specific shifts in the amount of unmodified, methylated, and hydroxymethylated cytosines are important for regulating gene expression in a tissue-specific manner. This is also supported by our work showing tissue-specific levels of 5hmC

at various loci in both mouse and human genomic DNA samples [32]. However, more detailed analysis of 5hmC patterning in various tissues and during development is required, which would help us to understand the roles of TET enzymes and 5hmC in differentiation and development.

3.6 Mutation of 5hmC Pathway Genes in Cancer and the Possible Consequences

TET1 is a common translocation partner of MLL histone methyltransferase at t(10;11)(q22;q23), in acute myeloid leukemia (AML) [13, 14]. The MLL-TET1 translocation has also been less commonly identified in acute lymphoblastic leukemia (ALL) [65]. Apart from the t(10;11)(q22;q23) translocation, no other mutations of TET1 have been reported. The MLL-TET1 fusion protein is predicted to have a molecular mass of approximately 204 kDa and is created by the fusion of the N-terminal part of MLL with the C-terminal part of TET1. The resulting protein contains the AT hooks, subnuclear localization domains, and the CXXC region of MLL fused to the core catalytic domain of TET1 [14]. The catalytic activity of the MLL-TET1 fusion protein is unknown, but it may be a gain of novel function of the fusion protein or loss of MLL and/or TET1 normal function that promotes oncogenesis. Regardless of the precise mechanism(s), MLL translocations correlate with a poor prognosis in ALL and AML patients [66–69].

Similar to TET1, it had been known that the 4q24 chromosomal region was commonly disrupted in hematologic malignancies, but the gene targeted within that region was not clear. It is now known that TET2 is the affected gene at 4q24 in many of these hematologic malignancies. TET2 mutations in myeloproliferative neoplasms (MPN) were identified recently [70–72]. Since then, mutations in TET2 have been observed in myelodysplastic syndrome (MDS), polycythemia vera, essential thrombocythemia, myelofibrosis, blastic plasmacytoid dendritic cell neoplasm (BPDCN), lymphomas, and different types of leukemia [23, 63, 70, 73–82]. Interestingly, certain TET2 mutations are found in specific subsets of these diseases [83].

TET2 mutations range from nonsense and missense mutations to frameshifts and deletions. Essentially all of these mutations are thought to result in loss of function of the TET2 enzyme and are generally somatic in nature. Several common mutations observed in MPN patients were tested for their effects on TET2 activity, including W1291R, E1318G, P1367S, I1873T, and G1913D [21]. All of these mutations are located in the cysteine-rich region or catalytic domain of human TET2. Overexpression of the mutant mouse counterpart of the W1291R (W1211R), P1367S (P1287S), and G1913D (C1834D) mutants in HEK293T cells results in reduced 5hmC as compared to overexpression of the WT Tet2 [21]. In addition, mutations of TET2 often occur on either one or both alleles suggesting that TET2 may either be haploinsufficient or gain an oncogenic function [70, 83]. These results indicate that TET2 functions as a tumor suppressor gene, especially in

hematopoietic cells. However, TET2 mutations may not be enough to cause transformation as it is commonly mutated along with genes in other important pathways, such as JAK and p53 [76, 84, 85].

Tet2 appears to have a direct role in myelopoiesis as *Tet2* knockdown alters differentiation of bone marrow stem cells when grown in the presence of specific cytokines [86]. Furthermore, conditional knockout or reduced expression of Tet2 in mice results in amplification of hematopoietic stem and progenitor cells with skewed numbers of differentiated myeloid and lymphoid lineages [63]. Several studies have attempted to evaluate the effect of TET2 mutations on patient prognosis, albeit in a limited number of samples. Mutations in TET2 correlate with reduced survival time in AML patients [77] and lower survival rate in patients with chronic myelomonocytic leukemia (CMML) [80]. Conversely, TET2 mutations in MDS patients appear to increase survival rate, as well as decrease progression to AML [79].

To date, there is only one report of a genetic aberration associated with TET3. A patient with refractory anemia with ringed sideroblasts (RARS), a specific form of MDS, and idiopathic myelofibrosis carried a deletion of 2p23 where the TET3 gene resides [87]. It is still unknown whether TET3 has a role in myeloproliferative diseases in a similar manner to TET1 and TET2. However, as TET3 is a catalytically active enzyme and has different tissue-specific expression patterns than TET1 and TET2, it remains a possibility that TET3 is involved in the development or progression of these and other diseases or disorders. Genetic studies will be required to test the functional role of TET enzymes in the development and progression of various diseases, including cancer.

As described above, the TET enzymes require cofactors for catalysis, one of which is 2-OG. Two enzymes that are involved in producing 2-OG are the cytosolic isocitrate dehydrogenase 1 (IDH1) and its mitochondrial homolog IDH2 [88]. Interestingly, IDH1 and IDH2 are commonly mutated in several diseases, including gliomas, astrocytomas, leukemias, and MPN [88], where 5hmC and TET expression are abundant. Furthermore, these mutations are not only mutually exclusive with each other but also with TET2 mutations in AML [88].

Mutations of IDH1 and IDH2 can result in a gain-of-function phenotype whereby 2-OG is further reduced by the mutant enzyme to 2-hydroxyglutarate (2-HG) [89, 90]. AML cells harboring mutations in IDH1, IDH2, or TET2 tend to have a hypermethylated phenotype (increased global and locus-specific methylation) and importantly a significant overlap of the genes that are hypermethylated [88]. Overexpression of mutant IDH enzyme results in a global increase in methylation and co-overexpression with TET2 does not result in increased 5hmC levels [88]. The above observation was confirmed in another study that showed inhibition of murine Tet1 and Tet2 in vitro by 2-HG and in vivo by mutant IDH1 [91]. In addition, glioma, astrocytoma, glioblastoma tissue samples harboring IDH1 mutations display decreased 5hmC staining and increased 5mC staining in immunohistological assays, as well as decreased 5hmC with LC-MS analysis [64, 91]. These studies suggest that alterations in 5hmC, either through directly disrupting the TET enzymes or changing availability of cofactors, may be involved in the development and progression of cancer and related diseases.

It is hypothesized that 5hmC is an intermediate in the process of demethylation (described fully in Sect. 3.7) and as a result disruption of the TET protein functions by translocation or mutation may result in a hypermethylated phenotype. Indeed, widespread locus-specific hypermethylation in AML patients with TET2 mutations has been reported [88]. Conversely, another study found that TET2 mutations in leukemia patients are associated with reduced 5hmC levels as expected, but also with global DNA hypomethylation [21]. Another recent report indicated that brain lesions, especially astrocytomas and glioblastomas, have significantly decreased global 5hmC with increasing tumor grade, although these samples did not display clear changes in 5mC levels [64]. Furthermore, several, tumor types appear to have decreased 5hmC when compared to matched normal tissue [39, 92, 93]. The mechanism of global hypo-hydroxymethylation in tumors and the relationship to mutations in TETs is not clear and may be dependent on tumor type and stage.

Hypomethylating agents were originally tested and approved for clinical use in MDS and leukemia patients [94]. The fact that these diseases have especially high rates of mutation in the TET proteins raises the question as to the correlation of TET mutations with treatment efficacy. One study on a very limited number of patients (two) did not confirm that TET2 mutations would improve the efficacy of DNMT inhibitors for the treatment of MDS [95]. In addition, a slightly larger study with AML patients reported that those with mutant TET2 had improved initial response, but did not yield better survival as compared to patients carrying the WT allele [96]. These results emphasize the necessity for studies to be completed using large cohorts of patients identify factors that categorize patients with myeloid disorders, harboring TET mutations, as likely or unlikely to benefit from treatment with demethylating agents. Finally, although TET mutations are clearly predominant in MPN it is still possible that they occur in any number of other diseases and this will likely be a focus of future research.

3.7 Demethylation Pathways of 5hmC and Possible Roles in Cancer Methylation

Reports of methylation cycling in the promoters of specific genes, active demethylation during certain stages of development, and global hypomethylation in tumors have left epigeneticists searching for a DNA demethylase [3]. Several possible demethylation mechanisms have been proposed in the past, including direct enzymatic removal of the methyl group by MBD2 [97], removal of the entire methylated base by a DNA glycosylase in a similar manner to the process of demethylation in plants [98], and deamination followed by base excision repair (BER), including deamination by DNMT3 enzymes in the presence of minimal *S*-adenosyl-L-methionine (AdoMet) [3, 99]. The stability of the carbon–carbon bond of the methyl group and the fifth carbon of the cytosine ring makes it unlikely that demethylation is due to direct removal of the methyl group from cytosine [3]. However, oxidation of methyl groups is a feasible mechanism for removal, especially as

histone demethylases function through oxidation to return histone proteins to an unmodified amino acid state [15].

Before 5hmC was found in mouse ESCs and brain DNA, several groups studied the effects of oxidation of 5mC on methyl binding proteins (MBD) and DNMT1 activity. For example, the MBD MeCP2 was shown to have decreased binding to 5hmC as compared to 5mC [100]. Altered binding of MeCP2 may have serious effects on transcriptional regulation, but would not lead to demethylation. However, DNMT1 was shown to have reduced catalytic activity when the DNA substrate was hemi-hydroxymethylated as opposed to the preferential hemi-methylated substrate [101]. This could have major effects on DNA methylation maintenance during replication, resulting in passive demethylation that is dependent on cell cycling. It is still unknown whether DNMT3a or DNMT3b expressed during S-phase is capable of methylating hemi-hydroxymethylated DNA.

Mammalian 5hmC glycosylases have been described as early as 1988 suggesting that this may be a possible mechanism for removal of this modified nucleobase [102]. Overexpression of TET genes causes increased 5hmC and then subsequent demethylation (based on digestion with methyl-sensitive restriction enzymes) of either endogenous or exogenous methylated DNA that requires a functional BER pathway [20, 103]. Additionally, overexpression of several of the Apobec family of cytidine deaminases causes further demethylation [103]. In fact, viral overexpression of Tet1 in the adult mouse dentate gyrus in the brain leads to substantial increases in global 5hmC, whereas viral overexpression of activation-induced deaminase (AID) in the same tissue causes a decrease in global 5hmC by more than 50%. Overexpression of either Tet1 or AID in adult mouse dentate granule cells results in demethylation and expression of neuronal genes known to display activity-induced DNA demethylation, but no demethylation occurs at non-neuronal promoters [103, 104]. Based on these findings, the following hypothesis has been proposed as one possible mechanism for 5hmC-stimulated demethylation: 5mC is first oxidized by TET enzymes to 5hmC, which is then deaminated by AID/APOBEC cytidine deaminases resulting in 5hmU, then 5hmU is targeted and removed by BER pathways (Fig. 3.2) [103].

Another possible mechanism of demethylation through 5hmC mimics the process of thymine conversion to uracil that is part of the thymidine salvage pathway in which successive oxidation of the 3-methyl group of thymine is completed to produce uracil by decarboxylation [3]. Previously, 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) (the further oxidized forms of 5hmC) could not be easily measured, but as more sensitive techniques were utilized it was clear that these forms of cytosine do exist in mammalian DNA (Fig. 3.2) [26–28]. Mouse ESC, mouse cortex DNA, and DNA from several other somatic tissues contain substantial amounts of each of these modifications, with 5caC being the lowest modified residue [27]. Interestingly, some tissue DNAs contained higher amounts of 5fC than 5hmC, such as liver and spleen [27]. The differences in the global amounts of each modified cytosine could be due to varied rate of conversion from one form to the next, as well as efficiency of removal for 5caC by thymine-DNA glycosylase (TDG) resulting in replacement with unmodified cytosine by

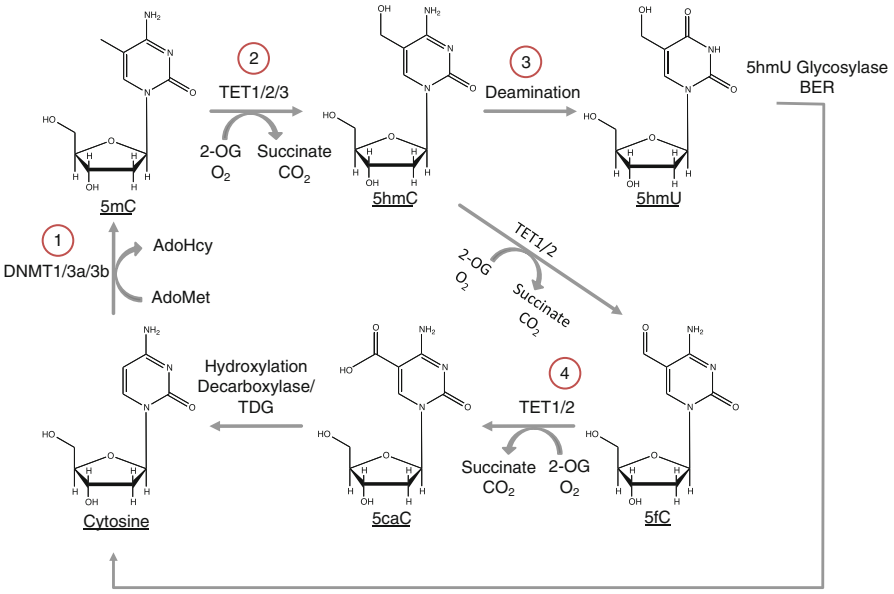

Fig. 3.2 5-Hydroxymethylcytosine and proposed demethylation pathways. (1) Cytosine in an unmodified state can be methylated by any of the three active DNMTs to 5-methylcytosine (5mC) to create the substrate for the TET enzymes. (2) 5mC can be oxidized by any of the three TET family enzymes to 5-hydroxymethylcytosine (5hmC). (3) 5hmC may then be deaminated by unknown enzymes to 5-hydroxymethyluracil (5hmU), which could then be removed by base excision repair pathway enzymes (BER). (4) 5hmC could also be further oxidized by the TET enzymes to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC), at which point the base can be removed by thymine-DNA glycosylase (TDG) or the carboxyl group can be removed by decarboxylases to produce unmethylated cytosine

DNA repair enzymes [28, 105]. Tet1 and Tet2 were both reported to oxidize 5hmC further to 5fC and 5caC both in vitro and in overexpression studies in cultured cells [27, 28].

The knowledge that 5hmC and its derivatives that are converted by the TET enzymes can result in demethylation provide some possible mechanisms for how aberrant methylation could occur in cancers. Loss-of-function mutations in TET2 correlate with hypermethylation and myeloid malignancies that commonly have TET mutations tend to be sensitive to hypomethylating agents [88, 94]. However, one study did correlate TET2 mutations with global hypomethylation in patients with myeloid malignancies [21]. For cancers that display hypomethylation, there are several potential explanations; one possibility is that hypomethylation by 5hmC is an earlier event during cancer progression than loss-of-function mutations that have been reported for TETs, or TET proteins (or other proteins involved in 5hmC-induced demethylation pathways) may be overexpressed or have gain-of-function mutations that are currently unknown. Clearly much research still needs to be done in this particular area to understand demethylation pathways of 5hmC and what enzymes are involved both in normal and disease states.

3.8 Future Perspectives

It was not long ago that the study of DNA methylation was uncharted territory, but now we have a basic understanding of how, when, and where DNA methylation occurs, as well as its role in many biological processes. The identification of 5hmC, and its oxidative products 5fC and 5caC, has complicated our understanding of this process, so now we have to tease out what past data (that may or may not include 5hmC, 5fC, and 5caC) means, and how to acquire more accurate data in the future. This has been and will continue to be a difficult process, but even in the short time since the identification of 5hmC, epigenetics research has moved forward by leaps and bounds, perhaps due to the past experiences with 5mC. Scientists have already developed several techniques to measure global and locus-specific 5hmC across the genome. It is known that there is tissue-specific expression of TETs and 5hmC levels, both globally and at specific loci, and that 5hmC may be involved in DNA demethylation pathways. Even so, there is certainly more research needed to determine the involvement of the TET enzymes and 5hmC in gene regulation, development, and disease.

3.9 Addendum

Two new methods have been reported that allow for single base resolution sequencing of 5hmC [106, 107]. Both techniques depend on the concept that 5fC and 5caC, unlike 5mC or 5hmC, are converted to uracil during sodium bisulfite treatment of the DNA. The first method utilizes potassium perruthenate (KRuO4) to chemically oxidize 5hmC to 5fC followed by rigorous bisulfite treatment and then sequencing of primarily CpG islands in mouse embryonic stem (ES) cell DNA [106]. The second method utilizes a three step process whereby the 5hmC sites are first glucosylated by beta-glucosyltransferase, which is followed by enzymatic oxidation of 5mC to 5caC by recombinant mouse Tet1 catalytic domain, and finally sodium bisulfite conversion and sequencing of human and mouse ES cell DNA. The glucosylated hydroxymethylcytosine residues are resistant to enzymatic oxidation and displayed as C in subsequent PCR based sequencing [107]. In both cases sequencing of both an oxidation pretreated DNA library and a control library must be completed to accurately map both 5mC and 5hmC sites across the genome. Considering that next generation sequencing analysis of bisulfite converted DNA is quite complicated, the data analysis for these methods could be especially difficult. However, these techniques should be useful for identification of 5mC and 5hmC at specific loci using a candidate gene approach in a similar manner to original bisulfite sequencing.

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References

1. Bird AP (1986) CpG-rich islands and the function of DNA methylation. Nature 321:209–213
2. Poulsen HE (2005) Oxidative DNA modifications. Exp Toxicol Pathol 57(Suppl 1):161–169
3. Wu SC, Zhang Y (2010) Active DNA demethylation: many roads lead to Rome. Nat Rev Mol Cell Biol 11:607–620
4. Wyatt GR, Cohen SS (1952) A new pyrimidine base from bacteriophage nucleic acids. Nature 170:1072–1073
5. Josse J, Kornberg A (1962) Glucosylation of deoxyribonucleic acid III alpha- and beta-glucosyl transferases from T4-infected Escherichia coli. J Biol Chem 237:1968–1976
6. Kornberg SR, Zimmerman SB, Kornberg A (1961) Glucosylation of deoxyribonucleic acid by enzymes from bacteriophage-infected Escherichia coli. J Biol Chem 236:1487–1493
7. Penn NW, Suwalski R, O'Riley C, Bojanowski K, Yura R (1972) The presence of 5-hydroxymethylcytosine in animal deoxyribonucleic acid. Biochem J 126:781–790
8. Penn NW (1976) Modification of brain deoxyribonucleic acid base content with maturation in normal and malnourished rats. Biochem J 155:709–712
9. Kothari RM, Shankar V (1976) 5-Methylcytosine content in the vertebrate deoxyribonucleic acids: species specificity. J Mol Evol 7:325–329
10. Tahiliani M, Koh KP, Shen Y, Pastor WA, Bandukwala H, Brudno Y, Agarwal S, Iyer LM, Liu DR, Aravind L, Rao A (2009) Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. Science 324:930–935
11. Kriaucionis S, Heintz N (2009) The nuclear DNA base 5-hydroxymethylcytosine is present in Purkinje neurons and the brain. Science 324:929–930
12. Shock LS, Thakkar PV, Peterson EJ, Moran RG, Taylor SM (2011) DNA methyltransferase 1, cytosine methylation, and cytosine hydroxymethylation in mammalian mitochondria. Proc Natl Acad Sci USA 108:3630–3635
13. Ono R, Taki T, Taketani T, Taniwaki M, Kobayashi H, Hayashi Y (2002) LCX, leukemia-associated protein with a CXXC domain, is fused to MLL in acute myeloid leukemia with trilineage dysplasia having t(10;11)(q22;q23). Cancer Res 62:4075–4080
14. Lorsbach RB, Moore J, Mathew S, Raimondi SC, Mukatira ST, Downing JR (2003) TET1, a member of a novel protein family, is fused to MLL in acute myeloid leukemia containing the t(10;11)(q22;q23). Leukemia 17:637–641
15. Loenarz C, Schofield CJ (2009) Oxygenase catalyzed 5-methylcytosine hydroxylation. Chem Biol 16:580–583
16. Yu Z, Genest PA, ter Riet B, Sweeney K, DiPaolo C, Kieft R, Christodoulou E, Perrakis A, Simmons JM, Hausinger RP, van Luenen HG, Rigden DJ, Sabatini R, Borst P (2007) The protein that binds to DNA base J in trypanosomatids has features of a thymidine hydroxylase. Nucleic Acids Res 35:2107–2115
17. Iyer LM, Tahiliani M, Rao A, Aravind L (2009) Prediction of novel families of enzymes involved in oxidative and other complex modifications of bases in nucleic acids. Cell Cycle 8:1698–1710
18. Mohr F, Dohner K, Buske C, Rawat VP (2011) TET genes: new players in DNA demethylation and important determinants for stemness. Exp Hematol 39:272–281
19. Frauer C, Rottach A, Meilinger D, Bultmann S, Fellinger K, Hasenoder S, Wang M, Qin W, Soding J, Spada F, Leonhardt H (2011) Different binding properties and function of CXXC zinc finger domains in Dnmt1 and Tet1. PLoS One 6:e16627
20. Zhang H, Zhang X, Clark E, Mulcahey M, Huang S, Shi YG (2010) TET1 is a DNA-binding protein that modulates DNA methylation and gene transcription via hydroxylation of 5-methylcytosine. Cell Res 20:1390–1393
21. Ko M, Huang Y, Jankowska AM, Pape UJ, Tahiliani M, Bandukwala HS, An J, Lamperti ED, Koh KP, Ganetzky R, Liu XS, Aravind L, Agarwal S, Maciejewski JP, Rao A (2010) Impaired hydroxylation of 5-methylcytosine in myeloid cancers with mutant TET2. Nature 468:839–843

22. Ito S, D'Alessio AC, Taranova OV, Hong K, Sowers LC, Zhang Y (2010) Role of Tet proteins in 5mC to 5hmC conversion, ES-cell self-renewal and inner cell mass specification. Nature 466:1129–1133
23. Langemeijer SM, Aslanyan MG, Jansen JH (2009) TET proteins in malignant hematopoiesis. Cell Cycle 8:4044–4048
24. Langemeijer SM, Kuiper RP, Berends M, Knops R, Aslanyan MG, Massop M, Stevens-Linders E, van Hoogen P, van Kessel AG, Raymakers RA, Kamping EJ, Verhoef GE, Verburgh E, Hagemeijer A, Vandenberghe P, de Witte T, van der Reijden BA, Jansen JH (2009) Acquired mutations in TET2 are common in myelodysplastic syndromes. Nat Genet 41:838–842
25. Ficz G, Branco MR, Seisenberger S, Santos F, Krueger F, Hore TA, Marques CJ, Andrews S, Reik W (2011) Dynamic regulation of 5-hydroxymethylcytosine in mouse ES cells and during differentiation. Nature 473:398–402
26. Globisch D, Munzel M, Muller M, Michalakis S, Wagner M, Koch S, Bruckl T, Biel M, Carell T (2010) Tissue distribution of 5-hydroxymethylcytosine and search for active demethylation intermediates. PLoS One 5:e15367
27. Ito S, Shen L, Dai Q, Wu SC, Collins LB, Swenberg JA, He C, Zhang Y (2011) Tet proteins can convert 5-methylcytosine to 5-formylcytosine and 5-carboxylcytosine. Science 333:1300–1303
28. He YF, Li BZ, Li Z, Liu P, Wang Y, Tang Q, Ding J, Jia Y, Chen Z, Li L, Sun Y, Li X, Dai Q, Song CX, Zhang K, He C, Xu GL (2011) Tet-mediated formation of 5-carboxylcytosine and its excision by TDG in mammalian DNA. Science 333:1303–1307
29. Huang Y, Pastor WA, Shen Y, Tahiliani M, Liu DR, Rao A (2010) The behaviour of 5-hydroxymethylcytosine in bisulfite sequencing. PLoS One 5:e8888
30. Wang H, Guan S, Quimby A, Cohen-Karni D, Pradhan S, Wilson G, Roberts RJ, Zhu Z, Zheng Y (2011) Comparative characterization of the PvuRts1I family of restriction enzymes and their application in mapping genomic 5-hydroxymethylcytosine. Nucleic Acids Res 39:9294–9305
31. Song CX, Yu M, Dai Q, He C (2011) Detection of 5-hydroxymethylcytosine in a combined glycosylation restriction analysis (CGRA) using restriction enzyme Taq(alpha)I. Bioorg Med Chem Lett 21:5075–5077
32. Kinney SM, Chin HG, Vaisvila R, Bitinaite J, Zheng Y, Esteve PO, Feng S, Stroud H, Jacobsen SE, Pradhan S (2011) Tissue-specific distribution and dynamic changes of 5-hydroxymethylcytosine in mammalian genomes. J Biol Chem 286:24685–24693
33. Szwagierczak A, Brachmann A, Schmidt CS, Bultmann S, Leonhardt H, Spada F (2011) Characterization of PvuRts1I endonuclease as a tool to investigate genomic 5-hydroxymethylcytosine. Nucleic Acids Res 39:5149–5156
34. Roberts RJ, Vincze T, Posfai J, Macelis D (2010) REBASE—a database for DNA restriction and modification: enzymes, genes and genomes. Nucleic Acids Res 38:D234–D236
35. Iqbal K, Jin SG, Pfeifer GP, Szabo PE (2011) Reprogramming of the paternal genome upon fertilization involves genome-wide oxidation of 5-methylcytosine. Proc Natl Acad Sci USA 08:3642–3647
36. Stroud H, Feng S, Morey Kinney S, Pradhan S, Jacobsen SE (2011) 5-Hydroxymethylcytosine is associated with enhancers and gene bodies in human embryonic stem cells. Genome Biol 12:R54
37. Le T, Kim KP, Fan G, Faull KF (2011) A sensitive mass spectrometry method for simultaneous quantification of DNA methylation and hydroxymethylation levels in biological samples. Anal Biochem 412:203–209
38. Szwagierczak A, Bultmann S, Schmidt CS, Spada F, Leonhardt H (2010) Sensitive enzymatic quantification of 5-hydroxymethylcytosine in genomic DNA. Nucleic Acids Res 38:e181
39. Terragni J, Bitinaite J, Zheng Y, Pradhan S (2012) Biochemical characterization of recombinant Beta-glucosyltransferase and analysis of global 5-hydroxymethylcytosine in unique genomes. Biochemistry 51:1009–1019
40. Wu H, D'Alessio AC, Ito S, Wang Z, Cui K, Zhao K, Sun YE, Zhang Y (2011) Genome-wide analysis of 5-hydroxymethylcytosine distribution reveals its dual function in transcriptional regulation in mouse embryonic stem cells. Genes Dev 25:679–684

41. Wu H, D'Alessio AC, Ito S, Xia K, Wang Z, Cui K, Zhao K, Sun YE, Zhang Y (2011) Dual functions of Tet1 in transcriptional regulation in mouse embryonic stem cells. Nature 473:389–393
42. Pastor WA, Pape UJ, Huang Y, Henderson HR, Lister R, Ko M, McLoughlin EM, Brudno Y, Mahapatra S, Kapranov P, Tahiliani M, Daley GQ, Liu XS, Ecker JR, Milos PM, Agarwal S, Rao A (2011) Genome-wide mapping of 5-hydroxymethylcytosine in embryonic stem cells. Nature 473:394–397
43. Song CX, Szulwach KE, Fu Y, Dai Q, Yi C, Li X, Li Y, Chen CH, Zhang W, Jian X, Wang J, Zhang L, Looney TJ, Zhang B, Godley LA, Hicks LM, Lahn BT, Jin P, He C (2011) Selective chemical labeling reveals the genome-wide distribution of 5-hydroxymethylcytosine. Nat Biotechnol 29:68–72
44. Flusberg BA, Webster DR, Lee JH, Travers KJ, Olivares EC, Clark TA, Korlach J, Turner SW (2010) Direct detection of DNA methylation during single-molecule, real-time sequencing. Nat Methods 7:461–465
45. Wanunu M, Cohen-Karni D, Johnson RR, Fields L, Benner J, Peterman N, Zheng Y, Klein ML, Drndic M (2010) Discrimination of methylcytosine from hydroxymethylcytosine in DNA molecules. J Am Chem Soc 133:486–492
46. Bair CL, Black LW (2007) A type IV modification dependent restriction nuclease that targets glucosylated hydroxymethyl cytosine modified DNAs. J Mol Biol 366:768–778
47. Freudenberg JM, Ghosh S, Lackford BL, Yellaboina S, Zheng X, Li R, Cuddapah S, Wade PA, Hu G, Jothi R (2011) Acute depletion of Tet1-dependent 5-hydroxymethylcytosine levels impairs LIF/Stat3 signaling and results in loss of embryonic stem cell identity. Nucleic Acids Res 40(8):3364–3377
48. Zhou GB, Meng QG, Li N (2010) In vitro derivation of germ cells from embryonic stem cells in mammals. Mol Reprod Dev 77:586–594
49. Koh KP, Yabuuchi A, Rao S, Huang Y, Cunniff K, Nardone J, Laiho A, Tahiliani M, Sommer CA, Mostoslavsky G, Lahesmaa R, Orkin SH, Rodig SJ, Daley GQ, Rao A (2011) Tet1 and Tet2 regulate 5-hydroxymethylcytosine production and cell lineage specification in mouse embryonic stem cells. Cell Stem Cell 8:200–213
50. Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126:663–676
51. Williams K, Christensen J, Pedersen MT, Johansen JV, Cloos PA, Rappsilber J, Helin K (2011) TET1 and hydroxymethylcytosine in transcription and DNA methylation fidelity. Nature 473:343–348
52. Xu Y, Wu F, Tan L, Kong L, Xiong L, Deng J, Barbera AJ, Zheng L, Zhang H, Huang S, Min J, Nicholson T, Chen T, Xu G, Shi Y, Zhang K, Shi YG (2011) Genome-wide regulation of 5hmC, 5mC, and gene expression by Tet1 hydroxylase in mouse embryonic stem cells. Mol Cell 42:451–464
53. Dawlaty MM, Ganz K, Powell BE, Hu YC, Markoulaki S, Cheng AW, Gao Q, Kim J, Choi SW, Page DC, Jaenisch R (2011) Tet1 is dispensable for maintaining pluripotency and its loss is compatible with embryonic and postnatal development. Cell Stem Cell 9:166–175
54. Yildirim O, Li R, Hung JH, Chen PB, Dong X, Ee LS, Weng Z, Rando OJ, Fazzio TG (2011) Mbd3/NURD Complex Regulates Expression of 5-Hydroxymethylcytosine Marked Genes in Embryonic Stem Cells. Cell 147:1498–1510
55. Reik W, Dean W, Walter J (2001) Epigenetic reprogramming in mammalian development. Science 293:1089–1093
56. Mayer W, Niveleau A, Walter J, Fundele R, Haaf T (2000) Demethylation of the zygotic paternal genome. Nature 403:501–502
57. Oswald J, Engemann S, Lane N, Mayer W, Olek A, Fundele R, Dean W, Reik W, Walter J (2000) Active demethylation of the paternal genome in the mouse zygote. Curr Biol 10:475–478
58. Santos F, Hendrich B, Reik W, Dean W (2002) Dynamic reprogramming of DNA methylation in the early mouse embryo. Dev Biol 241:172–182
59. Hajkova P, Jeffries SJ, Lee C, Miller N, Jackson SP, Surani MA (2010) Genome-wide reprogramming in the mouse germ line entails the base excision repair pathway. Science 329:78–82

60. Wossidlo M, Nakamura T, Lepikhov K, Marques CJ, Zakhartchenko V, Boiani M, Arand J, Nakano T, Reik W, Walter J (2011) 5-Hydroxymethylcytosine in the mammalian zygote is linked with epigenetic reprogramming. Nat Commun 2:241
61. Gu TP, Guo F, Yang H, Wu HP, Xu GF, Liu W, Xie ZG, Shi L, He X, Jin SG, Iqbal K, Shi YG, Deng Z, Szabo PE, Pfeifer GP, Li J, Xu GL (2011) The role of Tet3 DNA dioxygenase in epigenetic reprogramming by oocytes. Nature 477:606–610
62. Nakamura T, Arai Y, Umehara H, Masuhara M, Kimura T, Taniguchi H, Sekimoto T, Ikawa M, Yoneda Y, Okabe M, Tanaka S, Shiota K, Nakano T (2007) PGC7/Stella protects against DNA demethylation in early embryogenesis. Nat Cell Biol 9:64–71
63. Quivoron C, Couronne L, Della Valle V, Lopez CK, Plo I, Wagner-Ballon O, Do Cruzeiro M, Delhommeau F, Arnulf B, Stern MH, Godley L, Opolon P, Tilly H, Solary E, Duffourd Y, Dessen P, Merle-Beral H, Nguyen-Khac F, Fontenay M, Vainchenker W, Bastard C, Mercher T, Bernard OA (2011) TET2 inactivation results in pleiotropic hematopoietic abnormalities in mouse and is a recurrent event during human lymphomagenesis. Cancer Cell 20:25–38
64. Kraus TF, Globisch D, Wagner M, Eigenbrod S, Widmann D, Munzel M, Muller M, Pfaffeneder T, Hackner B, Feiden W, Schuller U, Carell T, Kretzschmar HA (2012) Low values of 5-hydroxymethylcytosine (5hmC), the "sixth base", are associated with anaplasia in human brain tumours. International journal of cancer. J Int Cancer Jan 10. doi: 10.1002/ijc.27429. [Epub ahead of print]
65. Burmeister T, Meyer C, Schwartz S, Hofmann J, Molkentin M, Kowarz E, Schneider B, Raff T, Reinhardt R, Gokbuget N, Hoelzer D, Thiel E, Marschalek R (2009) The MLL recombinome of adult CD10-negative B-cell precursor acute lymphoblastic leukemia: results from the GMALL study group. Blood 113:4011–4015
66. Pui CH, Chessells JM, Camitta B, Baruchel A, Biondi A, Boyett JM, Carroll A, Eden OB, Evans WE, Gadner H, Harbott J, Harms DO, Harrison CJ, Harrison PL, Heerema N, Janka-Schaub G, Kamps W, Masera G, Pullen J, Raimondi SC, Richards S, Riehm H, Sallan S, Sather H, Shuster J, Silverman LB, Valsecchi MG, Vilmer E, Zhou Y, Gaynon PS, Schrappe M (2003) Clinical heterogeneity in childhood acute lymphoblastic leukemia with 11q23 rearrangements. Leukemia 17:700–706
67. Pui CH, Gaynon PS, Boyett JM, Chessells JM, Baruchel A, Kamps W, Silverman LB, Biondi A, Harms DO, Vilmer E, Schrappe M, Camitta B (2002) Outcome of treatment in childhood acute lymphoblastic leukaemia with rearrangements of the 11q23 chromosomal region. Lancet 359:1909–1915
68. Liedtke M, Cleary ML (2009) Therapeutic targeting of MLL. Blood 113:6061–6068
69. Chou WC, Chou SC, Liu CY, Chen CY, Hou HA, Kuo YY, Lee MC, Ko BS, Tang JL, Yao M, Tsay W, Wu SJ, Huang SY, Hsu SC, Chen YC, Chang YC, Kuo KT, Lee FY, Liu MC, Liu CW, Tseng MH, Huang CF, Tien HF (2011) TET2 mutation is an unfavorable prognostic factor in acute myeloid leukemia patients with intermediate-risk cytogenetics. Blood 118:3803–3810
70. Delhommeau F, Dupont S, Della Valle V, James C, Trannoy S, Masse A, Kosmider O, Le Couedic JP, Robert F, Alberdi A, Lecluse Y, Plo I, Dreyfus FJ, Marzac C, Casadevall N, Lacombe C, Romana SP, Dessen P, Soulier J, Viguie F, Fontenay M, Vainchenker W, Bernard OA (2009) Mutation in TET2 in myeloid cancers. N Engl J Med 360:2289–2301
71. Tefferi A, Levine RL, Lim KH, Abdel-Wahab O, Lasho TL, Patel J, Finke CM, Mullally A, Li CY, Pardanani A, Gilliland DG (2009) Frequent TET2 mutations in systemic mastocytosis: clinical, KITD816V and FIP1L1-PDGFRA correlates. Leukemia 23:900–904
72. Tefferi A, Lim KH, Levine R (2009) Mutation in TET2 in myeloid cancers. N Engl J Med 361:1117
73. Tefferi A, Pardanani A, Lim KH, Abdel-Wahab O, Lasho TL, Patel J, Gangat N, Finke CM, Schwager S, Mullally A, Li CY, Hanson CA, Mesa R, Bernard O, Delhommeau F, Vainchenker W, Gilliland DG, Levine RL (2009) TET2 mutations and their clinical correlates in polycythemia vera, essential thrombocythemia and myelofibrosis. Leukemia 23:905–911
74. Tefferi A, Lim KH, Abdel-Wahab O, Lasho TL, Patel J, Patnaik MM, Hanson CA, Pardanani A, Gilliland DG, Levine RL (2009) Detection of mutant TET2 in myeloid malignancies other than myeloproliferative neoplasms: CMML, MDS, MDS/MPN and AML. Leukemia 23: 1343–1345

75. Makishima H, Jankowska AM, McDevitt MA, O'Keefe C, Dujardin S, Cazzolli H, Przychodzen B, Prince C, Nicoll J, Siddaiah H, Shaik M, Szpurka H, Hsi E, Advani A, Paquette R, Maciejewski JP (2011) CBL, CBLB, TET2, ASXL1, and IDH1/2 mutations and additional chromosomal aberrations constitute molecular events in chronic myelogenous leukemia. Blood 117:e198–e206
76. Jardin F, Ruminy P, Parmentier F, Troussard X, Vaida I, Stamatoullas A, Lepretre S, Penther D, Duval AB, Picquenot JM, Courville P, Capiod JC, Tilly H, Bastard C, Marolleau JP (2011) TET2 and TP53 mutations are frequently observed in blastic plasmacytoid dendritic cell neoplasm. Br J Haematol 153:413–416
77. Abdel-Wahab O, Mullally A, Hedvat C, Garcia-Manero G, Patel J, Wadleigh M, Malinge S, Yao J, Kilpivaara O, Bhat R, Huberman K, Thomas S, Dolgalev I, Heguy A, Paietta E, Le Beau MM, Beran M, Tallman MS, Ebert BL, Kantarjian HM, Stone RM, Gilliland DG, Crispino JD, Levine RL (2009) Genetic characterization of TET1, TET2, and TET3 alterations in myeloid malignancies. Blood 114:144–147
78. Jankowska AM, Szpurka H, Tiu RV, Makishima H, Afable M, Huh J, O'Keefe CL, Ganetzky R, McDevitt MA, Maciejewski JP (2009) Loss of heterozygosity 4q24 and TET2 mutations associated with myelodysplastic/myeloproliferative neoplasms. Blood 113:6403–6410
79. Kosmider O, Gelsi-Boyer V, Cheok M, Grabar S, Della-Valle V, Picard F, Viguie F, Quesnel B, Beyne-Rauzy O, Solary E, Vey N, Hunault-Berger M, Fenaux P, Mansat-De Mas V, Delabesse E, Guardiola P, Lacombe C, Vainchenker W, Preudhomme C, Dreyfus F, Bernard OA, Birnbaum D, Fontenay M (2009) TET2 mutation is an independent favorable prognostic factor in myelodysplastic syndromes (MDSs). Blood 114:3285–3291
80. Kosmider O, Gelsi-Boyer V, Ciudad M, Racoeur C, Jooste V, Vey N, Quesnel B, Fenaux P, Bastie JN, Beyne-Rauzy O, Stamatoulas A, Dreyfus F, Ifrah N, de Botton S, Vainchenker W, Bernard OA, Birnbaum D, Fontenay M, Solary E (2009) TET2 gene mutation is a frequent and adverse event in chronic myelomonocytic leukemia. Haematologica 94:1676–1681
81. Kohlmann A, Grossmann V, Klein HU, Schindela S, Weiss T, Kazak B, Dicker F, Schnittger S, Dugas M, Kern W, Haferlach C, Haferlach T (2010) Next-generation sequencing technology reveals a characteristic pattern of molecular mutations in 72.8% of chronic myelomonocytic leukemia by detecting frequent alterations in TET2, CBL, RAS, and RUNX1. J Clin Oncol 28:3858–3865
82. Nibourel O, Kosmider O, Cheok M, Boissel N, Renneville A, Philippe N, Dombret H, Dreyfus F, Quesnel B, Geffroy S, Quentin S, Roche-Lestienne C, Cayuela JM, Roumier C, Fenaux P, Vainchenker W, Bernard OA, Soulier J, Fontenay M, Preudhomme C (2010) Incidence and prognostic value of TET2 alterations in de novo acute myeloid leukemia achieving complete remission. Blood 116:1132–1135
83. Hellstrom-Lindberg E (2010) Significance of JAK2 and TET2 mutations in myelodysplastic syndromes. Blood Rev 24:83–90
84. Swierczek SI, Yoon D, Bellanne-Chantelot C, Kim SJ, Saint-Martin C, Delhommeau F, Najman A, Prchal JT (2011) Extent of hematopoietic involvement by TET2 mutations in JAK2V(1)F polycythemia vera. Haematologica 96:775–778
85. Rocquain J, Carbuccia N, Trouplin V, Raynaud S, Murati A, Nezri M, Tadrist Z, Olschwang S, Vey N, Birnbaum D, Gelsi-Boyer V, Mozziconacci MJ (2010) Combined mutations of ASXL1, CBL, FLT3, IDH1, IDH2, JAK2, KRAS, NPM1, NRAS, RUNX1, TET2 and WT1 genes in myelodysplastic syndromes and acute myeloid leukemias. BMC Cancer 10:401
86. Ko M, Bandukwala HS, An J, Lamperti ED, Thompson EC, Hastie R, Tsangaratou A, Rajewsky K, Koralov SB, Rao A (2011) Ten-Eleven-Translocation 2 (TET2) negatively regulates homeostasis and differentiation of hematopoietic stem cells in mice. Proc Natl Acad Sci USA 108:14566–14571
87. Gelsi-Boyer V, Trouplin V, Adelaide J, Bonansea J, Cervera N, Carbuccia N, Lagarde A, Prebet T, Nezri M, Sainty D, Olschwang S, Xerri L, Chaffanet M, Mozziconacci MJ, Vey N, Birnbaum D (2009) Mutations of polycomb-associated gene ASXL1 in myelodysplastic syndromes and chronic myelomonocytic leukaemia. Br J Haematol 145:788–800
88. Figueroa ME, Abdel-Wahab O, Lu C, Ward PS, Patel J, Shih A, Li Y, Bhagwat N, Vasanthakumar A, Fernandez HF, Tallman MS, Sun Z, Wolniak K, Peeters JK, Liu W, Choe

SE, Fantin VR, Paietta E, Lowenberg B, Licht JD, Godley LA, Delwel R, Valk PJ, Thompson CB, Levine RL, Melnick A (2010) Leukemic IDH1 and IDH2 mutations result in a hypermethylation phenotype, disrupt TET2 function, and impair hematopoietic differentiation. Cancer Cell 18:553–567
89. Dang L, White DW, Gross S, Bennett BD, Bittinger MA, Driggers EM, Fantin VR, Jang HG, Jin S, Keenan MC, Marks KM, Prins RM, Ward PS, Yen KE, Liau LM, Rabinowitz JD, Cantley LC, Thompson CB, Vander Heiden MG, Su SM (2009) Cancer-associated IDH1 mutations produce 2-hydroxyglutarate. Nature 462:739–744
90. Ward PS, Patel J, Wise DR, Abdel-Wahab O, Bennett BD, Coller HA, Cross JR, Fantin VR, Hedvat CV, Perl AE, Rabinowitz JD, Carroll M, Su SM, Sharp KA, Levine RL, Thompson CB (2010) The common feature of leukemia-associated IDH1 and IDH2 mutations is a neomorphic enzyme activity converting alpha-ketoglutarate to 2-hydroxyglutarate. Cancer Cell 17:225–234
91. Xu W, Yang H, Liu Y, Yang Y, Wang P, Kim SH, Ito S, Yang C, Xiao MT, Liu LX, Jiang WQ, Liu J, Zhang JY, Wang B, Frye S, Zhang Y, Xu YH, Lei QY, Guan KL, Zhao SM, Xiong Y (2011) Oncometabolite 2-hydroxyglutarate is a competitive inhibitor of alpha-ketoglutarate-dependent dioxygenases. Cancer Cell 19:17–30
92. Haffner MC, Chaux A, Meeker AK, Esopi DM, Gerber J, Pellakuru LG, Toubaji A, Argani P, Iacobuzio-Donahue C, Nelson WG, Netto GJ, De Marzo AM, Yegnasubramanian S (2011) Global 5-hydroxymethylcytosine content is significantly reduced in tissue stem/progenitor cell compartments and in human cancers. Oncotarget 2:627–637
93. Li W, Liu M (2011) Distribution of 5-hydroxymethylcytosine in different human tissues. J Nucleic Acids 2011:870726
94. Kantarjian H, O'Brien S, Cortes J, Wierda W, Faderl S, Garcia-Manero G, Issa JP, Estey E, Keating M, Freireich EJ (2008) Therapeutic advances in leukemia and myelodysplastic syndrome over the past 40 years. Cancer 113:1933–1952
95. Pollyea DA, Raval A, Kusler B, Gotlib JR, Alizadeh AA, Mitchell BS (2010) Impact of TET2 mutations on mRNA expression and clinical outcomes in MDS patients treated with DNA methyltransferase inhibitors. Hematol Oncol 29:157–160
96. Itzykson R, Kosmider O, Cluzeau T, Mansat-De Mas V, Dreyfus F, Beyne-Rauzy O, Quesnel B, Vey N, Gelsi-Boyer V, Raynaud S, Preudhomme C, Ades L, Fenaux P, Fontenay M (2011) Impact of TET2 mutations on response rate to azacitidine in myelodysplastic syndromes and low blast count acute myeloid leukemias. Leukemia 25:1147–1152
97. Detich N, Theberge J, Szyf M (2002) Promoter-specific activation and demethylation by MBD2/demethylase. J Biol Chem 277:35791–35794
98. Feng S, Jacobsen SE, Reik W (2010) Epigenetic reprogramming in plant and animal development. Science 330:622–627
99. Metivier R, Gallais R, Tiffoche C, Le Peron C, Jurkowska RZ, Carmouche RP, Ibberson D, Barath P, Demay F, Reid G, Benes V, Jeltsch A, Gannon F, Salbert G (2008) Cyclical DNA methylation of a transcriptionally active promoter. Nature 452:45–50
100. Valinluck V, Tsai HH, Rogstad DK, Burdzy A, Bird A, Sowers LC (2004) Oxidative damage to methyl-CpG sequences inhibits the binding of the methyl-CpG binding domain (MBD) of methyl-CpG binding protein 2 (MeCP2). Nucleic Acids Res 32:4100–4108
101. Valinluck V, Sowers LC (2007) Endogenous cytosine damage products alter the site selectivity of human DNA maintenance methyltransferase DNMT1. Cancer Res 67:946–950
102. Cannon SV, Cummings A, Teebor GW (1988) 5-Hydroxymethylcytosine DNA glycosylase activity in mammalian tissue. Biochem Biophys Res Commun 151:1173–1179
103. Guo JU, Su Y, Zhong C, Ming GL, Song H (2011) Hydroxylation of 5-methylcytosine by TET1 promotes active DNA demethylation in the adult brain. Cell 145:423–434
104. Ma DK, Jang MH, Guo JU, Kitabatake Y, Chang ML, Pow-Anpongkul N, Flavell RA, Lu B, Ming GL, Song H (2009) Neuronal activity-induced Gadd45b promotes epigenetic DNA demethylation and adult neurogenesis. Science 323:1074–1077

105. Maiti A, Drohat AC (2011) Thymine DNA glycosylase can rapidly excise 5-formylcytosine and 5-carboxylcytosine: Potential implications for active demethylation of CpG sites. J Biol Chem 286:35334–35338
106. Booth MJ, Branco MR, Ficz G, Oxley D, Krueger F, Reik W, Balasubramanian S. (2012) Quantitative sequencing of 5-methylcytosine and 5-hydroxymethylcytosine at single-base resolution. Science 336:934–937
107. Yu M, Hon GC, Szulwach KE, Song CX, Zhang L, Kim A, Li X, Dai Q, Shen Y, Park B, Min JH, Jin P, Ren B, He C (2012) Base-resolution analysis of 5-hydroxymethylcytosine in the mammalian genome. Cell 149:1368–1380

Chapter 4
Altered Histone Modifications in Cancer

Moray J. Campbell and Bryan M. Turner

Abstract In human health and disease the choreographed actions of a wide armory of transcription factors govern the regulated expression of coding and nonprotein coding genes. These actions are central to human health and are evidently aberrant in cancer. Central components of regulated gene expression are a variety of epigenetic mechanisms that include histone modifications. The post-translational modifications of histones are widespread and diverse, and appear to be spatial-temporally regulated in a highly intricate manner. The true functional consequences of these patterns of regulation are still emerging. Correlative evidence supports the idea that these patterns are distorted in malignancy on both a genome-wide and a discrete gene loci level. These patterns of distortion also often reflect the altered expression of the enzymes that control these histone states. Similarly gene expression patterns also appear to reflect a correlation with altered histone modifications at both the candidate loci and genome-

Chromatin-modifying enzymes: The nomenclature for enzymes involved in protein methylation, demethylation, and acetylation has recently been rationalized (Allis CD et al (2007) New nomenclature for chromatin-modifying enzymes. Cell 131:633–636). In this review, we use the new nomenclature for lysine methyltransferases (KMT), lysine demethylases (KDM), and lysine acetyltransferases (KAT). Histone deacetylases (HDACs) have retained their original nomenclature. To maintain a link between the new nomenclature and the literature, we use both the new designation and the original published designation(s), e.g., KDM5A/JARID1A/RBP2.

Histone modifications: We use the Brno nomenclature for histone modifications (Turner BM (2005) Reading signals on the nucleosome with a new nomenclature for modified histones. Nat Struct Mol Biol 12:110–112). For example, histone H3 tri-methylated at lysine 4 is shown as H3K4me3.

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wide level. Clarity is emerging in resolving these relationships between histone modification status and gene expression patterns. For example, altered transcription factor interactions with the key co-activator and co-repressors, which in turn marshal many of the histone-modifying enzymes, may distort regulation of histone modifications at specific gene loci. In turn these aberrant transcriptional processes can trigger other altered epigenetic events such as DNA methylation and underline the aberrant and specific gene expression patterns in cancer. Considered in this manner, altered expression and recruitment of histone-modifying enzymes may underline the distortion to transcriptional responsiveness observed in malignancy. Insight from understanding these processes addresses the challenge of targeted epigenetic therapies in cancer.

Abbreviations

AR	Androgen receptor
ChIP	Chromatin immunoprecipitation
CoA	Co-activator complex
E_2	Estradiol
ERα	Estrogen receptor alpha
ES	Embryonic stem cell
HDAC	Histone deacetylase
JMJD	Jumonji domain containing protein
JARID	Jumonji AT-rich interactive domain
KAT	Lysine acetyltransferase
KDM	Lysine demethylase
KMT	Lysine methyltransferase
LSD1	Lysine-specific demethylase 1
NCOR	Nuclear co-repressor
NR	Nuclear receptor
PSA	Prostate-specific antigen
SET	Su(var), enhancer of zeste and trithorax
TF	Transcription factor
TSA	Trichostatin A
TSS	Transcription start site

4.1 Altered Histone Modifications in Cancer

4.1.1 The Nucleosome and Its Modified Forms

Of the various protein–DNA interactions that are central to genome function, those between the histones and DNA are among the most intimate. A histone–DNA complex, the nucleosome, is the basic unit of chromatin structure in nearly

all eukaryotes, It comprises 146 bp of DNA wrapped in 1¾ superhelical turns around a core of eight histones, two each of H2A, H2B, H3, and H4. The structure of the nucleosome core particle has been defined in great detail by X-ray crystallography [3].

Despite its extreme conservation through evolution and its consistent crystal structure, the nucleosome in vivo is subject to a variety of enzyme-driven modifications that, potentially at least, alter its structure. Chromatin-modifying enzymes directly manipulate nucleosome structure or change nucleosome position along the DNA fiber [4]. DNA translocating enzymes such as polymerases, which pull and twist the DNA fiber as part of their normal activities, distort nucleosomes in their paths. Chromatin must deform reversibly in order to accommodate torsional and tensional stress generated by these enzymes ([5] and references therein). Nucleosome remodeling can dissociate the histone core, providing opportunities to enzymatically modify internal histone regions (see below), or to incorporate histone variants. All core histones, apart from H4, have nonallelic variant forms that differ in amino acid sequence and are associated with specific cellular and genomic functions [6].

4.1.1.1 Post-translational Modification of Histones

The most widespread and complex source of nucleosome variability is the enzyme catalyzed, post-translational modification of selected histone amino acids. All four core histones are subject to such changes, which include acetylation of lysines, methylation of lysines and arginines, phosphorylation of serines and threonines, and attachment of the small peptides ubiquitin and SUMO [7]. Advances in mass spectrometry and proteomics [8] have led to the identification of previously unsuspected chemical changes, including *O*-glycosylation of serines and threonines [9], formylation and crotonylation of lysines, and hydroxylation of serines [10]. They have also revealed that modifications occur both along the N-terminal tail domains, unstructured regions that are exposed on the nucleosome surface, and on residues in the globular internal regions that mediate histone–histone and histone–DNA interactions [11]. Histone modifications are put in place and removed by families (often large) of modifying and de-modifying enzymes and are consistently dynamic. The level of any particular modification reflects a steady-state balance between the actions of these two sets of enzymes.

The internal histone regions mediate the interactions that give the nucleosome its characteristic structure and their modification can, potentially, exert a direct structural effect. Yeast mutants with internal substitutions (some mimicking modifications) commonly cause functional changes, particularly altered gene silencing and increased sensitivity to DNA damaging agents [12, 13]. Acetylation of H3K56, on the lateral face of the nucleosome, is incorporated into chromatin at sites of DNA damage and repair [14, 15] and at replication forks [16]. These are all situations in which the nucleosome is partially dissociated, and during which internal residues will be accessible to modifying enzymes. Structural changes brought about by

H3K56 acetylation increase nucleosome mobility, thereby increasing DNA accessibility and, in the appropriate context, facilitating transcription [13, 17, 18].

Each core histone has an unstructured N-terminal tail domain that protrudes outside the nucleosomal DNA. These regions are not necessary for in vitro nucleosome assembly and crystallization [19] but contain many amino acids that are susceptible to post-translational modification [7]. How do these tail regions contribute to chromatin structure and function? Studies on the in vitro thermal mobility of nucleosomes [20] and earlier genetic and biochemical studies in yeast [21, 22] show that tails play a role in nucleosome mobility and higher order chromatin structure, but these roles are only revealed by removal of all, or most, of the tail, raising the question of how post-translational modifications could directly influence their function. Hyperacetylation of the tails of H2B, H3, and H4, each of which have 4–5 acetylatable lysines, will cause a significant loss of net positive charge and might influence higher order chromatin structures, even though the nucleosome itself is unaffected. An attempt to distinguish between the effects of lysine-specific and global acetylation of the H4 tail domain in yeast gave mixed results. For H4 lysines 5, 8, and 12, the level of acetylation (i.e., the number of lysines acetylated) seemed to be a more important determinant of transcription than the individual lysine involved, but H4 lysine 16 exerted independent effects [23]. Of course, methylation of lysines and arginines causes no change in net charge.

4.1.1.2 Chemical Signals on the Nucleosome Surface

An alternative explanation for the functional effects of histone tail modifications is that they act *indirectly* by generating, on the nucleosome surface, a variety of chemical signals that provide binding sites for nonhistone proteins. These binding proteins, in turn, regulate chromatin structure and function. This hypothesis was proposed 20 years ago [24, 25] and has since been extensively validated, not least by the identification of families of proteins carrying binding domains that recognize specific histone modifications [26, 27]. Bromodomains bind specifically to acetylated lysines, while chromodomains and several others bind to methylated lysines at selected positions on specific histones. Binding domains sometimes distinguish between lysines carrying one, two, or three methyl groups [26, 27].

A good example of how binding domains work is provided by the heterochromatin protein HP1, which is essential for heterochromatin formation in Drosophila and mammals. HP1 binds specifically, via its chromodomain, to H3 methylated at lysine 9 (H3K9me). H3K9me is located on heterochromatin in vivo and heterochromatin cannot form if the required methyl transferase is knocked out in mice [28]. Further, detailed studies of binding of HP1 to nucleosome arrays carrying methylated H3K9 provide likely mechanisms for both chromatin condensation and for the ability of heterochromatin to spread in vivo [29]. Other histone modifications have been associated with specific chromatin states. H4K36ac seems to be involved in the elongation phase of ongoing transcription [30], H4K20me3 is a marker for centric (constitutive) heterochromatin [31], and H3K27me3 is associated with long-term

gene silencing [32]. However, it is important to avoid oversimplifying a complex situation. Binding to any given modified residue will inevitably be influenced by modification at adjacent residues and functional outcomes are usually determined by the combinatorial action of different modifications. For example, phosphorylation of H3 serine 10 can displace HP1 bound to H3 methylated at lysine 9 [33]. Epigenomics approaches are beginning to reveal combinations of modifications that are consistently associated with functionally defined genomic regions, particularly promoters and enhancers [34–36].

The nucleosome can be seen as a gatekeeper that controls the access of transcription factors and other DNA binding proteins to DNA. Access is regulated by a variety of processes that change nucleosome structure, either directly (chromatin-remodeling enzymes, modification of internal amino acids) or indirectly (histone tail modifications). The enzyme families that carry out these processes are all susceptible to disruption, either through genetic mutation or environmental agents, triggering alterations in genome function that can sometimes precipitate changes in cell behavior and disease. Unraveling these complex chromatin-modifying enzyme systems will bring enormous benefits in the form of improved understanding of the etiology of diseases such as cancer and opening up new routes to therapy.

4.1.2 Histone Modification Status Is Regulated by Antagonistic Enzymes

Each histone modification is governed by antagonistic groups of enzymes that are able either to add or remove the modification in question. For example, histone acetyltransferases (KATs) catalyze the transfer of an acetyl group from acetyl-CoA to the ε-amino group of targeted lysine residues, and in this manner can neutralize the positive charge of the lysines. As a result the electrostatic interactions between histone and DNA are reduced. It is often suggested that this electrostatic effect can result in an open chromatin conformation that is more conducive to transcription [37, 38]. However, the role of the histone tails in maintaining higher order chromatin structure is not clear and while charge-mediated changes may be important in some contexts, they cannot provide a complete explanation for the functional affects of histone modifications. The actions of KATs are countered by HDACs. Broadly, acetylation is associated with gene activation and deacetylation with gene repression. However, for other modifications there is often not such a strict relationship between modification and function. For example, histone methyltransferases (KMTs) can either promote or inhibit transcription depending on the specific residue that is targeted and its genomic location relative to a gene's transcription start site (TSS). The functional identification of enzymes involved in setting and removing histone modifications has revealed an increasingly numerous battery of proteins and complexes. Many of these enzymes are either cofactors or binding partners for transcription factors (TF). Alternatively transcription regulatory factors can contain intrinsic histone-modifying capacity.

It is also apparent that at least some histone modifications can be regulated on a larger chromosomal scale or even globally, whereas other modifications have a much more restricted pattern. For example, H3 methylated at K79 (H3K79me3) is widely distributed across euchromatic regions in yeast and protects against the spreading of telomeric heterochromatin [39, 40] while H3K27me3, a mark put in place by the polycomb repressive complex, is spread across groups of genes (e.g., the HOX clusters) to bring about their coordinated silencing [41, 42]. Alternatively, marks such as H3K4me3 are closely associated with local genomic features, particularly promoters, enhancers, and TSSs [43, 44].

The KAT superfamily includes at least 20 different and diverse proteins including CLOCK and NCOA1. Several subfamilies exist including the P300/CBP family, e.g., p300; GCN5 family, e.g., KAT2A; the MYST family, e.g., MYST1; SRC/p160 nuclear receptor co-activator family, e.g., NCOA1. Eighteen HDAC are known in humans that are classified into four classes based on homology that include the HDAC1-11 and 7 SIRT members. Twenty-eight different KMT are known to act on histones, at least in vitro [1]. KMT are abundant and diverse reflecting the importance of the methylated state of key residues for the control of evolutionarily conserved transcriptional programs, for example, associated with development. There are at least 30 KMTs, including key families such as EZ, SUV39, and SET. At least 20 demethylases (KDM) are divided into two major groups that include the LSD family members, e.g., KDM1A/LSD1 and the Jumonji family, e.g., JHDM3 and JARID proteins containing ARID domains.

Two points are particularly important in considering the extent of redistribution and altered patterns of histone modifications in cancer. The first is that the steady-state level of each modification represents a dynamic balance between the effects of the modifying and de-modifying enzymes, with turnover likely to vary from one part of the genome to another, between cell types, and is intimately associated with cell cycle status, cell–cell interactions, and cell lineage commitment. Secondly, many, if not all, of the enzymes are either dependent upon, or influenced by, metabolites and components present in the intra- or extracellular environment. At the simplest level, many of these enzymes depend on cofactors such as acetyl CoA, NAD, and *S*-adenosyl methionine for their activity, and in turn these levels will depend on the metabolic and redox state of the cell. More subtle effects can be derived from metabolism. For example, naturally occurring inhibitors, such as short chain fatty acids (inhibitors of Class I HDACs) and nicotinamide (an inhibitor of the NAD-dependent deacetylase SIRT1) can be derived intrinsically within a cell or tissue and may naturally influence epigenetic status, for example, in the cell lining the lumen of the gut [45–47]. The effects of metabolic changes on gene expression are a strongly re-emergent area in cancer biology [48–50] and the generation of linked transcriptomic and metabolomics data is revealing the key functional associations in malignancy [51–53]. Thus the nucleosome, through the array of histone modifications it carries and the enzymes that put them in place, is a finely tuned sensor of the metabolic state of the cell and the composition of its environment. In this manner, nucleosome structure provides a platform through which external environmental and internal variables can influence genomic function.

4.2 Disruption of Histone Modifications in Cancer

Given that dynamic histone modifications are required for the precise control of DNA structure, during DNA repair and transcription, it is not surprising that there is significant evidence for the disruption of these events in malignancy. Understanding the differential recruitment and activity of proteins that govern histone modifications is key to understanding the roles that altered histone modifications can play in cancer initiation and development. Currently, a key focus in cancer biology is dissecting the mechanisms that alter the local and global recruitment and activity of histone-modifying complexes. It is anticipated that the insight generated will address the central challenge of separating which epigenetic processes directly drive cancer initiation and progression, from those that are merely a consequence of altered genomic structure such as mutation, copy number variation, and cytogenetic rearrangement. Insight into the contribution of altered histone functions to cancer progression can be gleamed by considering global and gene-loci specific alterations to histone modifications.

4.2.1 Global Distortions to Histone Modifications

A number of histone modifications are intimately associated with higher order chromatin structures and chromatin packaging and therefore changing the distribution of these global marks can have profound impact on the structure of chromatin in the nucleus. In turn such altered structures may be either more prone to aberrant DNA repair or promote genomic instability [54]. In prostate cancer, for example, quantifying global levels of five selected histone modifications in tissue sections by immunocytochemistry allowed discrimination between groups of patients with distinct risks of tumor recurrence [55, 56]. Quantitative analysis of just two modifications (H3K18ac and H3K4me2) was shown to provide useful prognostic information. The mechanisms underpinning these intriguing observations remain unknown.

The Polycomb complex (PcC) is a highly conserved inducer of repressive chromatin and sustains the H3K27me3 mark. This repression was shown to extend to multiple target genes associated with differentiation, often during development. Consequently, an emergent area in malignancy is the focus on aberrant PcC function to repress differentiation programs inappropriately. Increased H3K27me3 has been shown to have prognostic value in prostate and other cancers. These findings, however, reported the prognostic value to arise from the opposite patterns. Thus, increased levels of H3K27me3 are correlated with poor prognosis in esophageal cancer [57, 58], whereas in prostate cancer low levels have the poorer prognosis [59, 60].

The enzymes that control H3K27 methylation status are members of the enhancer of zeste homolog (EZH) that is the catalytic subunit of the polycomb repressive complex 2 [61]. These proteins are overexpressed in many cancers and in certain cases appear to correlate with poor prognosis or more aggressive disease.

However, although there are some correlations with increased H3K27me3 status, these are not universal in terms of the level of the mark. This may instead reflect the dynamics of turnover of the mark, and therefore the H3K27me3 status needs to be correlated with the enzymes that both add and subtract his mark.

Other modifications do appear to be altered in their global distribution in malignancy. For example, loss of H4K16 acetylation and H4K20me3 appears to be diminished globally in cancer cells, and indeed were some of the first histone marks to be characterized as being altered in malignancy [62, 63]. The consequence of these alterations probably reflect the role that certain modifications have in cross-talking with the mechanism of DNA methylation and indeed reduced levels of these marks were associated with DNA hypomethylation. Down-regulation of MYST1/MOF, one of the KAT that targets H4K16, may in part explain these altered patterns [64]. Reenforcing the concept of antagonistic enzymes, H4K16 is deacetylated by SIRT1 which is also up-regulated in several cancers and may have prognostic significance of its own [65]. Furthermore, the MYST family of KATs is associated with global changes in histone marks associated with chromatin packaging, DNA repair, and the control of developmental transcriptional programs (reviewed in [66]).

The control of lysine methylation states, however, is frequently more complex than acetylation states, and there are multiple enzymes controlling this modification. A major contributor to this complexity is the fact that the lysine epsilon amino group can accommodate one, two, or three methyl groups. All three methylation states are found in vivo and are often associated with distinct functional outcomes. Lysine methylation often proceeds in two steps, with mono and di-methylation governed by one class of enzyme and subsequent tri-methylation being regulated by a subsequent enzyme. For example, SET7 is able to catalyze the generation of H3K20me2, which then forms a substrate for the SUV class enzymes that generates the fully methylated state H3K20me3. Reflecting this, there is some evidence that levels of SUV family members are reduced in cancer in association with gene silencing [67, 68].

Further examples of a global alteration of histone status linked with cancer progression are those modifications that drive nucleosome movement. One of the key modifications in this regard is the internal lysine H3K56 that is targeted for acetylation by the KATs, CBP/p300 and GCN5, and has recently been shown to facilitate nucleosome disassembly and transcriptional activation. Inhibitor studies and expression profiling both suggest that the altered levels of H3K56ac distort the DNA damage response and maybe a trigger for genomic instability. Parallel studies have also revealed that H3K56ac is also involved in modulation of chromatin structure during DNA replication and repair; consequently, disruption to this process can also lead to genomic instability [18, 69–71]. Perhaps reflecting the importance of the regulation of this mark, multiple HDACs have been implicated in its control and include the NAD-dependent SIRTs.

Global changes in histone modifications have also been linked to stem cell differentiation. Undifferentiated embryonic stem (ES) cells show global enrichment in histone modifications associated with transcriptional activity and depletion in modifications associated with silent chromatin [72, 73]. By several criteria, ES cell

nuclear DNA is packaged in an unusual form of chromatin that appears to be more "open" than that in differentiated cells and is transcriptionally hyperactive [74]. How elevated histone modification levels are generated, and whether they are a cause or a consequence of open, hyperactive chromatin, remains to be determined.

Knocking down, individually, the histone demethylases KDM2A/JMJD1A and KDM4C/JMJD2C in mouse ES cells, globally increased the level of histone modifications usually associated with silent chromatin, namely, H3K9me2. In addition to their global effects, KDM2A/JMJD1A and KDM4C/JMJD2C were also shown to target, and regulate, specific genes, including *Tcl1*, a potential regulator of self-renewal, and *Nanog*, a key determinant of pluripotency [75]. Thus, key chromatin-modifying enzymes can exert both global and gene-specific effects that in turn influence differentiation. Intriguingly, both demethylase genes were themselves positively regulated by the key transcription factor Oct4, showing how a transcription factor might trigger a feed-forward signal to bring about a genome-wide change in the epigenetic landscape through regulation of genes encoding histone-modifying enzymes. In adult stem cell compartments, regulation of specific histone demethylating enzymes has also emerged as critical in activating differentiation programs, for example, the control of neural stem cell differentiation by the retinoic acid receptor, a member of the nuclear receptor (NR) superfamily [76]. A similar relationship between a transcription factor, global histone modifications, and adult stem cell differentiation is seen in studies of epidermal stem cells [77]. Quiescent stem cells are induced to leave their niche in the interfollicular epidermis and hair follicle bulge by activation of MYC, a process accompanied by globally increased H4 acetylation and di-methylation of H3K9 and H4K20. Together these studies illustrate how key transcription factors combine with environmental factors to influence and regulate the stem cell niche and control differentiation outputs.

Finally, the enzymes that govern histone methylation are also distorted in cancer with both loss and gain of function. Expression patterns of histone-modifying enzymes are even able to discriminate between tumor samples and their normal counterparts and cluster the tumor samples according to cell type [78]. This indicates that changes in the expression of histone-modifying enzymes have important and tumor-specific roles in cancer development. Thus, overexpression of G9a, an H3K9 KMT, occurs in lung and breast cancers and associates with aggressiveness [79]. Similarly enzymes that de-acetylate H3K9, and allow it to be methylated, are also overexpressed in cancers, including breast cancer. These enzymes may also be playing separate roles, and therefore expression is selected in malignancy on a different basis, for example, in gene regulation and DNA repair. It is possible that increases in HDAC levels are a homeostatic response in which the cell attempts to compensate for the aberrant increase in KAT activity (or vice versa). What is important from a functional point of view is not the absolute levels of KATs or HDACs, but the new steady-state levels of the (histone) modifications they regulate.

More precise specificity is dependent on the combination of both the enzyme *and* target gene(s). For example, mutation of KDM6A/UTX results in the inability to relieve H3K37me3 repression [80, 81]. Gain of function also occurs, for example, increased targeting of methyltransferases KMT1A/SUV39H1 to *CDKN1A* leads to

sustained H3K9me2 and transcriptional silencing that in turn can be targeted with the enzyme inhibitor, chaetocin [82, 83]. Similarly, the KMTs/MLLs are overexpressed in prostate cancer [84–86] and sustain levels of H3K27me3 at key targets such as DAP2IB, an RAS regulatory molecule, thereby leading to metastasis [87]. These observations illustrate deregulation of the enzymes that control histone lysine methylation is common but most likely highly targeted. This contextual nature is typified by KDM1A/LSD1 [88], which can target the demethylation of either H3K9me2 or H3K4me3 and thereby drive both gene activation [89, 90] and repression [91]. In this manner, KDM1A/LSD1 may mediate parallel repression and activation of target genes and play a key role in the malignant evolution of AR signaling in prostate cancer.

4.2.2 Altered Histone Modification Patterns at Discrete Gene Loci

Histone modifications therefore appear to operate at a level of restricted action, at discrete loci, exemplified by lysine methylation. Functional outcomes depend not only on which lysine on which histone is methylated, but also on whether the lysine carries one, two, or three methyl groups and its genomic position on a given loci with respect to the TSS. The different degrees of methylation are put in place, and removed, by a diverse group of enzymes. In particular, KDMs seem to have a particularly close association with key transcription factors that in turn are also implicated in malignancy such as MYC and members of the NR superfamily. Ligand binding or cofactor associations are able to influence the activity or even the specificity of these enzymes and thereby regulate functional outcomes (usually a change in gene expression) [92].

The modification of H3K9Ac and H3K9me2 serves to illustrate key concepts concerning histone status and specific gene expression. These marks are mutually exclusive and reciprocal, being associated with gene activation and repression, respectively. Loss of H3K9me2 is often associated with elevated gene expression. Recent studies have underscored the targeted changes in lysine methylation status and specifically illustrated that the KDM that targets H3K9me2 and the KMT that targets H3K4me at the gene TSS (to activate gene expression) are within the same complex associated with the ERα and therefore facilitate this two-step gene activation process [93]. Naturally, given that gene expression in cancer is uniformly neither up or down-regulated, the global expression of these marks is also not uniformly altered. Rather patterns are nuanced and suggest specific loci are deregulated.

Another example of this specificity emerges from considering KDM1A/LSD1 that can demethylate H3 mono- and di-methylated at either K4 or K9, and, remarkably, this specificity can be regulated in vitro by the protein cofactors, CoREST or BHC80, with which it is associated [94, 95]. Thus, KDM1A/LSD1 acts as an H3K4 demethylase (i.e., can remove a potentially activating modification) on NRSF targets and an H3K9 demethylase (i.e., can remove a potentially repressive modification)

on AR and ERα target promoters. Catalytic activity/specificity can also be regulated by adjacent histone modifications. H3K9 acetylation inhibits H3K4 demethylation (on the same tail) in vitro [96, 97]. Local patterns of modification are set by the combined actions of methylating and demethylating enzymes and the methylases too are influenced by other histone modifications. Further details of the gene-specific interactions have also emerged. JMJD2C demethylates H3K9me3, while KDM1A/LSD1 demethylates H3K9me2/me1 at promoters such as *PSA* and *KLK2* to remove H3K9 methylation associated with transcriptional silencing.

Therefore, the specific complex that KDM1A/LSD1 interacts with profoundly alters the transcriptional outputs, for example, of the AR, since demethylation of H3K9 has a gene activating effect, while demethylation of H3K4 has a gene silencing effect. The balance of these actions is in part controlled by the regulation of phosphorylation of H3 at threonine 6 (H3T6) by protein kinase C beta I. This prevents KDM1A/LSD1 from targeting H3K4me2 during AR-dependent gene activation and prevents it from limiting transcriptional activation. Also reflecting shared functions PKCbeta(I) co-localizes with AR and KDM1A/LSD1 on target gene promoters and phosphorylates H3T6 after androgen-induced gene expression. Therefore, it appears that androgen-dependent phosphorylation leads to the new chromatin mark H3T6ph, which in turn prevents removal of active methyl marks from H3K4 and forms a positive feed-forward loop of gene regulation [91]. More recently, KDM1A/LSD1 has been shown to drive AR-stimulated gene transrepression of the AR itself and thereby form a negative feedback loop of gene regulation [98]. Thus, the complex within which this one regulatory enzyme associates, its targeting to different genes, and the position of the response element, relative to the TSS, can all combine to determine how different H3K methylation states are governed.

4.2.3 Interplay Between Altered Transcriptional Signals and Epigenetic States

In normal cells a highly choreographed balance of histone modifications occurs during the dynamic regulation of coding and noncoding genes. These patterns are generated by the highly integrated actions of transcriptional networks [99] and are evident in many aspects of biology. For example, in development; in homeostasis to control the circadian rhythm [100], tissue self-renewal, and the response to hypoxia [83, 101]; in immune function to regulate inflammation [102]. Many of these processes are disrupted in malignancy and generally in cancer cells there is a loss of dynamic transcriptional patterns and signaling complexity is reduced [103]. Consequently, an area where altered histone modifications appear to associate with the cancer phenotype is in distortion of transcriptional control of key cellular processes.

Epigenetic events play a central role for transcriptional complexes and the various components in these multimeric complexes sequentially initiate, sustain, and

finally terminate transcription [104]. In this manner, transcription can work as a type of biological ratchet, with histone modifications being associated with the various states by generating chromatin states that are either receptive or resistant to transcription (reviewed in [27]). For example, different histone modifications can control the rate and magnitude of transcription (reviewed in [105]). These events are intertwined with low-level CpG methylation [106–108]. Thus, the histone modifications and other epigenetic events including DNA methylation processes combine during transcription to generate highly flexible chromatin states that are either transcriptionally receptive and resistant [101]. That is, the specific transcriptional potential of a gene is flexibly controlled by the combination of epigenetic events. These events are varied in space across the gene loci, and in time through the course of the transcriptional cycle. Current challenges in the field of cancer epigenetics, therefore, are to reveal how altered histone modifications directly drive distorted transcriptional programs, and what patterns exist on a genome-wide scale to distort networks of transcription. This will help to define how these altered histone states are genuine drivers in cancer progression.

Precisely how transcriptional programs evolve during malignancy is emerging. Genome-wide approaches are now allowing workers to ascribe broader views of the biology of transcription factor families, now that all members are known, and questions can be addressed in more detailed biological contexts. These findings suggest that the actions of the many key transcription factors are distilled through interactions with multiple cellular processes thereby generating an extremely flexible and integrated signaling module. In malignancy, however, these transcriptional choices and phenotypic outputs become restricted, for example, as seen with the emergence of a novel AR-transcriptome in androgen deprivation therapy-resistant prostate cancer [109].

Importantly, these epigenetic regulatory mechanisms operate in response to signals from the cellular microenvironment of the tumor, including signals from associated stromal (noncancerous) cells [110, 111]. The “niche” in which cells find themselves is an important determinant of their epigenetic properties [112] and raises the possibility that histone marks can be modified by environmental conditions that alter metabolic and redox status, leading to a heritable alteration in cell phenotype, an “epigenetic mutation.” Such lesions are not restricted to single nucleotides, but rather can be targeted to larger regions and therefore comparable to genetic deletions and amplifications. They can act alongside conventional genetic and cytogenetic alterations, either inherited or de novo, to cause the bi-allelic silencing of tumor suppressor genes that can be the first step in development of a cancer [113]. These concepts are illustrated by considering key transcription factor families implicated strongly in cancer initiation and progression.

4.2.3.1 The MYC/MAX/MAD Family

The MYC/MAX/MAD family forms heterodimeric complexes with MAX as the central partner to activate the expression of a diverse range of genes. Deregulated

and elevated expression of c-MYC has been documented in a wide range of human malignancies, associated frequently with aggressive and poorly differentiated tumors [114]. MYC has the potential to target a large proportion (11%) of all genes in the human genome [115], but the set of genes to which it actually binds in any particular cell is regulated by a variety of factors, including interacting proteins. For example, the MAD family of transcritpional repressors is , like MYC, able to bind MAX proteins and antagonize the activity of MYC by competing for MAX binding at E-box sequences in target gene promoters, actively repressing transcription of MYC target genes [116].

The specificity and affinity of MYC binding is influenced by the configuration of the chromatin packaging at potential binding sites, and particularly by patterns of histone modification [117]. MYC was found to bind E-boxes in regions enriched for several histone modifications generally associated with euchromatin, such as acetylated H3 (specifically H3K9ac, H3K14ac, and H3K18ac), but showed the strongest association with H3K4me3. Reciprocally, MYC was inversely correlated with the repressive polycomb group mark H3K27me3. On some promoters, MYC associated with both H3K4me3 and H3K27me3, a bivalent state that is common in ES cells but seems rarer in lineage committed cells [118]. Overall, it seems more likely that H3K4me3 recruits MYC rather than H3K27me3 excluding MYC binding. [117].

MYC function can be controlled interactions with JARID1A/RBP2 and JARID1B/PLU-1 [119, 120]. These enzymes are both specific for H3 methylated at lysine 4 (H3K4me1,2,3) and may help to regulate this modification at MYC binding sites. There is emerging evidence that this process is disrupted by increased association with histone demethylase NO66/MAPJD to alter the potential interactions with genes involved in proliferation of lung cancer cells [121]. A gene encoding a related protein, MINA53 (myc-induced nuclear antigen) is a MYC target that is overexpressed in lung cancer, for example [122, 123]. Together these findings suggest that the co-association of MYC with different histone-modifying enzymes, for example, through the consequence of altered enzyme expression, distorts and restricts the MYC transcriptome in malignancy.

In the light of these developments, MYC function has been reassessed to reveal the regulation of unexpected gene targets, some of which inhibit proliferation and induce programmed cell death [124], contrary to the accepted view of MYC as an oncogene promoting growth and survival. These findings suggest that the malignant function of MYC represents selection for a subset of its potential actions.

4.2.3.2 The NR Superfamily

The NR superfamily also illustrates the key concepts of distorted and selected transcription in cancer due to altered regulation of histone modifications. NRs are the largest superfamily of transcription factors in humans and generally form active heterodimers to control networks that regulate homeostasis, energy metabolism, and xenobiotic handling. These receptors are intimately associated with the control of self-renewal in a number of epithelial systems, notably the prostate and mammary

glands. For example, studies in the prostate have established that the androgen receptor (AR) cooperates with WNT and mTOR pathways [125, 126] to induce proliferation. Equally other receptors, such VDR, PPARs, and RARs, exert mitotic restraint, at least in part by antagonizing WNT signaling and activation of cell cycle arrest through regulation of gene targets such as *CDKN1A* (encodes $p21^{(waf1/cip1)}$) and *IGFBP3* [127–134].

Cancer is typified by the actions of individual receptors becoming selective and the NR network collectively displaying a loss of transcriptional plasticity. The AR transcriptional program evolves towards increased targeting of proliferative gene promoters and decreased targeting of pro-differentiation genes [135, 136]. Similarly, within breast cancer the transcriptional actions of the ERα appear to become increasingly selective for gene targets associated with proliferation and survival and away from targets associated with differentiation [137–139]. Equally in a range of solid tumors and myeloid leukemia, NRs that normally exert mitotic restraint, such as the VDR, RARs, and PPARs, become skewed, with selective silencing of antiproliferative target genes [129, 140–144]. Combined, oncogenic transcriptional rigidity reflects the simultaneous distorted regulation of target loci such that proliferative and survival signals are enhanced and antimitotic inputs are either limited or lost. This filtering of transcriptional choices during cancer progression has significant therapeutic implications. For example, the oncogenic actions of the TMPRSS2/ETS fusion, a common event in prostate cancer [145], are critical precisely because the *TMPRSS2* promoter is sustained in an AR-responsive state.

More recently, genome-wide ChIP approaches have revealed considerable variability in the networks of interactions capable of bringing about varied transcriptional responses [146–148]. For example, in prostate cancer, as the disease progresses, there are altered levels of H3K4me1 and 2 on gene enhancer regions in the so-called AR-independent state, where cells have evolved resistance to antiandrogen therapies. In this new state, the targeted increase of H3K4Me1 and 2 at different enhancer regions allows the cells to initiate a different AR transcriptional program [109].

4.2.3.3 Hypoxia-Inducible Factor-1 Alpha

The hypoxia response of hypoxia-inducible factor-1 alpha (HIF-1A) also illustrates how transcriptional actions are selectively distorted by epigenetic processes in cancer cells. Within a normal cell, the levels of oxygen are monitored sensitively by a transcriptional circuit that governs the function of HIF-1A. In normoxic conditions, HIF levels are kept low level by destruction by an E3 ubiquitin ligase containing the VHL tumor suppressor protein, where oxygen serves as a co-substrate. Also oxygen impedes the interactions of HIF1α with the KATs CBP/p300 thus limiting the capacity to initiate activating histone marks. In hypoxia, HIF-1α becomes stabilized and active, and promotes a stable interaction with CBP/p300 and therefore facilitates transcription [83]. Genome-wide analyses of HIF binding sites identified a number of KDMs as downstream targets, notably

JMJD1A and JMJD2B, thus providing the capacity to affect the epigenetic status of the cell. In part, this may contribute to maintenance of transcriptional activity under stress. It may also support the observed aberrant and selective HIF1α transcriptional responses [149].

Taken together these findings support the concept that the actions of major transcription factor families are selective at several levels to govern the expression of sub-transcriptomes that are phenotypically related. The flexibility of transcriptional actions includes the exact choice of target sequence, the timing, amplitude, and magnitude of transcription and integration with other transcriptional programs and signal transduction events. In malignancy, the dexterity of targeting and regulation is blunted and instead transcription factors become addicted to specific sub-transcriptomes, for example, those associated with blockade of programmed cell death and progression through the cell cycle.

4.2.4 Loss- and Gain-of-Function of Transcriptional Co-activators and Co-repressors

One means by which transcriptional actions are distorted is through the altered expression of associated cofactors that either have an intrinsic or associated capacity to regulate histone modifications. The diversity of co-activator and co-repressors is extreme and they have been the subject of numerous reviews [150–154]. Several examples are strongly illustrative of underlying mechanisms of transcriptional regulation. In essence, the altered expression and function of these key proteins alters the equilibrium of key histone modifications and thereby allowing the gene regulatory actions of a given transcription factor to become more or less pronounced.

Co-activators and co-repressors each display both loss and gain of function, and can result in similar phenotypes. Thus, the loss of a co-activator can lead to suppressed ability of a transcription factor to transactivate a given target. Similarly, the gain of function of co-repressors can limit transactivation ability and enhance transrepression. The opposite patterns will in turn enhance the transactivation function.

For example, NCOA3/SRC3 is situated within a common area of chromosomal amplification in breast cancer on chromosome 20q. Initially, cDNAs were isolated from this region that contained a putative target gene that was termed AIB1 (for "amplified in breast cancer-1"). Subsequently, this gene was found to be a member of the SRC co-activator family and was amplified and overexpressed in breast and ovarian cancer cell lines, as well as in breast cancer biopsies [155]. NCOA3/SRC3/AIB1 interacts with ERs in a ligand-dependent fashion and enhances the regulation of target genes. Specifically the protein has intrinsic KAT activity and also acts to recruit other CBP/p300 in an allosteric manner [156]. Therefore, increased expression increases the ability of the ERα to transactivate a given gene target. Subsequently, this protein was identified NCOA3 and shown to be a potent histone acetyl transferase able to enhance the function of multiple NRs [157–159].

Compared to their co-activator cousins, the co-repressors are somewhat under-explored. Again, these key proteins, originally identified for their repressive interactions with NR illustrate how deregulated functions can alter chromatin and thereby attenuate gene regulation. NCOR1 and NCOR2/SMRT were cloned in 1995 using NR as bait [160, 161], and both proteins exist in large multimeric complexes (~2.0 MDa) [162] with histone deactylases and other histone-modifying enzymes (reviewed in [153]). These complexes are recruited to many different transcription factors to repress gene activity during the transcriptional cycle. These transcription factors include: NR, MAD/MXI, MYOD, ETO, CBF, FOXP, AP-1, and NF-κB factors. The importance of targeted *basal repression* by co-repressors is evident in the lethality of the *Ncor1*$^{-/-}$ and *Ncor2/Smrt*$^{-/-}$ mice. These models reveal enhanced function of transcription factors, notably Pparγ in adipocytes [163] and FoxP in cardiomyocytes [164]. Dynamic mechanisms have also emerged whereby NCOR1 and NCOR2/SMRT complexes can be recruited to activate transcription factors leading to transrepression [165, 166]. Finally, an emerging theme is the pattern of active de-repression where loss of co-repressor association, following activated transcription factor, leads to up-regulation of target genes independently of the sustained presence of the transcription factor [167].

Well-established oncogenic roles for NCOR1 and NCOR2/SMRT have been elucidated in acute promyelocytic leukemia (PML) that results from a fusion between the NR, RARα, and either the PML or promyelocytic leukemia zinc finger (PLZF) genes [142]. Both chimeric proteins sustain NCOR1 interactions and consequently RARα-mediated cell differentiation is blocked, in part, as a result of maintaining a condensed chromatin structure around the promoters of RARα target genes that govern normal hematopoietic differentiation [168, 169]. In the PML-RAR fusion, this can be overcome by pharmacological dosing with retinoic acid. The PLZF-RAR fusion is resistant to retinoic acid alone and treatment with a combination of retinoic acid and HDAC inhibitors has shown promising results. Similarly, in acute myeloid leukemia (AML), the AML1/ETO fusion protein promotes leukemogenesis by recruiting NCOR1 and again impeding transcriptional regulation [170]. The importance of NCOR1 binding in the treatment of these disease states exemplifies the relevance of the co-repressors in firstly driving critical oncogenic events, but secondly providing a rational targeted strategy towards HDACs.

Expression profiling in solid tumors has revealed altered NCOR1 and NCOR2/SMRT expression and localization, for example, in breast, bladder, and prostate cancers [129, 141, 143, 171–173]. However, to date, uncertainty remains over their precise role in solid tumors, especially in the case of breast and prostate cancers where the etiology of disease is intimately driven by the actions of steroid hormone NRs. Indeed, the ability of the ligand-free NR to bind NCOR1 and NCOR2/SMRT is important to therapeutic exploitation with receptor antagonists such as Tamoxifen in the case of breast cancer. Therefore, ambiguity exists over the extent and timing of NCOR1 and NCOR2/SMRT expression changes, as they relate to initiation and progression of disease. Secondly, it remains unclear how changes in NCOR1 and NCOR2/SMRT expression relate to different NRs and other transcription factors that exert either pro- or antimitotic and survival effects. Resolving these ambiguities

has significant therapeutic implications in terms of targeting co-repressors as either epigenetic mono-therapies using HDAC inhibitors or in combinations with transcription factor targeting.

In prostate cancer cells, elevated levels of NCOR2/SMRT have been detected and suppress VDR responsiveness [129]. Similarly, PPAR actions are disrupted and can be targeted selectively by using HDAC inhibitor co-treatments [174, 175]. More specifically, elevated NCOR1, and to a lesser extent NCOR2/SMRT correlated with, and functionally drove, the selective insensitivity of PPARα/γ receptors towards dietary derived and therapeutic ligands [175] most clearly in androgen-independent disease. Similar roles for NCOR1 and NCOR2/SMRT appear in the development of breast cancer and Tamoxifen resistance [171]. Elevated levels of NCOR1 occur in ERα negative disease and in turn attenuate antimitotic actions of VDR. Again, this molecular lesion can be targeted in ERα negative breast cancer cell lines with co-treatments of VDR ligand (e.g., $1\alpha,25(OH)_2D_3$) plus HDAC inhibitors resulting in selective re-expression of VDR target genes, notably *VDUP1* and *GADD45A* [143]. Together, the studies in breast and prostate cancer suggest that NR show specificity in their interactions with co-repressors. NCOR1 appears to be involved in the regulation of receptors such as the VDR and PPARs and NCOR2/SMRT with steroid hormone receptors, reflecting the emergent specificities of NR interactions in the murine knockout models.

4.3 Consequences of Altered Histone Modification States

4.3.1 Higher Order Chromatin Interactions Associated with Transcription

Another theme that has emerged concerning epigenetic regulation of transcription is higher order chromosomal interactions. It seems that large-scale chromatin rearrangement, through looping, is frequent and widespread. Loops can be inter- or intra-chromosomal and are guided by transcription factors, key pioneer factors, and chromatin-modifying enzymes [176, 177]. Improved microscopy techniques have recently shown nascent RNA on the surface of protein dense transcription factories ("gene hubs") that seem to correspond to structures previously termed "nuclear speckles" [178].

A clear example of these interactions has been illustrated in the transcriptional responses of B-cells where translocation of genes occurs from separate chromosomes and nuclear regions to common sites referred to as transcription factories. These sites contain significant levels of RNA Pol II, and other proteins, including factors required for elongation, chromatin remodeling, capping, splicing, and nonsense-mediated decay. Recruitment of genes to transcription factories is highly selective, with certain genes and chromosome regions co-localizing far more frequently than expected by chance. Intriguingly, sites of chromosome translocation

associated with various cancers often co-localize. For example, *Myc* and *Igh* tend to co-localize and their fusion, in human lymphoid cells, is a common cause of Burkitt's Lymphoma. These rapid movements are associated with movements of the nuclear architecture and involve ATP-dependent mechanisms that involve a chromosome locus usually located at the nuclear periphery being rapidly translocated to the interior in a direction perpendicular to the nuclear membrane [179].

Again, the NR superfamily illustrates these aspects of the deregulation of epigenetic states. NRs appear to interact with more dominant more widely binding pioneer factors. For example, ERα interacts with pioneer factors and KDMs. This interaction is involved with micro-chromatin reorganization at response elements, and also with higher order chromatin reorganization. Active ATP-dependent transport mechanisms have recently been shown to be an essential intermediate step in gene activation by ERα and act to move discrete chromosomal regions together into interchromatin hubs. These granules are subsequently joined to the surface of nuclear structures rich in splicing and transcriptional machinery that may reflect the previously termed "nuclear speckles" [180].

This suggests a role for KDM1A/LSD1 in directing docking of the ERα-gene hub complex with the nuclear speckles, but the exact function of KDM1/LSD1 in this process remains unclear. If this role is catalytic rather than purely structural, it is possible that the substrate involved is a nonhistone protein. It will also be of interest to determine whether KDM1/LSD1 or related enzymes play a role in directing *MYC* and *IGH* alleles to transcription factories. The recent development of improved microscopy techniques which has shown nascent RNA appearing on the surface of protein dense transcription factories should aid in clarifying this situation [178], as well as further work investigating the relationship between nuclear speckles and transcription factories.

4.3.2 Directing DNA Methyltransferase Specificity and Stable Gene Silencing

There is compelling evidence that histone and DNA methylation processes disrupt transcriptional actions, both alone and together. For example, one consequence of NCOR1 and NCOR2/SMRT association at target genes is the loss of H3K9ac and accumulation of H3K9me2, allowing the potential for hypermethylation at adjacent CpG regions. Further links exist between NCOR1 and DNA methylation through its interaction with KAISO [181]. Correlative studies reveal that a number of key AR and VDR target genes are silenced by increased CpG methylation [182, 183]. At high density regions of CpG methylation, spanning hundreds of base pairs, the entire region acquires H3K9 and -K27 methylation, loses H3K4 methylation, and recruits heterochromatin binding protein 1 (HP1) [101]. The recruitment of HP1 through interaction with MBD1 leads to recruitment of both an H3K9 methylase (KMT1A/SUV39H1) [184] and DNA methyltransferases (DNMTs) [185]; enzymes that add repressive methylation marks to histones and CpG.

DNMT3L and UHFR1 also provide potential links between DNA methylation and absence of H3K4 methylation and presence of H3K9 methylation, respectively (reviewed in [186]).

Thus, these processes become self-reinforcing. It is not precisely clear, however, in mammalian cells whether either the H3K9 methylation or the high density of CpG methylation is required first to set up this heterochromatic structure. However in *Neurospora crassa*, loss of HP1 (which requires H3K9 methylation for binding to chromatin) leads to loss of DNA methylation [187]. This situation describes stable heterochromatic silencing of genomic regions and is in contrast to the dynamic changes at a locus with active epigenetic regulation of transcription in response to NR activation. However, even in such actively regulated regions, dynamic changes in DNA methylation appear to occur. For example, these have been measured in response to NR actions [106–108].

This differential regulation of histone methylation has profound implications for transcriptional control. DNA methylation and H3K4 methylation are mutually exclusive, while H3K9 methylation is strongly associated with DNA methylation, for example, through the formation of heterochromatin by HP1 binding and histone deacetylation. In the absence of DNA methylation, these inter-relationships are highly dynamic, with target gene promoters often poised to be subsequently pushed towards a fully active, or a more stably repressed state. For example, CpG island promoter regions of non-expressed genes do in fact show low-level RNA POLII association and modest transcriptional initiation. It seems that the presence of H3K4me3 methylation holds these promoters in a chromatin structure that is accessible to the transcriptional machinery, poised to recruit specific transcription factors to drive high level, efficient transcription. In turn this prevents H3K9me2 and DNA methylation. Aberrant DNA methylation of these CpG islands in cancer cells reduces this plasticity and coincides with loss of H3K4 methylation, gain of H3K9 methylation along with other heterochromatin marks, and stable transcriptional silencing [101].

The distributions of these histone modifications and DNA methylation patterns in cell line models are being organized by research consortia, for example, ENCODE [188]. Again, these genome-wide datasets also appear to support the idea that these histone marks are strongly associated with features of genomic architecture, such as gene regions, TSS, and enhancer regions where regulatory transcription factors can bind.

The links between sustained repressive histone modifications in the enhancer or promoter regions of a gene locus and altered DNA methylating events are targets for exploitation. Importantly, these epigenetic lesions are individually highly targetable with clinically available small molecular weight inhibitors targeted to specific histone deacetylation events and more recently this has been extended to include histone methylation events [189], coupled with agents that target CpG methylation (reviewed in [190]). Thus, comprehensive understanding of the key co-repressors in malignancy, delineating the key transcription factors interactions and the critical targets that are thereby dysregulated, may have considerable prognostic utility, specifically through the capacity to stratify patients for specific tailored epigenetic therapies.

References

1. Allis CD et al (2007) New nomenclature for chromatin-modifying enzymes. Cell 131: 633–636
2. Turner BM (2005) Reading signals on the nucleosome with a new nomenclature for modified histones. Nat Struct Mol Biol 12:110–112
3. Luger K, Mader AW, Richmond RK, Sargent DF, Richmond TJ (1997) Crystal structure of the nucleosome core particle at 2.8 A resolution. Nature 389:251–260
4. Flaus A, Owen-Hughes T (2011) Mechanisms for ATP-dependent chromatin remodelling: the means to the end. FEBS J 278:3579–3595
5. Recouvreux P et al (2011) Linker histones incorporation maintains chromatin fiber plasticity. Biophys J 100:2726–2735
6. Talbert PB, Henikoff S (2010) Histone variants—ancient wrap artists of the epigenome. Nat Rev Mol Cell Biol 11:264–275
7. Kouzarides T (2007) Chromatin modifications and their function. Cell 128:693–705
8. Ngara R, Ndimba R, Borch-Jensen J, Jensen ON, Ndimba B (2012) Identification and profiling of salinity stress-responsive proteins in Sorghum bicolor seedlings. J Proteomics 75: 4139–4150
9. Zhang S, Roche K, Nasheuer HP, Lowndes NF (2011) Modification of histones by sugar beta-N-acetylglucosamine (GlcNAc) occurs on multiple residues, including histone H3 serine 10, and is cell cycle-regulated. J Biol Chem 286:37483–37495
10. Tan M et al (2011) Identification of 67 histone marks and histone lysine crotonylation as a new type of histone modification. Cell 146:1016–1028
11. Cosgrove MS (2007) Histone proteomics and the epigenetic regulation of nucleosome mobility. Expert Rev Proteomics 4:465–478
12. Hyland EM et al (2005) Insights into the role of histone H3 and histone H4 core modifiable residues in Saccharomyces cerevisiae. Mol Cell Biol 25:10060–10070
13. Xu F, Zhang K, Grunstein M (2005) Acetylation in histone H3 globular domain regulates gene expression in yeast. Cell 121:375–385
14. Masumoto H, Hawke D, Kobayashi R, Verreault A (2005) A role for cell-cycle-regulated histone H3 lysine 56 acetylation in the DNA damage response. Nature 436:294–298
15. Vempati RK (2012) DNA damage in the presence of chemical genotoxic agents induce acetylation of H3K56 and H4K16 but not H3K9 in mammalian cells. Mol Biol Rep 39:303–308
16. Clemente-Ruiz M, Gonzalez-Prieto R, Prado F (2011) Histone H3K56 acetylation, CAF1, and Rtt106 coordinate nucleosome assembly and stability of advancing replication forks. PLoS Genet 7:e1002376
17. Watanabe S et al (2010) Structural characterization of H3K56Q nucleosomes and nucleosomal arrays. Biochim Biophys Acta 1799:480–486
18. Xie W et al (2009) Histone h3 lysine 56 acetylation is linked to the core transcriptional network in human embryonic stem cells. Mol Cell 33:417–427
19. Luger K, Richmond TJ (1998) The histone tails of the nucleosome. Curr Opin Genet Dev 8:140–146
20. Ferreira H, Somers J, Webster R, Flaus A, Owen-Hughes T (2007) Histone tails and the H3 alphaN helix regulate nucleosome mobility and stability. Mol Cell Biol 27:4037–4048
21. Lenfant F, Mann RK, Thomsen B, Ling X, Grunstein M (1996) All four core histone N-termini contain sequences required for the repression of basal transcription in yeast. EMBO J 15:3974–3985
22. Ling X, Harkness TA, Schultz MC, Fisher-Adams G, Grunstein M (1996) Yeast histone H3 and H4 amino termini are important for nucleosome assembly in vivo and in vitro: redundant and position-independent functions in assembly but not in gene regulation. Genes Dev 10:686–699
23. Dion MF, Altschuler SJ, Wu LF, Rando OJ (2005) Genomic characterization reveals a simple histone H4 acetylation code. Proc Natl Acad Sci USA 102:5501–5506

24. Turner BM (1993) Decoding the nucleosome. Cell 75:5–8
25. Turner BM, Birley AJ, Lavender J (1992) Histone H4 isoforms acetylated at specific lysine residues define individual chromosomes and chromatin domains in Drosophila polytene nuclei. Cell 69:375–384
26. Kutateladze TG (2011) SnapShot: histone readers. Cell 146:842–842.e1
27. Taverna SD, Li H, Ruthenburg AJ, Allis CD, Patel DJ (2007) How chromatin-binding modules interpret histone modifications: lessons from professional pocket pickers. Nat Struct Mol Biol 14:1025–1040
28. Liu Z et al (2010) Jmjd1a demethylase-regulated histone modification is essential for cAMP-response element modulator-regulated gene expression and spermatogenesis. J Biol Chem 285:2758–2770
29. Canzio D et al (2011) Chromodomain-mediated oligomerization of HP1 suggests a nucleosome-bridging mechanism for heterochromatin assembly. Mol Cell 41:67–81
30. Kolasinska-Zwierz P et al (2009) Differential chromatin marking of introns and expressed exons by H3K36me3. Nat Genet 41:376–381
31. Kourmouli N et al (2004) Heterochromatin and tri-methylated lysine 20 of histone H4 in animals. J Cell Sci 117:2491–2501
32. Ringrose L, Paro R (2007) Polycomb/trithorax response elements and epigenetic memory of cell identity. Development 134:223–232
33. Mateescu B, England P, Halgand F, Yaniv M, Muchardt C (2004) Tethering of HP1 proteins to chromatin is relieved by phosphoacetylation of histone H3. EMBO Rep 5:490–496
34. Ernst J et al (2011) Mapping and analysis of chromatin state dynamics in nine human cell types. Nature 473:43–49
35. Kharchenko PV et al (2011) Comprehensive analysis of the chromatin landscape in Drosophila melanogaster. Nature 471:480–485
36. Rada-Iglesias A et al (2011) A unique chromatin signature uncovers early developmental enhancers in humans. Nature 470:279–283
37. Korolev N, Lyubartsev AP, Nordenskiold L (2006) Computer modeling demonstrates that electrostatic attraction of nucleosomal DNA is mediated by histone tails. Biophys J 90: 4305–4316
38. Perico A, La Penna G, Arcesi L (2006) Electrostatic interactions with histone tails may bend linker DNA in chromatin. Biopolymers 81:20–28
39. Takahashi YH et al (2011) Dot1 and histone H3K79 methylation in natural telomeric and HM silencing. Mol Cell 42:118–126
40. Jones B et al (2008) The histone H3K79 methyltransferase Dot1L is essential for mammalian development and heterochromatin structure. PLoS Genet 4:e1000190
41. Lee MG et al (2007) Demethylation of H3K27 regulates polycomb recruitment and H2A ubiquitination. Science 318:447–450
42. Agger K et al (2007) UTX and JMJD3 are histone H3K27 demethylases involved in HOX gene regulation and development. Nature 449:731–734
43. Pekowska A et al (2011) H3K4 tri-methylation provides an epigenetic signature of active enhancers. EMBO J 30:4198–4210
44. Robertson AG et al (2008) Genome-wide relationship between histone H3 lysine 4 mono- and tri-methylation and transcription factor binding. Genome Res 18:1906–1917
45. Zeissig S et al (2007) Butyrate induces intestinal sodium absorption via Sp3-mediated transcriptional up-regulation of epithelial sodium channels. Gastroenterology 132:236–248
46. Augenlicht LH et al (2002) Short chain fatty acids and colon cancer. J Nutr 132: 3804S–3808S
47. Tanaka Y, Bush KK, Klauck TM, Higgins PJ (1989) Enhancement of butyrate-induced differentiation of HT-29 human colon carcinoma cells by 1,25-dihydroxyvitamin D3. Biochem Pharmacol 38:3859–3865
48. Cuezva JM et al (2002) The bioenergetic signature of cancer: a marker of tumor progression. Cancer Res 62:6674–6681

49. Racker E, Spector M (1981) Warburg effect revisited: merger of biochemistry and molecular biology. Science 213:303–307
50. Hsu PP, Sabatini DM (2008) Cancer cell metabolism: Warburg and beyond. Cell 134:703–707
51. Cavill R et al (2011) Consensus-phenotype integration of transcriptomic and metabolomic data implies a role for metabolism in the chemosensitivity of tumour cells. PLoS Comput Biol 7:e1001113
52. Su G, Burant CF, Beecher CW, Athey BD, Meng F (2011) Integrated metabolome and transcriptome analysis of the NCI60 dataset. BMC Bioinformatics 12(Suppl 1):S36
53. Sharon D, Chen R, Snyder M (2010) Systems biology approaches to disease marker discovery. Dis Markers 28:209–224
54. Misteli T, Soutoglou E (2009) The emerging role of nuclear architecture in DNA repair and genome maintenance. Nat Rev Mol Cell Biol 10:243–254
55. Seligson DB et al (2005) Global histone modification patterns predict risk of prostate cancer recurrence. Nature 435:1262–1266
56. Kurdistani SK (2007) Histone modifications as markers of cancer prognosis: a cellular view. Br J Cancer 97:1–5
57. He LR et al (2009) Prognostic impact of H3K27me3 expression on locoregional progression after chemoradiotherapy in esophageal squamous cell carcinoma. BMC Cancer 9:461
58. Tzao C et al (2009) Prognostic significance of global histone modifications in resected squamous cell carcinoma of the esophagus. Mod Pathol 22:252–260
59. Yu J et al (2007) A polycomb repression signature in metastatic prostate cancer predicts cancer outcome. Cancer Res 67:10657–10663
60. Wei Y et al (2008) Loss of trimethylation at lysine 27 of histone H3 is a predictor of poor outcome in breast, ovarian, and pancreatic cancers. Mol Carcinog 47:701–706
61. Hansen KH et al (2008) A model for transmission of the H3K27me3 epigenetic mark. Nat Cell Biol 10:1291–1300
62. Fraga MF et al (2005) Loss of acetylation at Lys16 and trimethylation at Lys20 of histone H4 is a common hallmark of human cancer. Nat Genet 37:391–400
63. Tryndyak VP, Kovalchuk O, Pogribny IP (2006) Loss of DNA methylation and histone H4 lysine 20 trimethylation in human breast cancer cells is associated with aberrant expression of DNA methyltransferase 1, Suv4-20h2 histone methyltransferase and methyl-binding proteins. Cancer Biol Ther 5:65–70
64. Pfister S et al (2008) The histone acetyltransferase hMOF is frequently downregulated in primary breast carcinoma and medulloblastoma and constitutes a biomarker for clinical outcome in medulloblastoma. Int J Cancer 122:1207–1213
65. Bosch-Presegue L, Vaquero A (2011) The dual role of sirtuins in cancer. Genes Cancer 2:648–662
66. Rius M, Lyko F (2011) Epigenetic cancer therapy: rationales, targets and drugs. Oncogene Dec 19. doi:10.1038/onc.2011.601
67. Pogribny IP et al (2006) Histone H3 lysine 9 and H4 lysine 20 trimethylation and the expression of Suv4-20h2 and Suv-39h1 histone methyltransferases in hepatocarcinogenesis induced by methyl deficiency in rats. Carcinogenesis 27:1180–1186
68. Lakshmikuttyamma A, Scott SA, DeCoteau JF, Geyer CR (2010) Reexpression of epigenetically silenced AML tumor suppressor genes by SUV39H1 inhibition. Oncogene 29:576–588
69. Das C, Lucia MS, Hansen KC, Tyler JK (2009) CBP/p300-mediated acetylation of histone H3 on lysine 56. Nature 459:113–117
70. Williams SK, Truong D, Tyler JK (2008) Acetylation in the globular core of histone H3 on lysine-56 promotes chromatin disassembly during transcriptional activation. Proc Natl Acad Sci USA 105:9000–9005
71. Adkins MW, Williams SK, Linger J, Tyler JK (2007) Chromatin disassembly from the PHO5 promoter is essential for the recruitment of the general transcription machinery and coactivators. Mol Cell Biol 27:6372–6382

72. Meshorer E, Misteli T (2006) Chromatin in pluripotent embryonic stem cells and differentiation. Nat Rev Mol Cell Biol 7:540–546
73. Spivakov M, Fisher AG (2007) Epigenetic signatures of stem-cell identity. Nat Rev Genet 8:263–271
74. Meshorer E, Gruenbaum Y (2008) Gone with the Wnt/Notch: stem cells in laminopathies, progeria, and aging. J Cell Biol 181:9–13
75. Loh YH, Zhang W, Chen X, George J, Ng HH (2007) Jmjd1a and Jmjd2c histone H3 Lys 9 demethylases regulate self-renewal in embryonic stem cells. Genes Dev 21:2545–2557
76. Jepsen K et al (2007) SMRT-mediated repression of an H3K27 demethylase in progression from neural stem cell to neuron. Nature 450:415–419
77. Frye M, Fisher AG, Watt FM (2007) Epidermal stem cells are defined by global histone modifications that are altered by Myc-induced differentiation. PLoS One 2:e763
78. Ozdag H et al (2006) Differential expression of selected histone modifier genes in human solid cancers. BMC Genomics 7:90
79. Watanabe H et al (2008) Deregulation of histone lysine methyltransferases contributes to oncogenic transformation of human bronchoepithelial cells. Cancer Cell Int 8:15
80. Wang JK et al (2010) The histone demethylase UTX enables RB-dependent cell fate control. Genes Dev 24:327–332
81. van Haaften G et al (2009) Somatic mutations of the histone H3K27 demethylase gene UTX in human cancer. Nat Genet 41:521–523
82. Cherrier T et al (2009) p21(WAF1) gene promoter is epigenetically silenced by CTIP2 and SUV39H1. Oncogene 28:3380–3389
83. Pollard PJ et al (2008) Regulation of Jumonji-domain-containing histone demethylases by hypoxia-inducible factor (HIF)-1alpha. Biochem J 416:387–394
84. Scharer CD et al (2009) Genome-wide promoter analysis of the SOX4 transcriptional network in prostate cancer cells. Cancer Res 69:709–717
85. Varambally S et al (2002) The polycomb group protein EZH2 is involved in progression of prostate cancer. Nature 419:624–629
86. Kondo Y et al (2008) Gene silencing in cancer by histone H3 lysine 27 trimethylation independent of promoter DNA methylation. Nat Genet 40:741–750
87. Min J et al (2010) An oncogene-tumor suppressor cascade drives metastatic prostate cancer by coordinately activating Ras and nuclear factor-kappaB. Nat Med 16:286–294
88. Shi Y et al (2004) Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. Cell 119:941–953
89. Wissmann M et al (2007) Cooperative demethylation by JMJD2C and LSD1 promotes androgen receptor-dependent gene expression. Nat Cell Biol 9:347–353
90. Metzger E et al (2005) LSD1 demethylates repressive histone marks to promote androgen-receptor-dependent transcription. Nature 437:436–439
91. Metzger E et al (2010) Phosphorylation of histone H3T6 by PKCbeta(I) controls demethylation at histone H3K4. Nature 464:792–796
92. Shi Y (2007) Histone lysine demethylases: emerging roles in development, physiology and disease. Nat Rev Genet 8:829–833
93. Shi L et al (2011) Histone demethylase JMJD2B coordinates H3K4/H3K9 methylation and promotes hormonally responsive breast carcinogenesis. Proc Natl Acad Sci USA 108:7541–7546
94. Lee MG, Wynder C, Cooch N, Shiekhattar R (2005) An essential role for CoREST in nucleosomal histone 3 lysine 4 demethylation. Nature 437:432–435
95. Shi YJ et al (2005) Regulation of LSD1 histone demethylase activity by its associated factors. Mol Cell 19:857–864
96. Forneris F, Binda C, Vanoni MA, Battaglioli E, Mattevi A (2005) Human histone demethylase LSD1 reads the histone code. J Biol Chem 280:41360–41365
97. Forneris F et al (2006) A highly specific mechanism of histone H3-K4 recognition by histone demethylase LSD1. J Biol Chem 281:35289–35295

98. Cai C et al (2011) Androgen receptor gene expression in prostate cancer is directly suppressed by the androgen receptor through recruitment of lysine-specific demethylase 1. Cancer Cell 20:457–471
99. Yosef N, Regev A (2011) Impulse control: temporal dynamics in gene transcription. Cell 144:886–896
100. Alenghat T et al (2008) Nuclear receptor corepressor and histone deacetylase 3 govern circadian metabolic physiology. Nature 456(7224):997–1000
101. Mohn F, Schubeler D (2009) Genetics and epigenetics: stability and plasticity during cellular differentiation. Trends Genet 25:129–136
102. De Santa F et al (2007) The histone H3 lysine-27 demethylase Jmjd3 links inflammation to inhibition of polycomb-mediated gene silencing. Cell 130:1083–1094
103. Cui Q et al (2007) A map of human cancer signaling. Mol Syst Biol 3:152
104. Dobrzynski M, Bruggeman FJ (2009) Elongation dynamics shape bursty transcription and translation. Proc Natl Acad Sci USA 106(8):2583–2588
105. Goldberg AD, Allis CD, Bernstein E (2007) Epigenetics: a landscape takes shape. Cell 128:635–638
106. Le May N et al (2010) NER factors are recruited to active promoters and facilitate chromatin modification for transcription in the absence of exogenous genotoxic attack. Mol Cell 38:54–66
107. Metivier R et al (2008) Cyclical DNA methylation of a transcriptionally active promoter. Nature 452:45–50
108. Kangaspeska S et al (2008) Transient cyclical methylation of promoter DNA. Nature 452:112–115
109. Wang Q et al (2009) Androgen receptor regulates a distinct transcription program in androgen-independent prostate cancer. Cell 138:245–256
110. Hu M et al (2005) Distinct epigenetic changes in the stromal cells of breast cancers. Nat Genet 37:899–905
111. Orimo A, Weinberg RA (2006) Stromal fibroblasts in cancer: a novel tumor-promoting cell type. Cell Cycle 5:1597–1601
112. Li L, Neaves WB (2006) Normal stem cells and cancer stem cells: the niche matters. Cancer Res 66:4553–4557
113. Feinberg AP, Ohlsson R, Henikoff S (2006) The epigenetic progenitor origin of human cancer. Nat Rev Genet 7:21–33
114. Vita M, Henriksson M (2006) The Myc oncoprotein as a therapeutic target for human cancer. Semin Cancer Biol 16:318–330
115. Fernandez PC et al (2003) Genomic targets of the human c-Myc protein. Genes Dev 17:1115–1129
116. Adhikary S, Eilers M (2005) Transcriptional regulation and transformation by Myc proteins. Nat Rev Mol Cell Biol 6:635–645
117. Guccione E et al (2006) Myc-binding-site recognition in the human genome is determined by chromatin context. Nat Cell Biol 8:764–770
118. Bernstein BE et al (2006) A bivalent chromatin structure marks key developmental genes in embryonic stem cells. Cell 125:315–326
119. Secombe J, Li L, Carlos L, Eisenman RN (2007) The Trithorax group protein Lid is a trimethyl histone H3K4 demethylase required for dMyc-induced cell growth. Genes Dev 21:537–551
120. Secombe J, Eisenman RN (2007) The function and regulation of the JARID1 family of histone H3 lysine 4 demethylases: the Myc connection. Cell Cycle 6:1324–1328
121. Suzuki C et al (2007) Identification of Myc-associated protein with JmjC domain as a novel therapeutic target oncogene for lung cancer. Mol Cancer Ther 6:542–551
122. Ogasawara S et al (2010) Accelerated expression of a Myc target gene Mina53 in aggressive hepatocellular carcinoma. Hepatol Res 40:330–336
123. Komiya K et al (2010) Mina53, a novel c-Myc target gene, is frequently expressed in lung cancers and exerts oncogenic property in NIH/3T3 cells. J Cancer Res Clin Oncol 136:465–473

124. Watt FM, Frye M, Benitah SA (2008) MYC in mammalian epidermis: how can an oncogene stimulate differentiation? Nat Rev Cancer 8:234–242
125. Li H, Kim JH, Koh SS, Stallcup MR (2004) Synergistic effects of coactivators GRIP1 and beta-catenin on gene activation: cross-talk between androgen receptor and Wnt signaling pathways. J Biol Chem 279:4212–4220
126. Yang X et al (2006) Complex regulation of human androgen receptor expression by Wnt signaling in prostate cancer cells. Oncogene 25(24):3436–3444
127. Campbell MJ, Elstner E, Holden S, Uskokovic M, Koeffler HP (1997) Inhibition of proliferation of prostate cancer cells by a 19-nor-hexafluoride vitamin D3 analogue involves the induction of p21waf1, p27kip1 and E-cadherin. J Mol Endocrinol 19:15–27
128. Degenhardt T, Matilainen M, Herzig KH, Dunlop TW, Carlberg C (2006) The insulin-like growth factor binding protein 1 gene is a primary target of peroxisome proliferator-activated receptors. J Biol Chem 281(51):39607–39619
129. Khanim FL et al (2004) Altered SMRT levels disrupt vitamin D(3) receptor signalling in prostate cancer cells. Oncogene 23:6712–6725
130. Kubota T et al (1998) 19-nor-26,27-bishomo-vitamin D3 analogs: a unique class of potent inhibitors of proliferation of prostate, breast, and hematopoietic cancer cells. Cancer Res 58:3370–3375
131. Liu M, Lee MH, Cohen M, Bommakanti M, Freedman LP (1996) Transcriptional activation of the Cdk inhibitor p21 by vitamin D3 leads to the induced differentiation of the myelomonocytic cell line U937. Genes Dev 10:142–153
132. Palmer HG et al (2004) The transcription factor SNAIL represses vitamin D receptor expression and responsiveness in human colon cancer. Nat Med 10:917–919
133. Saramaki A, Banwell CM, Campbell MJ, Carlberg C (2006) Regulation of the human p21(waf1/cip1) gene promoter via multiple binding sites for p53 and the vitamin D3 receptor. Nucleic Acids Res 34:543–554
134. Thorne J, Campbell MJ (2008) The vitamin D receptor in cancer. Proc Nutr Soc 67:115–127
135. Hendriksen PJ et al (2006) Evolution of the androgen receptor pathway during progression of prostate cancer. Cancer Res 66:5012–5020
136. Taneja SS et al (2004) ART-27, an androgen receptor coactivator regulated in prostate development and cancer. J Biol Chem 279:13944–13952
137. Ross-Innes CS et al (2012) Differential oestrogen receptor binding is associated with clinical outcome in breast cancer. Nature 481(7381):389–393
138. Ceschin DG et al (2011) Methylation specifies distinct estrogen-induced binding site repertoires of CBP to chromatin. Genes Dev 25:1132–1146
139. Welboren WJ et al (2009) ChIP-Seq of ERalpha and RNA polymerase II defines genes differentially responding to ligands. EMBO J 28:1418–1428
140. Rashid SF et al (2001) Synergistic growth inhibition of prostate cancer cells by 1 alpha,25 Dihydroxyvitamin D(3) and its 19-nor-hexafluoride analogs in combination with either sodium butyrate or trichostatin A. Oncogene 20:1860–1872
141. Abedin SA et al (2009) Elevated NCOR1 disrupts a network of dietary-sensing nuclear receptors in bladder cancer cells. Carcinogenesis 30(3):449–456
142. Lin RJ et al (1998) Role of the histone deacetylase complex in acute promyelocytic leukaemia. Nature 391:811–814
143. Banwell CM et al (2006) Altered nuclear receptor corepressor expression attenuates vitamin D receptor signaling in breast cancer cells. Clin Cancer Res 12:2004–2013
144. Ting HJ, Bao BY, Reeder JE, Messing EM, Lee YF (2007) Increased expression of corepressors in aggressive androgen-independent prostate cancer cells results in loss of 1alpha,25-dihydroxyvitamin D3 responsiveness. Mol Cancer Res 5:967–980
145. Tomlins SA et al (2005) Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. Science 310:644–648
146. Anderson SP et al (2004) Overlapping transcriptional programs regulated by the nuclear receptors peroxisome proliferator-activated receptor {alpha}, retinoid X receptor and liver X receptor in mouse liver. Mol Pharmacol 66(6):1440–1452

147. Bookout AL et al (2006) Anatomical profiling of nuclear receptor expression reveals a hierarchical transcriptional network. Cell 126:789–799
148. Handschin C, Meyer UA (2005) Regulatory network of lipid-sensing nuclear receptors: roles for CAR, PXR, LXR, and FXR. Arch Biochem Biophys 433:387–396
149. Xia X et al (2009) Integrative analysis of HIF binding and transactivation reveals its role in maintaining histone methylation homeostasis. Proc Natl Acad Sci USA 106:4260–4265
150. Malik S, Roeder RG (2010) The metazoan Mediator co-activator complex as an integrative hub for transcriptional regulation. Nat Rev Genet 11:761–772
151. Xu J, Wu RC, O'Malley BW (2009) Normal and cancer-related functions of the p160 steroid receptor co-activator (SRC) family. Nat Rev Cancer 9:615–630
152. Taatjes DJ, Marr MT, Tjian R (2004) Regulatory diversity among metazoan co-activator complexes. Nat Rev Mol Cell Biol 5:403–410
153. Perissi V, Jepsen K, Glass CK, Rosenfeld MG (2010) Deconstructing repression: evolving models of co-repressor action. Nat Rev Genet 11:109–123
154. Battaglia S, Maguire O, Campbell MJ (2010) Transcription factor co-repressors in cancer biology: roles and targeting. Int J Cancer 126:2511–2519
155. Anzick SL et al (1997) AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer. Science 277:965–968
156. Demarest SJ et al (2002) Mutual synergistic folding in recruitment of CBP/p300 by p160 nuclear receptor coactivators. Nature 415:549–553
157. Zhao C et al (2003) Elevated expression levels of NCOA3, TOP1, and TFAP2C in breast tumors as predictors of poor prognosis. Cancer 98:18–23
158. Doi M, Hirayama J, Sassone-Corsi P (2006) Circadian regulator CLOCK is a histone acetyltransferase. Cell 125:497–508
159. Esteyries S et al (2008) NCOA3, a new fusion partner for MOZ/MYST3 in M5 acute myeloid leukemia. Leukemia 22:663–665
160. Horlein AJ et al (1995) Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor. Nature 377:397–404
161. Chen JD, Evans RM (1995) A transcriptional co-repressor that interacts with nuclear hormone receptors. Nature 377:454–457
162. Li J et al (2000) Both corepressor proteins SMRT and N-CoR exist in large protein complexes containing HDAC3. EMBO J 19:4342–4350
163. Yu C et al (2005) The nuclear receptor corepressors NCoR and SMRT decrease PPARgamma transcriptional activity and repress 3T3-L1 adipogenesis. J. Biol, Chem
164. Jepsen K, Gleiberman AS, Shi C, Simon DI, Rosenfeld MG (2008) Cooperative regulation in development by SMRT and FOXP1. Genes Dev 22:740–745
165. Tiefenbach J et al (2006) SUMOylation of the corepressor N-CoR modulates its capacity to repress transcription. Mol Biol Cell 17:1643–1651
166. Surjit M et al (2011) Widespread negative response elements mediate direct repression by agonist-liganded glucocorticoid receptor. Cell 145:224–241
167. Heikkinen S et al (2011) Nuclear hormone 1{alpha},25-dihydroxyvitamin D3 elicits a genome-wide shift in the locations of VDR chromatin occupancy. Nucleic Acids Res 39(21):9181–9193
168. Muller-Tidow C et al (2010) Profiling of histone H3 lysine 9 trimethylation levels predicts transcription factor activity and survival in acute myeloid leukemia. Blood 116:3564–3571
169. Hoemme C et al (2008) Chromatin modifications induced by PML-RARalpha repress critical targets in leukemogenesis as analyzed by ChIP-Chip. Blood 111:2887–2895
170. Minucci S et al (2000) Oligomerization of RAR and AML1 transcription factors as a novel mechanism of oncogenic activation. Mol Cell 5:811–820
171. Girault I et al (2003) Expression analysis of estrogen receptor alpha coregulators in breast carcinoma: evidence that NCOR1 expression is predictive of the response to tamoxifen. Clin Cancer Res 9:1259–1266
172. Zhang Z et al (2005) NCOR1 mRNA is an independent prognostic factor for breast cancer. Cancer Lett 237(1):123–129

173. Kim JY, Son YL, Lee YC (2009) Involvement of SMRT corepressor in transcriptional repression by the vitamin D receptor. Mol Endocrinol 23:251–264
174. Chang TH, Szabo E (2002) Enhanced growth inhibition by combination differentiation therapy with ligands of peroxisome proliferator-activated receptor-gamma and inhibitors of histone deacetylase in adenocarcinoma of the lung. Clin Cancer Res 8:1206–1212
175. Battaglia S et al (2010) Elevated NCOR1 disrupts PPAR signaling in prostate cancer and forms a targetable epigenetic lesion. Carcinogenesis 31(9):1650–1660
176. Bau D et al (2011) The three-dimensional folding of the alpha-globin gene domain reveals formation of chromatin globules. Nat Struct Mol Biol 18:107–114
177. Li Q, Barkess G, Qian H (2006) Chromatin looping and the probability of transcription. Trends Genet 22:197–202
178. Eskiw CH, Rapp A, Carter DR, Cook PR (2008) RNA polymerase II activity is located on the surface of protein-rich transcription factories. J Cell Sci 121:1999–2007
179. Mitchell JA, Fraser P (2008) Transcription factories are nuclear subcompartments that remain in the absence of transcription. Genes Dev 22:20–25
180. Hu Q et al (2008) Enhancing nuclear receptor-induced transcription requires nuclear motor and LSD1-dependent gene networking in interchromatin granules. Proc Natl Acad Sci USA 105:19199–19204
181. Yoon HG, Chan DW, Reynolds AB, Qin J, Wong J (2003) N-CoR mediates DNA methylation-dependent repression through a methyl CpG binding protein Kaiso. Mol Cell 12:723–734
182. Yegnasubramanian S et al (2004) Hypermethylation of CpG islands in primary and metastatic human prostate cancer. Cancer Res 64:1975–1986
183. Asatiani E et al (2005) Deletion, methylation, and expression of the NKX3.1 suppressor gene in primary human prostate cancer. Cancer Res 65:1164–1173
184. Fujita N et al (2003) Methyl-CpG binding domain 1 (MBD1) interacts with the Suv39h1-HP1 heterochromatic complex for DNA methylation-based transcriptional repression. J Biol Chem 278:24132–24138
185. Esteve PO et al (2006) Direct interaction between DNMT1 and G9a coordinates DNA and histone methylation during replication. Genes Dev 20:3089–3103
186. Cheng X, Blumenthal RM (2010) Coordinated chromatin control: structural and functional linkage of DNA and histone methylation. Biochemistry 49:2999–3008
187. Freitag M, Hickey PC, Khlafallah TK, Read ND, Selker EU (2004) HP1 is essential for DNA methylation in Neurospora. Mol Cell 13:427–434
188. Birney E et al (2007) Identification and analysis of functional elements in 1% of the human genome by the ENCODE pilot project. Nature 447:799–816
189. Schulte JH et al (2009) Lysine-specific demethylase 1 is strongly expressed in poorly differentiated neuroblastoma: implications for therapy. Cancer Res 69:2065–2071
190. Graham JS, Kaye SB, Brown R (2009) The promises and pitfalls of epigenetic therapies in solid tumours. Eur J Cancer 45:1129–1136

Chapter 5
Nucleosome Occupancy and Gene Regulation During Tumorigenesis

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Abstract Nucleosomes are the basic structural units of eukaryotic chromatin. In recent years, it has become evident that nucleosomes and their position, in concert with other epigenetic mechanisms (such as DNA methylation, histone modifications, changes in histone variants, as well as small noncoding regulatory RNAs) play essential roles in the control of gene expression. Here, we discuss the mechanisms and factors that regulate nucleosome position and gene expression in normal and cancer cells.

5.1 Introduction

Nucleosomes are the basic units of eukaryotic chromatin, each one containing ~146 bp of DNA wrapped around an octamer of histone core proteins (H3, H4, H2A, and H2B), which in turn are separated by linker DNA of variable length [1]. At least five epigenetic mechanisms have been shown to act in concert to regulate gene expression by modifying chromatin structure, namely DNA methylation, histone modifications, nucleosome remodeling, and changes in histone variants as well as small noncoding regulatory RNAs [2]. In addition to playing a pivotal role in chromatin structure, nucleosomes display differential occupancy at promoter regions, thereby regulating gene expression by altering DNA accessibility. For instance, a nucleosome-depleted region (NDR) at transcriptional start sites correlates with gene expression, whereas the positioning of a nucleosome over the transcriptional start site results in gene repression [2, 3]. The position of nucleosomes is determined and influenced by a number of factors, including DNA sequence, DNA methylation, histone modifications and histone variants, chromatin remodelers, and

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transcription factor binding [4]. We discuss how these factors act in normal cells and how abnormalities in these factors impact nucleosome occupancy and gene expression in cancer cells.

5.2 Regulation of Nucleosome Position in Normal Cells

5.2.1 DNA Sequence Preferences

The sequences that regulate nucleosome position fall into two categories: motifs that are preferred (included within the nucleosome) and motifs that avoided (excluded from the nucleosome) [5]. Preferred sequences were originally characterized as particular dinucleotides, including CG and GC dinucleotides, occurring with approximately 10 bp periodicity, although nucleosomes may also prefer longer DNA motifs [4, 6]. The sequences that are disfavored by nucleosomes include various 5-mers and long tracts of As (10–20 bp or more), possibly due to their resistance to the structural distortions required for DNA wrapping and nucleosome formation [4, 7]. Such organization helps restrict nucleosome access to those regions to ensure proper gene expression pattern [7]. An example of regions containing both preferred and disfavored sequences with restricted nucleosome positioning are the Alu repeats [6, 8]. More recently, however, the concept of intrinsically DNA-encoded positioning as an organizational determinant of the 5′ end of genes has been challenged. In this regard, studies showed that the majority of the human genome displays great flexibility in nucleosome positioning, although DNA sequence can strongly drive the organization of nucleosomes at specific sites [9]. It has also been shown that these intrinsic signals can be overridden, confirming that additional factors are involved in nucleosome organization [9, 10].

5.2.2 Nucleosomes and DNA Methylation

DNA methylation in mammals occurs at CpG dinucleotides, which are distributed along the genome in clusters (CpG islands) or in regions containing high concentration of repeat sequences, and acts as a relatively stable gene silencing mechanism [2]. The majority of isolated CpGs tend to be methylated in mammals. In contrast, the majority of the CpG islands, which represent 60% of all human promoters, remain largely unmethylated [2, 3]. However, a number of CpG island promoters, such as those of imprinted genes, are methylated resulting in monoallelic gene expression in normal cells [3]. CpG islands can also be found within or in between transcriptional units (orphan CpG islands) [3] and can be associated with novel promoter regions and to be active in a tissue-specific manner [3]. DNA methylation also appears to be important for the regulation of non-CpG island promoters and the

tissue-specific expression of the genes that they control including *MASPIN*, *OCT-4*, *LAMB3*, and *RUNX3* promoter 1 [11–14]. Methylation is also observed in repetitive genomic sequences, which include transposable elements and noncoding DNA, where it helps maintain genomic stability [15, 16]. DNA methylation is established by the activity of three DNA methyltransferases (DNMTs): DNMT1, which preferentially methylates hemimethylated DNA during replication, and DNMT3A and DNMT3B, which are replication-independent, have been shown to establish de novo DNA methylation. Furthermore, DNMT3A and 3B were shown to be recruited to sites methylated by DNMT1 thereby contributing to propagate the methylated state [17, 18].

CpG DNA methylation causes steric interference in the formation of nucleosomes in vitro, suggesting that methylation may play a role in nucleosome occupancy [4]. However, more recent in vivo studies demonstrate that the nucleosome architecture plays a role in the shaping of DNA methylation patterns [19]. This is in agreement with studies from our laboratory showing that nucleosomes are required for stable DNMT3A/3B anchoring [17, 18, 20] and that nucleosome occupancy precedes de novo DNA methylation in vivo [14]. While the direction of the relationship is still under investigation, it is clear that nucleosome position and methylation are interrelated.

5.2.3 Nucleosomes and Histone Modifications

The N-terminus of histones can undergo a variety of modifications in specific residues, including acetylation, methylation, ubiquitination, sumoylation, and phosphorylation [21]. Histone modifications work in a combinatorial fashion to alter chromatin accessibility by disrupting interactions between nucleosomes or by regulating the recruitment of nonhistone proteins [4, 22]. Specific patterns of histone modifications characterize genomic regions. For instance, active promoter regions are enriched in trimethylated H3 at lysine 4 (H3K4me3), whereas inactive promoters are enriched in trimethylated H3 at lysine 27 and trimethylated H3 at lysine 9 (H3K27me3 and H3K9me3), and regulatory enhancers are enriched in monomethylated H3 at lysine 4 (H3K4me1) [21]. Such patterns are dynamic and regulated by enzymes that can add or remove the modifications. These include histone methyltransferases (HMTs) and demethylases (HDMTs), which introduce and remove methyl groups, respectively, and histone acetyltransferases (HATs) and deacetylases (HDACs), which introduce and remove acetyl groups, respectively. Histone acetylation is an important marker of transcriptional activity; for instance, acetylated histone H3 (acH3) can also be found at well-positioned nucleosomes flanking the AR binding site of 20% of AR enhancers, upon hormone stimulation [23]. In addition, acH4K16 can be found at well-positioned nuclesomes flanking unmethylated CpG islands at the promoter regions of some tumor-suppressor genes [24]. In addition, although histone modifications themselves are not likely to have a direct impact in nucleosome

positioning, their ability to recruit chromatin remodeler proteins and other factors may have a substantial impact in nucleosome organization [4].

5.2.4 ATPase-Dependent Chromatin Remodelers and Histone Variants

ATP-dependent chromatin remodelers can be grouped in families based on subunit composition and activity: the SWI/SNF family includes the SWI/SNF, INO80, and SWR1 complexes; the ISWI family comprises the RSF, ACF/CHRAC, WICH, and NURF complexes; and the CHD family which includes NURD complexes [25, 26]. These complexes directly affect nucleosome positioning by actively mobilizing nucleosomes or introducing histone variants.

5.2.4.1 SWItch/Sucrose Non-fermenting

These complexes consist of 9–12 subunits, which include one of two ATPases: Brahma homologue (BRM/SMARCA2) or Brahma-related gene 1 (BRG1/SMARCA4), a set of "core" subunits, including SNF5 and BAF53a/b, and a number of variable subunits [27]. A number of the variable subunits are mutually exclusive; for example, AT-rich interactive proteins (ARID) 1A and ARID1B (BAF250a and BAF250b) [25, 27] do not coexist in the same complex and Polybromo 1 (PBRM1 or BAF180), bromodomain-containing 7 (BRD7), and BAF200 are only present in complexes lacking ARID1 proteins [27]. Complexes containing ARID1 proteins are named BAF whereas complexes containing PBRM1 are known as PBAF [27]. The variety of subunits allows for a combinatorial assemblage that leads to functional diversity as evidenced by the developmental stage-specific composition of SWItch/sucrose non-fermenting (SWI/SNF) complexes [25]. SWI/SNF complexes remodel chromatin by sliding or by ejecting or inserting nucleosomes thereby contributing to either transcriptional activation or repression [27, 28]; interestingly, they are primarily enriched at distal regulatory regions rather than at promoters [25]. SWI/SNF complexes also associate and act in concert with histone modifying complexes, including HDACs, HATs, and protein arginine methyltransferases (PRMT4/CARM1 and 5), to regulate gene expression [27, 29, 30].

5.2.4.2 INO80 and SWR1

These complexes consist of core proteins (the ATPase, helicases, and actin-related proteins) and additional subunits [31]. INO80 complexes contain the INO80 ATPase [31] whereas the SWR1 complexes (SRCAP and TRAAP/Tip60) contain the ATPases SRCAP or p400 and share a number of subunits [31]. The INO80 complex displays helicase activity and catalyzes nucleosome sliding *in cis*, and is involved in

chromosome segregation [32], the DNA, and damage repair response, and facilitates recombination-mediated events [25, 33, 34]. INO80 recruitment to damaged sites has been recently shown to depend on actin-related protein 8 [35]. SRCAP complex directs the incorporation of H2A.Z into nucleosomes by exchange of H2A/H2B dimers for H2A.Z/H2B dimers in a replication-independent manner [36, 37]. SRCAP-mediated deposition of H2A.Z is required for gene reactivation in colon cancer cells treated with the DNA methylase inhibitor Azacitidine [38]. P400-containing complexes play a role in DNA repair by destabilizing nucleosomes and promoting chromatin ubiquitination [39]. It has been suggested that TRAAP/Tip60 (p400) complexes are involved in the deposition of H2A.Z into chromatin in an acetylation-dependent manner. P400-mediated H2A.Z deposition is important for estrogen receptor-mediated gene expression [40] whereas SRCAP appears to be important for the androgen receptor-stimulated expression of Kallikrein 3/prostate specific antigen (KLK3/PSA) and cell proliferation in prostate cancer cells [41].

H2A.Z deposition is associated with several nucleosomes surrounding the transcriptional start site of active and poised promoters, and nucleosomes and H2A.Z are lost preferentially at the −1 nucleosome upon gene activation [42]. In addition, enrichment in H2A.Z, and also the histone H3 variant histone H3.3, has been found at distal regulatory regions such as enhancers [42, 43]. During mitosis, the H2A.Z-containing +1 nucleosome of active genes shift upstream to occupy the transcriptional start site of genes silenced during mitosis, significantly reducing NDRs [44]. Interestingly, H2A.Z has also been shown to play an inhibitory role in cell cycle arrest, providing evidence that H2A.Z localization at regulatory regions may contribute to the positive or negative regulation of gene transcription [42]. Differential H2A.Z acetylation patterns at promoters may contribute to the opposing functions of H2A.Z, as the presence of acetylated H2A.Z has been shown to correlate with gene activation in prostate cancer cells [45] and to be anti-correlated with DNA methylation [46, 47].

5.2.4.3 ISWI Complexes

Similar to SWI/SNF complexes, the combinatorial assembly of subunits allows for a multiplicity of ISWI complexes that display specific functions, including transcriptional repression, DNA replication, and heterochromatin formation. The remodeling spacing factor (RSF), ATP-utilizing chromatin assembly and remodeling factor (ACF), chromatin accessibility (CHRAC), and WICH complexes share the hSNF2H ATPase [25], while the nucleosome remodeling factor (NURF) complexes contain the hSNF2L ATPase. All ISWI complexes display ATPase and nucleosome spacing and remodeling activities and RSF, in particular, promotes regular spacing between nucleosomes and stimulates transcriptional activation [25]. In addition, WICH complexes are important for DNA replication of pericentromeric heterochromatin and the WSTF subunit of this complex binds and stabilizes H2A.X by phosphorylation after DNA damage [25]. NURF complexes have also been shown to play a role in the regulation of chromatin barriers; for example, the

transcription factor USF1 (upstream stimulatory factor 1) recruits NURF and an HMT to the insulator of the beta-globin gene to retain its active configuration [48].

5.2.4.4 NURD Complexes

These complexes are formed by the CHD ATPases CHD3 or CHD4 (or Mi-2a or Mi2b), HDACs, and additional subunits and contain both HDAC and remodeling activity [25]. NURD complexes play a role in transcription, cell differentiation, cell cycle checkpoint control, and metastasis, and are recruited to sites of DNA damage by poly-ADP-ribose polymerase (PARP) [25, 49, 50]. The methyl CpG binding domain 2/3 (MBD2 and 3) subunits of these complexes are thought to be involved in protein–protein interaction and are mutually exclusive, whereas the metastasis associated gene 1 and 2 (MTA1 and 2) subunits bind to specific transcription factors thereby targeting the complex to different genomic loci [50].

5.2.5 Transcription Factor Binding

The position of nucleosomes can be directly affected by transcription factors as they compete for DNA access [4]. Transcription factors often bind at NDRs. For example, OCT-4 is required for establishing and maintaining of an NDR at the distal OCT-4 enhancer and the proximal NANOG promoter regions, which are necessary for gene expression [14]. We have recently reported that a percentage of androgen receptor (AR) enhancers show a NDR in the absence of ligand, and that androgen treatment and subsequent AR recruitment increase the number of enhancers with NDRs without changes in footprint [51]. The pioneering factor GATA-2 is required for the maintenance of the NDR at the AR enhancer of TMPRSS2 in the absence of ligand [51]. The presence of GATA-2 at the enhancer may facilitate AR binding, as proposed by the model of transcription factor cooperativity of Segal and Widom [4]. In contrast, other transcription factors are frequently bound to nucleosome occupied regions; for instance, P53 binding occurs preferentially to regions with high intrinsic nucleosome occupancy [52]. Thus, the relationship between nucleosome occupancy and transcription factor binding is context-specific.

5.3 Aberrant Epigenetic Regulation and Epigenetic Switching in Cancer Cells

Genetic and epigenetic changes play important roles in cancer initiation and progression [53, 54]. During tumorigenesis, the cell epigenome undergoes global changes, including a genome-wide reduction in DNA methylation, an increase in localized DNA methylation at CpG island promoters, and changes in histone

modification profiles [55]; in addition, cancer cells display aberrant expression of chromatin-modifying enzymes [56]. The events leading to these epigenetic abnormalities are still not fully understood. Epigenetic changes are mitotically inherited and may promote tumorigenesis by either silencing tumor suppressor genes [57] or by activating oncogenes [2].

Because of the interaction amongst chromatin remodeling complexes [58] and between these complexes and DNMTs [59, 60], genetic mutations in enzymes or other subunits of chromatin remodeling complexes may lead to profound epigenetic changes, including aberrant nucleosome position, DNA methylation, histone composition, and/or histone modifications [2]. In addition, deregulated expression of proteins involved in the recruitment of remodeling complexes to specific loci may alter nucleosome localization and/or retention at such sites, contributing to the propagation of abnormal epigenetic states [2]. All these changes will in turn lead to aberrant gene expression patterns and genomic instability, which ultimately may predispose or give rise to disease [2]. The mechanisms contributing to the altered epigenetic landscape of cancer cells are discussed below.

5.3.1 Mutations in DNA Methylation Enzymes

CpG island methylation at gene promoters affects gene expression and abnormal patterns of DNA methylation have been implicated in carcinogenesis [53, 54]. Hypomethylation of retrotransposons may lead to their reactivation and genomic translocation or to the activation of alternative transcripts. These DNA methylation changes have also been shown to correlate with changes in nucleosome occupancy [2]. For instance, LINE-1 is hypomethylated and nucleosome depleted in colon cancer [61] and bladder cancer, where it induced the expression of an alternate transcript of the *MET* oncogene [16]. Hypomethylation of centromeric regions and/or of pericentromeric satellite sequences may lead to abnormal chromosome segregation and genomic instability [62]. Perhaps the best example of chromosome instability is a germ line mutation in DNMT3B, which underlies a chromosome instability and immunodeficiency syndrome [63]. In addition, DNA hypomethylation may lead to loss of imprinting (LOI), resulting in biallelic expression of a monoallelic gene [2, 64], which often occurs in a variety of cancer types [64]. Re-expression of normally silenced genes or microRNAs (miRNA) can also occur due to DNA hypomethylation; examples of these events are *R-RAS*, *MASPIN*, and *Cyclin D2* in gastric cancer; *MAGE* in melanoma; *HPV16* (human papillomavirus 16) in cervical cancer; *S100A4* in colon cancer; and the *let-7a-3* miRNA in lung adenocarcinomas [2, 62].

Site-specific hypermethylation and silencing of tumor suppressor genes has also reported in cancer and correlates with changes in nucleosome occupancy [65]. Genes that regulate cell cycle progression, and DNA repair, such as *RB* (retinoblastoma), *MLH1* (endometrial cancer), *p16* (glioma, lymphoma, multiple myeloma), and *p15* (lymphoma and multiple myeloma), *BRCA1* and *BRCA2* (lung and ovarian cancer), *APC* (lung, breast, and colorectal cancer), *PTEN* (brain and thyroid gland

cancers), *XRCC5* (lung and ovarian cancer), and estrogen receptor (prostate cancer) have all been reported to be hypermethylated in cancer [2, 62]. DNA hypermethylation can also indirectly inactivate other genes by silencing transcription factors that control their expression. For example, hypermethylation has been found at the *RUNX3* promoter in esophageal cancer and at the *GATA-4* and *-5* promoters in colorectal and gastric cancers [2, 62]. In addition, inactivation of miRNAs by hypermethylation has been observed in a variety of cancer types including bladder and prostate (mir-127), endometrial (mir-152, mir-129-2), pancreatic (mir-132), oral (mir-137 and miR-193a), gastrointestinal (mir-34b/c), and colorectal (mir-137) cancers, and in ALL (mir-124a), and other hematological malignancies (mir-124-1) [66–75]. A new class of noncoding RNA (mirtrons) has been also shown to be susceptible to epigenetic silencing in urothelial cell carcinoma [73]. DNMT1 mutations have been described in colorectal cancer and DNMT3A mutations and decreased protein levels have been shown to occur in myelodysplastic syndromes (MDS) and AML, and in primary prostate tumors, respectively [76–80], *DNMT1*, *DNMT3A,* and *DNMT3B* appear to be largely overexpressed in a variety of cancer types and may contribute to ectopic hypermethylation [81].

Recent studies have pointed to the existence of both passive and active mechanisms of DNA demethylation [82]. Active demethylation occurs during early embryogenesis and is mediated by the formation of cytosine intermediaries, for instance 5-hydroxy-methyl cytosine or 5-methyl uracil, via the action of enzymes such as ten-eleven-translocation (TET) or activation-induced cytidine deaminase (AID), respectively [82].

TET1 translocations have been reported to occur in AML [83] and TET2 mutations have been frequently found in myelodysplasia and in myeloid malignancies [84–90]. In addition, TET2 promoter hypermethylation was observed in a fraction of gliomas [91].

AID promotes somatic hypermutation and class switch recombination of immunoglobulin (Ig) genes in germinal center (GC) B cells and aberrant AID expression has been implicated in the progression of chronic myeloid leukemia (CML) into fatal blast crisis [92].

Because DNA methylation stabilizes nucleosome occupancy, mutations in DNMTs and in enzymes involved in DNA demethylation are likely to cause large-scale epigenetic alterations in cancer cells; in addition, de novo functions generated by fusion with their translocation partners may also contribute to tumorigenesis [93].

5.3.2 Mutations in Genes Encoding Histone Modifiers

Genome-wide analyses of histone modifications in cancer cells have revealed global changes in various histone marks [2]. These changes may affect the recruitment of transcription factors and chromatin remodeler complexes to specific genomic loci, thereby affecting nucleosome positioning.

5.3.2.1 HATs and HDACs

In cancer cells, there is a global reduction in the active acH4K16 and H3K4me3 marks, and in the repressive H4K20me3 mark [94] as well as a gain in the repressive H3K27me3 mark [95]. Acetylation patterns are disrupted in colon, uterus, lung tumors, and in leukemias as a result of translocations or mutations in the genes that encode some of the HATs and HDACs (for instance, HDAC2) or due to mistargeting of the fusion products [94]. HDAC overexpression has also been observed; for example, the levels of the dedicated H4K16 HDAC SIRT1 were found to be high in hepatocellular carcinoma [96] and colon cancer [97, 98].

5.3.2.2 HMTs and HDMTs

Alterations in HMTs and HDMTs have also been shown to be involved in tumorigenesis. Mixed lineage leukemia (MLL) 1–4, SETD1A, and SETD1B are H3K4 HMTs that exist as multiprotein complexes that contain core subunits and various unique subunits including HATs, tumor suppressor gene products, mRNA-processing factors, and nuclear hormone receptors. MLLs play critical roles during development and in adult tissues; they regulate gene transcription directly by introducing the active H3K4me3 mark, and indirectly via their partnership with other chromatin remodeling complexes and co-regulators [99]. In addition, a potential role for MLL complexes in alternative splicing has been proposed [99]. Mutations in MLL1 and MLL3 genes have been reported in 59% of bladder cancer patients [100]. Chromosomal rearrangements in the *MLL1* gene occur preferentially in hematopoietic cells [101] and result in a multiplicity of fusion proteins with new properties and binding partners that contribute to the development of hematological malignancies [101]. Mutations in *MLL2* [102, 103] and MLL2 decreased expression levels as well as mutations and deletions in *MLL3* have also been reported (Table 5.1) [79, 104–106]. Deletions in MLL5, a member of the MLL family that lacks the HMT and DNA binding domains [107], have been shown in leiomyomata (benign uterine fibroids) [108] and low expression of MLL5 was associated with poorer outcome in acute myeloid leukemia (AML) patients [109]. Genomic alterations in other HMTs have also been reported; for instance, mutations in SETD2, an H3K36 HMT, were found in renal clear cell carcinoma [110].

Members of the polycomb group (Pc-G) of repressor proteins have been shown to be deregulated in cancer. The Pc-G HMT EZH2 (enhancer of zeste homologue 2), a subunit of the polycomb repressor complexes (PRC) 2 and PRC3, is not expressed in adult tissues [111]. However, it is overexpressed in several tumor types (Table 5.1) [112, 113]. EZH2 has been shown to interact with DNMTs in human cell lines, suggesting that it may also play a role in controlling DNA methylation [114]. Overexpression of BMI-1 (B lymphoma Mo-MLV insertion region 1 homolog), a component of PRC1, was also observed in a variety of tumors (Table 5.1) [115–117].

Table 5.1 Summary of epigenetic abnormalities found in cancer

Gene	Function	Alteration	Tumor type	Reference
DNMT1	DNA methylase	Mutation	7% of colorectal	[78]
DNMT3A	DNA methylase	Mutation	MDS; 22% AML	[74–77]
TET1	5'methylcytosine hydroxylase	Chromosome translocation	AML	[81]
TET2	5'methylcytosine hydroxylase	Mutation	MDS, myeloid malignancies	[82–88]
		Silencing	Gliomas	[89]
AID	5'cytidine deaminase	Aberrant expression	CML	[90]
SIRT1	H4K16 HDAC	Overexpression	HCC, colon	[94–96]
MLL1	H3K4 HMT	Mutation	Bladder TCC	[98]
		Chromosome translocation	Hematopoietic	[99]
MLL2	H3K4 HMT	Mutation	Non-Hodgkin lymphoma, B-cell lymphoma	[100, 101]
		Low expression	Prostate (primary)	[77]
MLL3	H3K4 HMT	Mutation	Bladder TCC, glioblastoma, breast, pancreas	[98, 102–104]
		Deletion	Colon	[99]
		Low expression	Prostate (primary)	[77]
MLL5	tumor suppressor gene	Deletion	Uterine fibroids	[106]
		Low expression	AML	[107]
SETD2	H3K36 HMT	Mutation	RCCC	[108]
EZH2	H3K27 HMT	Overexpression	Breast, prostate, bladder, colon, pancreas, liver, gastric, uterine tumors, melanoma, lymphoma, myeloma, and Ewing's sarcoma	[110, 111]
BMI-1	PRC1 subunit	Overexpression	Ovarian, mantle cell lymphomas, and Merkel cell carcinomas	[113–115]
NSD1	H3K36/H4K20 HMT	Chromosome rearrangement	AML	[116]
		Amplification	Lung	[117]
		Silencing	Neuroblastoma	[118]
		Low expression	Prostate (primary)	[77]

G9a	H3K9 HMT	Overexpression	HCC	[111]
LSD1	H3K4/H3K9 HDMT	Mutation	Prostate	[119]
UTX	H3K27 HDMT	Mutation	Bladder, breast, kidney, lung, pancreas, esophagus, colon, uterus, brain, hematological malignancies	[98, 108, 120]
JARID1C	H3K4 HDMT	Mutation	RCCC	[108]
JMJD2C	H3K9/H3K36 HDMT	Overexpression	Esophageal cancer	[121]
JARID1B	H3K4 HDMT	Overexpression	Testicular and breast	[121]
GASC1	H3K9	Amplification	Squamous cell carcinoma	[122]
SNF5	BAF subunit	Mutation	Kidney malignant rhabdoid tumors, atypical rhabdoid/teratoid tumors (extra-renal), epithelioid sarcomas, small cell hepatoblastomas, extraskeletal myxoid chondrosarcomas, and undifferentiated sarcomas	[130–134]
		Loss of expression	Renal medullary carcinoma, metastatic melanoma	[135]
BRG1	ATPase of BAF	Mutations	Lung, rhabdoid, medulloblastoma	[138–141]
BRM	ATPase of BAF	Low expression	Prostate	[142]
		Mutations	Basal cell carcinoma	[143, 144]
ARID1A	Subunit of BAF	Mutations	50% of ovarian clear cell carcinomas, 30% of endometrioid carcinomas, endometrial carcinomas	[150–154]
		Deletions	33% of primary pancreatic adenocarcinomas	[155]
		Genomic rearrangements	Bladder TCC	[98]
		Low expression	56% of ER-/PR-/HER2-breast tumors	[156]
ARID2	PBAF subunit	Mutation	HCC	[159]
BRD7	PBAF subunit	Mutations and deletions	Breast	[161]
		Hypermethylation	Nasopharyngeal	[165]
PBRM1	PBAF subunit	Mutations	41% RCCC, breast	[166, 167]
SRCAP	ATPase of SWR1	Overexpression	Prostate (primary)	[77]
		Low expression	Prostate (metastatic)	[77]

(continued)

Table 5.1 (continued)

Gene	Function	Alteration	Tumor type	Reference
Tip60	Acetylase of SWR1	Low expression (monoallelic expression loss)	Lymphomas, head and neck, breast	[170]
		Low expression	Colon	[171]
P400	ATPase of SWR1	Low expression	Colon	[172]
hSNF2	ATPase of RSF, ACF, CHRAC, WICH and NURF	Overexpression	AML	[175]
BPTF	NURD subunit	Amplification	55% of neuroblastomas, 27% lung tumors	[173, 174]
RBBP7/RbAp46	Helicase	Overexpression	79% of breast tumors	[175]
RBBP4/RbAp48	Helicase	Overexpression	AML	[176]
CHD4	ATPase of NURD	Mutation with loss of expression	55.7% colorectal and 56.4% gastric cancer	[178]
MTA1	NURD subunit	Overexpression	Breast, colorectal, gastric, pancreatic, ovarian, prostate, esophageal, endometrial, NSCLC, HCC, and diffuse B cell lymphoma	[49]
MTA3	NURD subunit	Loss of expression	Advanced breast carcinoma	[49]
CHD5	ATP-dependent helicase	Deletion and mutations	Ovarian, prostate, neuroblastoma, hematopoietic	[178–181]
		Hypermethylation	Gliomas, laryngeal squamous carcinoma, colon, gastric, ovarian, and breast	[178, 182–185]
CHD7	ATP-dependent helicase	Mutations	Gastric and colorectal	[177]

HCC Hepatocellular carcinoma; *NSCLC* non-small cell lung carcinoma, *AML* acute myeloid leukemia; *MDS* myelodysplastic syndromes; *CML* chronic myeloid leukemia; *TCC* transitional cell carcinoma; *RCCC* renal clear cell carcinoma; *ER* estrogen receptor; *PR* progesterone receptor; *HER2* human epidermal growth factor receptor 2; *HMT* histone methyltransferase; *HDMT* histone demethylase; *DNMT* DNA methylase, *PRC1* polycomb repressor complex 1

Other HMTs have been shown to display aberrant expression patterns or chromosome rearrangements. Nuclear receptor binding SET domain protein 1 (NSD1) has been reported to undergo chromosome rearrangements in pediatric AML [118], to be amplified in some lung cancer cases [119] and to be silenced by DNA methylation in neuroblastomas [120]. In addition, the H3K9me3 HMT G9a was found to be upregulated in hepatocellular carcinoma [113].

Lysine-specific histone demethylases, such as LSD1, lysine (K)-specific demethylase 6A (KDM6A/UTX), and Jumonji C-domain containing proteins (JARID1A-D), have been implicated in cancer progression (Table 5.1). For instance, mutations in LSD1 have been reported in prostate cancer [121], whereas KDM6A/UTX was found mutated in many tumors (Table 5.1) [100, 110, 122]. Mutations in KDM5C/JARID1C were observed in renal cell carcinoma lacking VHL [110]. In addition, overexpression of KDM4C/JMJD2C and JARID1B/PLU-1 was found in esophageal cancer and in breast and testicular tumors, respectively, whereas genomic amplification of GASC1 was observed in squamous cell carcinoma [123, 124]. Thus, mutations and aberrant expression of histone modifiers may alter or block the recruitment of chromatin remodelers and transcription factors to specific loci, thereby affecting nucleosome positioning and gene expression patterns.

5.3.3 Mutations in Genes Encoding Subunits of Chromatin Remodeler Complexes

ATPase-dependent chromatin remodeler complexes directly control the position of nucleosomes or alter their stability by introducing histone variants. Thus, aberrant expression of their subunits will cause changes in nucleosome composition, location, and stability.

5.3.3.1 SWItch/Sucrose Non-fermenting

Because of their important role in controlling fundamental processes such as cell proliferation, migration, and differentiation [27], the aberrant expression of SWI/SNF components will have profound effects on cell function. Indeed, mutations in several subunits have been recently identified in tumors of various origins. Since genomic instability is largely absent in tumors harboring defective SWI/SNF complexes, it is likely that perturbations in nucleosome positioning, misslocalization, and excessive formation of complexes with opposing functions contribute to the development of these aggressive cancers [27].

The SWI/SNF subunit SNF5 helps recruit this complex to specific genomic sites and is required for the expression of genes associated with cell proliferation, including *P53* and the cell cycle inhibitor *p16INK4a* [125–127], adipocyte differentiation [128], and inhibition of cell migration [129]. *SNF5* loss, however, does not result in genome instability [130] nor does it inactivate SWI/SNF complexes completely, as

tumorigenesis in the absence of *SNF5* is dependent on BRG1 activity [131]. Thus, it is thought that tumorigenesis arises from aberrant activity of the remaining complexes [131]. *SNF5* mutations have been found in rhabdoid and other tumors (Table 5.1) [132–136]. Loss of the SNF5 protein was also observed in renal medullary carcinomas and in advanced and metastatic melanomas, where it correlated with poor survival rates [137].

Although complexes containing the catalytic subunits BRM or BRG1 display some functional redundancy, they also play distinct roles [27, 28]. *BRG1* mutations have been shown to occur in cancer cell lines of various origins [138, 139] and in primary lung tumors [140, 141], medulloblastoma [142], and rhabdoid tumors [143]. Reduced BRM protein levels occur in prostate tumors [144], and mutations have been found in basal cell carcinoma [145, 146]. In addition, BRM has been shown to be postranslationally regulated in cancer cell lines [28].

BAF250A/ARID1A binds to DNA without sequence specificity [147, 148] and its recruitment represses the expression of cell cycle-related genes in differentiated mouse calvaria cells [149, 150]. In addition, BAF250A/ARID1A is required for normal cell cycle arrest in senescent human fibroblasts [151]. *ARID1A/BAF250a* mutations have been recently described in ovarian clear cell [152–154] and endometrioid carcinomas (Table 5.1) [153]. Frequent mutations in low- and high grade endometrial carcinomas have also been observed [155, 156]. Heterozygous deletions and mutations in *ARID1A/BAF250a* have been reported to exist in 33% of primary pancreatic adenocarcinomas [157]. Genetic aberrations in *ARID1A* were recently reported in transitional cell carcinoma (TCC) of the bladder [100] and low ARID1A expression was found to be significantly associated with larger tumor size and grade and the ER-/PR-/HER2-phenotype in breast cancer cases (Table 5.1) [158]. ARID1A/BAF250a expression was also found to be severely reduced in breast (T47D), renal clear cell (Caki-1 and Caki-2), and cervical (C33A) cancer cell lines [159]. BAF250b/ARID1B containing complexes include components of an E3 ubiquitin ligase that was found to target H2BK20 for monoubiquitination in a nucleosomal context, an upstream event for trimethylation of H3K4 and gene activation [160]. BAF250b/ARID1B and BAF250a/ARID1A have also been shown to play opposing roles in the control of cell cycle genes in osteoblast differentiation in mice [149, 150]; however, no mutations in human *BAF250b/ARID1B* have been described to date. In contrast, inactivating mutations in *ARID2*, which encodes a component of PBAF that facilitates transcriptional activation by nuclear receptors, have been reported in four subtypes of hepatocellular carcinomas (HCC) [161].

BRD7 and BAF180/PBRM1 are regulators of replicative senescence in human cells by controlling P53 transcriptional activity towards a subset of its target genes required for replicative and oncogenic stress senescence induction [162]. BRD7 has also been shown to either activate or repress the expression of a number of genes by protein–protein interaction. BRD7 physically interacts with P53 and the acetylase P300 [162, 163], disheveled-1 [164], and TRIM24 [165], as well as with BRCA1 thereby regulating genes involved in DNA repair [166]. *BRD7* deletions and reduced expression levels have been observed in breast tumors [163] (Table 5.1). In addition, the *BRD7* promoter has also been shown to be silenced by DNA methylation in

nasopharyngeal cancer cell lines and tumors [167]. Mutations in *BAF180/PBRM1* have been recently described in renal clear cell carcinomas [168] and breast tumors (Table 5.1) [169]. BAF57 is required to maintain the proper subunit composition of the PBAF complex and to regulate the transcription of a subset of cell cycle-related genes in Hela cells [170]. Thus far, loss of *BAF57* has only been reported in the breast cancer cell line BT-549 [171]. Thus, aberrant expression of SWI/SNF subunits is a frequent event in a variety of cancer types. Although SWI/SNF complexes control nucleosome positioning, the extent of the changes caused by the mutation of specific subunits remains to be elucidated.

5.3.3.2 INO80 and SWR1

Deregulated expression of the subunits of these complexes may affect H2A.Z deposition and nucleosome dynamics as well as nucleosome position and DNA repair. SRCAP deregulated expression has been found in primary and metastatic prostate cancer, although the mechanisms underlying such dysregulation are unclear [79]. Monoallelic loss of the acetylase Tip60 (a subunit of TRAAP/Tip60/p400) has been reported in lymphomas, and head-and-neck and mammary carcinomas, with decreased mRNA and protein expression levels, suggesting that critical levels of Tip60 are required for normal cell function [172]. Tip60 and P400 expression is also decreased in colorectal tumors compared to normal colon, although no mutations were found in these two genes [173]. Finally, single nucleotide polymorphisms (SNPs) in Tip49a/RUVBL1 have been recently associated with higher risk of serous epithelial ovarian cancer [174].

5.3.3.3 RSF, ACF, CHRAC, WICH, and NURF

To date no mutations in the ATPase subunits of ISWI complexes have been described. However, genomic amplification of bromodomain PHD finger transcription factor (BPTF), a subunit of NURD, has been reported in neuroblastomas and lung cancer cases (Table 5.1) [175, 176]. In addition, increased expression of other subunits of the NURF complex, including Retinoblastoma-related protein 46 (RBBP7/RbAp46), as well as Retinoblastoma-related protein 48 (RBBP4/RbAp48) and hSNF2 have been reported in breast carcinomas [177] and in AML [178], respectively (Table 5.1).

5.3.3.4 NURD

Mutations and loss of expression of the CHD4 ATPase subunit have been recently described in colorectal and gastric cancers (Table 5.1) [179]. MTA1 expression is high in a number of cancer types (Table 5.1) [50]. In contrast, MTA3 expression is lost in advanced breast epithelial carcinoma (Table 5.1) [50].

5.3.3.5 Mutations in Other CHD Proteins

Recent studies have identified the helicase CHD5 as a tumor suppressor involved in the transactivation of *p16Ink4a/p19arf* and deleted or mutated in ovarian and prostate cancer [180, 181], neuroblastomas [182], and hematopoietic malignancies [183]. Silencing of the *CHD5* promoter by DNA hypermethylation has also been observed in various tumor types (Table 5.1) [180, 184–187]. CHD7 plays a role in pluripotency [25] and mutations in CHD7 have been found in more than 50% of the cases of CHARGE syndrome, which is characterized by nonrandom congenital abnormalities in several tissues [188, 189]. In addition, gastric and colorectal cancers also showed mutations in *CHD7* [179].

5.4 Epigenetic Switching

The gene silencing events that take place during tumorigenesis as a consequence of aberrant DNA methylation or histone modification result in a reduction of cellular plasticity. A subset of genes becomes repressed by the action of Pc-G proteins through the establishment of the H3K27me3 mark at their promoters when stem cells differentiate into developmental lineages [2]. After differentiation, this mark and, thus, the repressive state are maintained by the action of EZH2. In cancer cells, H3K27me3 is replaced by de novo DNA methylation likely through the recruitment of DNMTs [114, 190–192]. This process is termed "epigenetic switching" and results in permanent silencing of genes that may be implicated in tumorigenesis by locking nucleosome positions.

5.5 Epigenetic Therapy and Gene Reactivation

Epigenetic therapy aims to reverse epigenetic aberrations that occur in cancer in order to restore a more normal epigenetic state [55]. The first characterized DNA methylation inhibitors, namely 5-Azacitidine (5′-aza-CR, Azacitidine) and 5-aza-2-deoxy-cytidine (5′-aza-CdR, Decitabine) [193], are incorporated into the DNA of proliferating cells during DNA replication and inhibit DNA methylation by trapping DNMTs onto the DNA, leading to their depletion [2, 56]. The resulting DNA hypomethylation causes nucleosome depletion at the promoters of tumor suppressor genes that were silenced during tumorigenesis, leading to gene reactivation and growth arrest [2, 65]. Azacitidine and decitabine have been approved by the FDA for the treatment of myelodysplastic syndromes and have shown great promise in the treatment of AML and myeloid leukemia [194]. Decitabine has also been tested in clinical trials for the treatment of epithelial ovarian cancer, alone or in combination with chemotherapy [195]. These studies have shown that combination therapies are more effective, particularly in patients with platinum resistance, likely due to re-sensitization [195]. Clinical applications for Zebularine, a newer generation

DNMT inhibitor that can be orally administered, are currently under investigation [196]. Alternative approaches include small molecule DNMT inhibitors, such as SGI110, RG108, and MG98, which block DNMT enzyme activity or target regulatory messenger RNA sequences [2].

Loss of histone acetylation at promoter regions occurs concomitant to DNA hypermethylation, and therefore HDAC inhibitors (HDACI) have also been tested as potential therapeutic agents. HDACIs induce growth arrest, apoptosis, cell differentiation, and tumor suppressor gene reactivation. Suberoylanilide hydroxamic acid (SAHA, Vorinostat) has been recently approved for the treatment of T-cell cutaneous lymphoma [197]; however, it was not successful for the treatment of recurrent ovarian cancer [195]. Treatment with another HDACI, belinostat (PDX, 101), has shown to lead to disease stabilization in patients with different malignancies, including sarcoma, renal cancer, thymoma and melanoma, and ovarian cancer [195]. Other HDACIs are currently under investigation [2, 197]. The lysine HMT inhibitors described to date, chaetocin, DZNep, and BIX-01294, have shown some antitumor properties in vitro [197]. Combined epigenetic therapies have also been tested; for instance, chemotherapeutic agents have been successfully used in combination with HDAC, SIRT, DNMT inhibitors [197]. Thus, epigenetic drugs currently in use or under investigation target histone modifiers or DNMTs to restore chromatin plasticity, thereby affecting nucleosome positioning in an indirect manner. Targeting subunits of the ATPase-dependent chromatin remodeler complexes may provide a more efficient and direct way to restore nucleosome position and composition.

5.6 Challenges and Future Prospects

In recent years, high-throughput technologies have been successfully applied to the field of epigenetics allowing for the mapping of histone modifications, proteins binding to DNA, nucleosome positioning, and DNA methylation. The emerging picture is that nucleosome positioning and occupancy is determined by the combined action of DNA sequence, transcription factors, and chromatin remodelers, and that the resulting nucleosome configuration has direct effects in sequence accessibility and gene transcription (Fig. 5.1). Recent studies show that the genes more frequently mutated in various types of cancers encode for subunits of chromatin remodeler complexes [197], further highlighting the relevance of nucleosome positioning in tumorigenesis (Fig. 5.1). As most of these genes regulate multiple cellular processes, they are likely to be important therapeutic targets.

Although the wealth of information generated by epigenomic studies has greatly improved our understanding of chromatin regulation, the integration of epigenetic, genetic, and transcriptional changes will be essential to advance our knowledge of cancer development and progression. Several challenges lay ahead as we explore further the development of epigenetic therapies, although a combinatorial approach holds promise. Key issues to be resolved include type of agent combinations and optimal doses, agent specificity, the sequence of agent delivery, and the method of

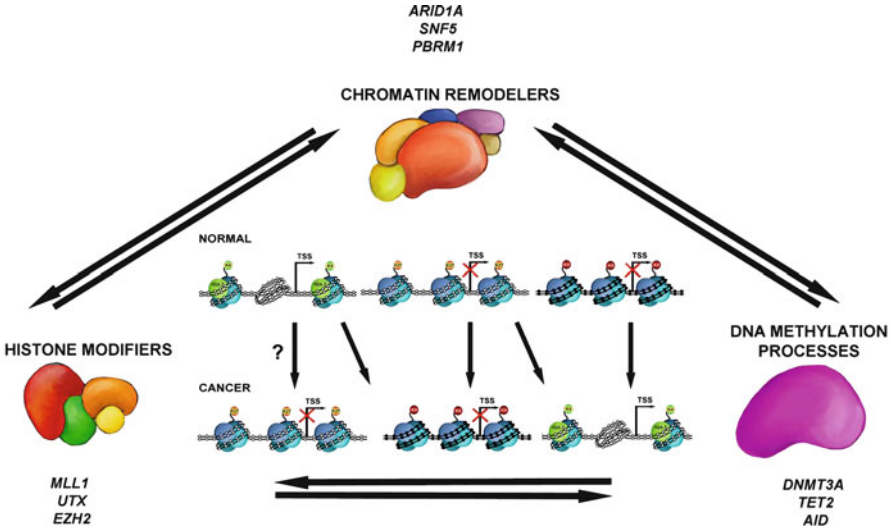

Fig. 5.1 The emerging picture is that nucleosome positioning and occupancy, which is influenced by chromatin remodelers, histone modifiers and DNA methylating enzymes, has direct effects in sequence accessibility and gene transcription and that. In normal cells, gene promoter regions exist in three epigenetic states: open (left), which shows nucleosome depletion at the transcriptional start site (TSS) and contains active histone marks (e.g. H3K4me3) and the histone variant H2A.Z; repressed (center), which shows nucleosome occupancy at the TSS and contains repressive histone marks (e.g. H3K27me3); or silenced (right), which shows nucleosome occupancy at the TSS and DNA methylation, and contains silencing histone marls (e.g.H3K9me3). These epigenetic states correlate with transcriptional activity (left) or lack thereof (center and right). In cancer, epigenetic states are altered, and active promoters may become silenced by DNA methylation, or, potentially, repressed; repressed promoters may become reactivated or silenced by DNA methylation; and silenced promoters may become reactivated, thereby causing profound changes in gene expression patterns. Recent studies show that the genes more frequently mutated in various types of cancers encode for subunits of chromatin remodeler complexes (e.g. *ARID1A, SNF5, PBRM1*), histone modifying enzymes (e.g. *MLL1, UTX, EZH2*) or enzymes involved in the DNA methylation pathway (e.g. *DNMT3A, TET2, AID*). These studies provide evidence for a link between genetic mutation and epigenetic alterations

delivery. Given the current multi-institutional and multinational efforts to map the human epigenome in all cancer types, it is likely that therapeutic development will be significantly advanced in the near future.

References

1. Luger K, Mader AW, Richmond RK, Sargent DF, Richmond TJ (1997) Crystal structure of the nucleosome core particle at 2.8 A resolution. Nature 389:251–260
2. Sharma S, Kelly TK, Jones PA (2010) Epigenetics in cancer. Carcinogenesis 31:27–36
3. Deaton AM, Bird A (2011) CpG islands and the regulation of transcription. Genes Dev 25:1010–1022

4. Segal E, Widom J (2009) What controls nucleosome positions? Trends Genet 25:335–343
5. Sadeh R, Allis CD (2011) Genome-wide "re"-modeling of nucleosome positions. Cell 147:263–266
6. Bettecken T, Frenkel ZM, Trifonov EN (2011) Human nucleosomes: special role of CG dinucleotides and Alu-nucleosomes. BMC Genomics 12:273
7. Tillo D et al (2010) High nucleosome occupancy is encoded at human regulatory sequences. PLoS One 5:e9129
8. Tanaka Y, Yamashita R, Suzuki Y, Nakai K (2010) Effects of Alu elements on global nucleosome positioning in the human genome. BMC Genomics 11:309
9. Valouev A et al (2011) Determinants of nucleosome organization in primary human cells. Nature 474:516–520
10. Zhang Z et al (2011) A packing mechanism for nucleosome organization reconstituted across a eukaryotic genome. Science 332:977–980
11. Han H et al (2011) DNA methylation directly silences genes with non-CpG island promoters and establishes a nucleosome occupied promoter. Hum Mol Genet 20(22):4299–4310
12. Hattori N et al (2004) Epigenetic control of mouse Oct-4 gene expression in embryonic stem cells and trophoblast stem cells. J Biol Chem 279:17063–17069
13. Futscher BW et al (2002) Role for DNA methylation in the control of cell type specific maspin expression. Nat Genet 31:175–179
14. You JS et al (2011) OCT4 establishes and maintains nucleosome-depleted regions that provide additional layers of epigenetic regulation of its target genes. Proc Natl Acad Sci USA 108:14497–14502 [AU4]
15. Taberlay PC, Jones PA (2010) DNA methylation and cancer. Prog Drug Res 67:1–23
16. Wolff EM et al (2010) Hypomethylation of a LINE-1 promoter activates an alternate transcript of the MET oncogene in bladders with cancer. PLoS Genet 6:e1000917
17. Sharma S, De Carvalho DD, Jeong S, Jones PA, Liang G (2011) Nucleosomes containing methylated DNA stabilize DNA methyltransferases 3A/3B and ensure faithful epigenetic inheritance. PLoS Genet 7:e1001286
18. Jones PA, Liang G (2009) Rethinking how DNA methylation patterns are maintained. Nat Rev Genet 10:805–811
19. Chodavarapu RK et al (2010) Relationship between nucleosome positioning and DNA methylation. Nature 466:388–392
20. Jeong S et al (2009) Selective anchoring of DNA methyltransferases 3A and 3B to nucleosomes containing methylated DNA. Mol Cell Biol 29:5366–5376
21. Mills AA (2010) Throwing the cancer switch: reciprocal roles of polycomb and trithorax proteins. Nat Rev Cancer 10:669–682
22. Bell O, Tiwari VK, Thoma NH, Schubeler D (2011) Determinants and dynamics of genome accessibility. Nature reviews 12:554–564
23. Berman BP, Frenkel B, Coetzee GA, Jia L (2010) Androgen receptor responsive enhancers are flanked by consistently-positioned H3-acetylated nucleosomes. Cell Cycle 9:2249–2250
24. Kapoor-Vazirani P, Kagey JD, Powell DR, Vertino PM (2008) Role of hMOF-dependent histone H4 lysine 16 acetylation in the maintenance of TMS1/ASC gene activity. Cancer Res 68:6810–6821
25. Hargreaves DC, Crabtree GR (2011) ATP-dependent chromatin remodeling: genetics, genomics and mechanisms. Cell Res 21:396–420
26. Clapier CR, Cairns BR (2009) The biology of chromatin remodeling complexes. Annu Rev Biochem 78:273–304
27. Wilson BG, Roberts CW (2011) SWI/SNF nucleosome remodellers and cancer. Nat Rev Cancer 11:481–492
28. Reisman D, Glaros S, Thompson EA (2009) The SWI/SNF complex and cancer. Oncogene 28:1653–1668
29. Pal S, Sif S (2007) Interplay between chromatin remodelers and protein arginine methyltransferases. J Cell Physiol 213:306–315

30. Choi HK et al (2007) The functional role of the CARM1-SNF5 complex and its associated HMT activity in transcriptional activation by thyroid hormone receptor. Exp Mol Med 39:544–555
31. Morrison AJ, Shen X (2009) Chromatin remodelling beyond transcription: the INO80 and SWR1 complexes. Nat Rev Mol Cell Biol 10:373–384
32. Hur SK et al (2010) Roles of human INO80 chromatin remodeling enzyme in DNA replication and chromosome segregation suppress genome instability. Cell Mol Life Sci 67: 2283–2296
33. van Attikum H, Gasser SM (2009) Crosstalk between histone modifications during the DNA damage response. Trends Cell Biol 19:207–217
34. Park EJ, Hur SK, Kwon J (2010) Human INO80 chromatin-remodelling complex contributes to DNA double-strand break repair via the expression of Rad54B and XRCC3 genes. Biochem J 431:179–187
35. Kashiwaba S et al (2010) The mammalian INO80 complex is recruited to DNA damage sites in an ARP8 dependent manner. Biochem Biophys Res Commun 402:619–625
36. Ruhl DD et al (2006) Purification of a human SRCAP complex that remodels chromatin by incorporating the histone variant H2A.Z into nucleosomes. Biochemistry 45:5671–5677
37. Wong MM, Cox LK, Chrivia JC (2007) The chromatin remodeling protein, SRCAP, is critical for deposition of the histone variant H2A.Z at promoters. J Biol Chem 282:26132–26139
38. Yang X et al (2012) Gene reactivation by 5-Aza-2'-deoxycytidine requires H2A.z insertion to establish but not to maintain nucleosome depleted regions. PLoS Genet 8:e1002604
39. Xu Y et al (2010) The p400 ATPase regulates nucleosome stability and chromatin ubiquitination during DNA repair. J Cell Biol 191:31–43
40. Gevry N et al (2009) Histone H2A.Z is essential for estrogen receptor signaling. Genes Dev 23:1522–1533
41. Slupianek A, Yerrum S, Safadi FF, Monroy MA (2010) The chromatin remodeling factor SRCAP modulates expression of prostate specific antigen and cellular proliferation in prostate cancer cells. J Cell Physiol 224:369–375
42. Svotelis A, Gevry N, Gaudreau L (2009) Regulation of gene expression and cellular proliferation by histone H2A.Z. Biochem Cell Biol 87:179–188
43. Ong CT, Corces VG (2011) Enhancer function: new insights into the regulation of tissue-specific gene expression. Nature reviews 12:283–293
44. Kelly TK et al (2010) H2A.Z maintenance during mitosis reveals nucleosome shifting on mitotically silenced genes. Mol Cell 39:901–911
45. Valdes-Mora F et al (2011) Acetylation of H2A.Z is a key epigenetic modification associated with gene deregulation and epigenetic remodeling in cancer. Genome Res 20(22):4299–4310
46. Zemach A, McDaniel IE, Silva P, Zilberman D (2010) Genome-wide evolutionary analysis of eukaryotic DNA methylation. Science 328:916–919
47. Zilberman D, Coleman-Derr D, Ballinger T, Henikoff S (2008) Histone H2A.Z and DNA methylation are mutually antagonistic chromatin marks. Nature 456:125–129
48. Li X et al (2011) Chromatin boundaries require functional collaboration between the hSET1 and NURF complexes. Blood 118:1386–1394
49. Sims JK, Wade PA (2011) Mi-2/NuRD complex function is required for normal S phase progression and assembly of pericentric heterochromatin. Mol Biol Cell 22:3094–3102
50. Lai AY, Wade PA (2011) Cancer biology and NuRD: a multifaceted chromatin remodelling complex. Nat Rev Cancer 11:588–596
51. Andreu-Vieyra C et al (2011) Dynamic nucleosome-depleted regions at androgen receptor enhancers in the absence of ligand in prostate cancer cells. Mol Cell Biol 31:4648–4662
52. Lidor Nili E et al (2010) p53 binds preferentially to genomic regions with high DNA-encoded nucleosome occupancy. Genome Res 20:1361–1368
53. Jones PA, Baylin SB (2002) The fundamental role of epigenetic events in cancer. Nature reviews 3:415–428
54. Jones PA, Baylin SB (2007) The epigenomics of cancer. Cell 128:683–692

55. Baylin SB, Jones PA (2011) A decade of exploring the cancer epigenome—biological and translational implications. Nat Rev Cancer 11:726–734
56. Egger G, Liang G, Aparicio A, Jones PA (2004) Epigenetics in human disease and prospects for epigenetic therapy. Nature 429:457–463
57. Jones PA, Laird PW (1999) Cancer epigenetics comes of age. Nat Genet 21:163–167
58. Xu W et al (2004) A methylation-mediator complex in hormone signaling. Genes Dev 18:144–156
59. Geiman TM et al (2004) DNMT3B interacts with hSNF2H chromatin remodeling enzyme, HDACs 1 and 2, and components of the histone methylation system. Biochem Biophys Res Commun 318:544–555
60. Geiman TM et al (2004) Isolation and characterization of a novel DNA methyltransferase complex linking DNMT3B with components of the mitotic chromosome condensation machinery. Nucleic Acids Res 32:2716–2729
61. Sunami E, de Maat M, Vu A, Turner RR, Hoon DS (2011) LINE-1 hypomethylation during primary colon cancer progression. PLoS One 6:e18884
62. Hatziapostolou M, Iliopoulos D (2011) Epigenetic aberrations during oncogenesis. Cell Mol Life Sci 68:1681–1702
63. Xu GL et al (1999) Chromosome instability and immunodeficiency syndrome caused by mutations in a DNA methyltransferase gene. Nature 402:187–191
64. Jelinic P, Shaw P (2007) Loss of imprinting and cancer. J Pathol 211:261–268
65. Lin JC et al (2007) Role of nucleosomal occupancy in the epigenetic silencing of the MLH1 CpG island. Cancer Cell 12:432–444
66. Tsuruta T et al (2011) miR-152 is a tumor suppressor microRNA that is silenced by DNA hypermethylation in endometrial cancer. Cancer Res 71(20):6450–6462
67. Agirre X et al (2009) Epigenetic silencing of the tumor suppressor microRNA Hsa-miR-124a regulates CDK6 expression and confers a poor prognosis in acute lymphoblastic leukemia. Cancer Res 69:4443–4453
68. Balaguer F et al (2010) Epigenetic silencing of miR-137 is an early event in colorectal carcinogenesis. Cancer Res 70:6609–6618
69. Zhang S et al (2011) Downregulation of miR-132 by promoter methylation contributes to pancreatic cancer development. Carcinogenesis 32:1183–1189
70. Huang YW et al (2009) Epigenetic repression of microRNA-129-2 leads to overexpression of SOX4 oncogene in endometrial cancer. Cancer Res 69:9038–9046
71. Wong KY et al (2011) Epigenetic inactivation of the miR-124-1 in haematological malignancies. PLoS One 6:e19027
72. Saito Y et al (2006) Specific activation of microRNA-127 with downregulation of the proto-oncogene BCL6 by chromatin-modifying drugs in human cancer cells. Cancer Cell 9:435–443
73. Dudziec E et al (2011) Hypermethylation of CpG islands and shores around specific microRNAs and mirtrons is associated with the phenotype and presence of bladder cancer. Clin Cancer Res 17:1287–1296
74. Kozaki K, Imoto I, Mogi S, Omura K, Inazawa J (2008) Exploration of tumor-suppressive microRNAs silenced by DNA hypermethylation in oral cancer. Cancer Res 68:2094–2105
75. Suzuki H et al (2010) Methylation-associated silencing of microRNA-34b/c in gastric cancer and its involvement in an epigenetic field defect. Carcinogenesis 31:2066–2073
76. Yan XJ et al (2011) Exome sequencing identifies somatic mutations of DNA methyltransferase gene DNMT3A in acute monocytic leukemia. Nat Genet 43:309–315
77. Tiu RV, Visconte V, Traina F, Schwandt A, Maciejewski JP (2011) Updates in cytogenetics and molecular markers in MDS. Curr Hematol Malig Rep 6:126–135
78. Tan PT, Wei AH (2011) The epigenomics revolution in myelodysplasia: a clinico-pathological perspective. Pathology 43:536–546
79. Bianco-Miotto T et al (2010) Global levels of specific histone modifications and an epigenetic gene signature predict prostate cancer progression and development. Cancer Epidemiol Biomarkers Prev 19:2611–2622

80. Kanai Y, Ushijima S, Nakanishi Y, Sakamoto M, Hirohashi S (2003) Mutation of the DNA methyltransferase (DNMT) 1 gene in human colorectal cancers. Cancer Lett 192:75–82
81. Miremadi A, Oestergaard MZ, Pharoah PD, Caldas C (2007) Cancer genetics of epigenetic genes. Hum Mol Genet 16(Spec No 1):R28–R49
82. Bhutani N et al (2011) Reprogramming towards pluripotency requires AID-dependent DNA demethylation. Nature 463:1042–1047
83. Guo JU, Su Y, Zhong C, Ming GL, Song H (2011) Emerging roles of TET proteins and 5-hydroxymethylcytosines in active DNA demethylation and beyond. Cell Cycle 10:2662–2668
84. Mullighan CG (2009) TET2 mutations in myelodysplasia and myeloid malignancies. Nat Genet 41:766–767
85. Jardin F et al (2011) TET2 and TP53 mutations are frequently observed in blastic plasmacytoid dendritic cell neoplasm. Br J Haematol 153:413–416
86. Quivoron C et al (2011) TET2 inactivation results in pleiotropic hematopoietic abnormalities in mouse and is a recurrent event during human lymphomagenesis. Cancer Cell 20:25–38
87. Bacher U et al (2010) Mutations of the TET2 and CBL genes: novel molecular markers in myeloid malignancies. Ann Hematol 89:643–652
88. Tefferi A et al (2009) Frequent TET2 mutations in systemic mastocytosis: clinical, KITD816V and FIP1L1-PDGFRA correlates. Leukemia 23:900–904
89. Tefferi A et al (2009) Detection of mutant TET2 in myeloid malignancies other than myeloproliferative neoplasms: CMML, MDS, MDS/MPN and AML. Leukemia 23:1343–1345
90. Tefferi A et al (2009) TET2 mutations and their clinical correlates in polycythemia vera, essential thrombocythemia and myelofibrosis. Leukemia 23:905–911
91. Kim YH et al (2011) TET2 promoter methylation in low-grade diffuse gliomas lacking IDH1/2 mutations. J Clin Pathol 64(10):850–852
92. Klemm L et al (2009) The B cell mutator AID promotes B lymphoid blast crisis and drug resistance in chronic myeloid leukemia. Cancer Cell 16:232–245
93. Tan AY, Manley JL (2009) The TET family of proteins: functions and roles in disease. J Mol Cell Biol 1:82–92
94. Esteller M (2007) Cancer epigenomics: DNA methylomes and histone-modification maps. Nature reviews 8:286–298
95. Kondo Y et al (2008) Gene silencing in cancer by histone H3 lysine 27 trimethylation independent of promoter DNA methylation. Nat Genet 40:741–750
96. Choi HN et al (2011) Expression and role of SIRT1 in hepatocellular carcinoma. Oncol Rep 26:503–510
97. Stunkel W et al (2007) Function of the SIRT1 protein deacetylase in cancer. Biotechnol J 2:1360–1368
98. Kim YR, Kim SS, Yoo NJ, Lee SH (2010) Frameshift mutation of SIRT1 gene in gastric and colorectal carcinomas with microsatellite instability. APMIS 118:81–82
99. Ansari KI, Mandal SS (2010) Mixed lineage leukemia: roles in gene expression, hormone signaling and mRNA processing. FEBS J 277:1790–1804
100. Gui Y et al (2011) Frequent mutations of chromatin remodeling genes in transitional cell carcinoma of the bladder. Nat Genet 43:875–878
101. Marschalek R (2010) Mixed lineage leukemia: roles in human malignancies and potential therapy. FEBS J 277:1822–1831
102. Morin RD et al (2011) Frequent mutation of histone-modifying genes in non-Hodgkin lymphoma. Nature 476:298–303
103. Shaknovich R, Melnick A (2011) Epigenetics and B-cell lymphoma. Curr Opin Hematol 18:293–299
104. Ashktorab H et al (2010) Distinct genetic alterations in colorectal cancer. PLoS One 5:e8879
105. Balakrishnan A et al (2007) Novel somatic and germline mutations in cancer candidate genes in glioblastoma, melanoma, and pancreatic carcinoma. Cancer Res 67:3545–3550

106. Wang XX et al (2011) Somatic mutations of the mixed-lineage leukemia 3 (MLL3) gene in primary breast cancers. Pathol Oncol Res 17:429–433
107. Liu H, Westergard TD, Hsieh JJ (2009) MLL5 governs hematopoiesis: a step closer. Blood 113:1395–1396
108. Hodge JC et al (2009) Identifying the molecular signature of the interstitial deletion 7q subgroup of uterine leiomyomata using a paired analysis. Genes Chromosomes Cancer 48:865–885
109. Damm F et al (2011) Prognostic importance of histone methyltransferase MLL5 expression in acute myeloid leukemia. J Clin Oncol 29:682–689
110. Dalgliesh GL et al (2010) Systematic sequencing of renal carcinoma reveals inactivation of histone modifying genes. Nature 463:360–363
111. Portela A, Esteller M (2010) Epigenetic modifications and human disease. Nat Biotechnol 28:1057–1068
112. Tsang DP, Cheng AS (2010) Epigenetic regulation of signaling pathways in cancer: role of the histone methyltransferase EZH2. J Gastroenterol Hepatol 26:19–27
113. Kondo Y et al (2007) Alterations of DNA methylation and histone modifications contribute to gene silencing in hepatocellular carcinomas. Hepatol Res 37:974–983
114. Vire E et al (2006) The Polycomb group protein EZH2 directly controls DNA methylation. Nature 439:871–874
115. Yang GF et al (2010) Intensive expression of Bmi-1 is a new independent predictor of poor outcome in patients with ovarian carcinoma. BMC Cancer 10:133
116. Bea S et al (2001) BMI-1 gene amplification and overexpression in hematological malignancies occur mainly in mantle cell lymphomas. Cancer Res 61:2409–2412
117. Brunner M et al (2008) Expression of VEGF-A/C, VEGF-R2, PDGF-alpha/beta, c-kit, EGFR, Her-2/Neu, Mcl-1 and Bmi-1 in Merkel cell carcinoma. Mod Pathol 21:876–884
118. Jaju RJ et al (2001) A novel gene, NSD1, is fused to NUP98 in the t(5;11)(q35;p15.5) in de novo childhood acute myeloid leukemia. Blood 98:1264–1267
119. Job B et al (2010) Genomic aberrations in lung adenocarcinoma in never smokers. PLoS One 5:e15145
120. Berdasco M et al (2009) Epigenetic inactivation of the Sotos overgrowth syndrome gene histone methyltransferase NSD1 in human neuroblastoma and glioma. Proc Natl Acad Sci USA 106:21830–21835
121. Kahl P et al (2006) Androgen receptor coactivators lysine-specific histone demethylase 1 and four and a half LIM domain protein 2 predict risk of prostate cancer recurrence. Cancer Res 66:11341–11347
122. van Haaften G et al (2009) Somatic mutations of the histone H3K27 demethylase gene UTX in human cancer. Nat Genet 41:521–523
123. Shi Y (2007) Histone lysine demethylases: emerging roles in development, physiology and disease. Nature reviews 8:829–833
124. Liu G et al (2009) Genomic amplification and oncogenic properties of the GASC1 histone demethylase gene in breast cancer. Oncogene 28:4491–4500
125. Xu Y, Yan W, Chen X (2010) SNF5, a core component of the SWI/SNF complex, is necessary for p53 expression and cell survival, in part through eIF4E. Oncogene 29:4090–4100
126. Oruetxebarria I et al (2004) P16INK4a is required for hSNF5 chromatin remodeler-induced cellular senescence in malignant rhabdoid tumor cells. J Biol Chem 279:3807–3816
127. Sansam CG, Roberts CW (2006) Epigenetics and cancer: altered chromatin remodeling via Snf5 loss leads to aberrant cell cycle regulation. Cell Cycle 5:621–624
128. Caramel J, Medjkane S, Quignon F, Delattre O (2008) The requirement for SNF5/INI1 in adipocyte differentiation highlights new features of malignant rhabdoid tumors. Oncogene 27:2035–2044
129. Caramel J, Quignon F, Delattre O (2008) RhoA-dependent regulation of cell migration by the tumor suppressor hSNF5/INI1. Cancer Res 68:6154–6161
130. McKenna ES et al (2008) Loss of the epigenetic tumor suppressor SNF5 leads to cancer without genomic instability. Mol Cell Biol 28:6223–6233

131. Wang X et al (2009) Oncogenesis caused by loss of the SNF5 tumor suppressor is dependent on activity of BRG1, the ATPase of the SWI/SNF chromatin remodeling complex. Cancer Res 69:8094–8101
132. Gadd S, Sredni ST, Huang CC, Perlman EJ (2010) Rhabdoid tumor: gene expression clues to pathogenesis and potential therapeutic targets. Lab Invest 90:724–738
133. Modena P et al (2005) SMARCB1/INI1 tumor suppressor gene is frequently inactivated in epithelioid sarcomas. Cancer Res 65:4012–4019
134. Kreiger PA et al (2009) Loss of INI1 expression defines a unique subset of pediatric undifferentiated soft tissue sarcomas. Mod Pathol 22:142–150
135. Trobaugh-Lotrario AD, Tomlinson GE, Finegold MJ, Gore L, Feusner JH (2009) Small cell undifferentiated variant of hepatoblastoma: adverse clinical and molecular features similar to rhabdoid tumors. Pediatr Blood Cancer 52:328–334
136. Cheng JX et al (2008) Renal medullary carcinoma: rhabdoid features and the absence of INI1 expression as markers of aggressive behavior. Mod Pathol 21:647–652
137. Lin H, Wong RP, Martinka M, Li G (2009) Loss of SNF5 expression correlates with poor patient survival in melanoma. Clin Cancer Res 15:6404–6411
138. Medina PP et al (2005) Transcriptional targets of the chromatin-remodelling factor SMARCA4/BRG1 in lung cancer cells. Hum Mol Genet 14:973–982
139. Wong AK et al (2000) BRG1, a component of the SWI-SNF complex, is mutated in multiple human tumor cell lines. Cancer Res 60:6171–6177
140. Rodriguez-Nieto S, Sanchez-Cespedes M (2009) BRG1 and LKB1: tales of two tumor suppressor genes on chromosome 19p and lung cancer. Carcinogenesis 30:547–554
141. Medina PP et al (2004) Genetic and epigenetic screening for gene alterations of the chromatin-remodeling factor, SMARCA4/BRG1, in lung tumors. Genes Chromosomes Cancer 41:170–177
142. Parsons DW et al (2010) The genetic landscape of the childhood cancer medulloblastoma. Science 331:435–439
143. Schneppenheim R et al (2010) Germline nonsense mutation and somatic inactivation of SMARCA4/BRG1 in a family with rhabdoid tumor predisposition syndrome. Am J Hum Genet 86:279–284
144. Sun A et al (2007) Aberrant expression of SWI/SNF catalytic subunits BRG1/BRM is associated with tumor development and increased invasiveness in prostate cancers. Prostate 67:203–213
145. de Zwaan SE, Haass NK (2010) Genetics of basal cell carcinoma. Australas J Dermatol 51:81–92; quiz 93–94
146. Moloney FJ et al (2009) Hotspot mutation of Brahma in non-melanoma skin cancer. J Invest Dermatol 129:1012–1015
147. Patsialou A, Wilsker D, Moran E (2005) DNA-binding properties of ARID family proteins. Nucleic Acids Res 33:66–80
148. Dallas PB et al (2000) The human SWI-SNF complex protein p270 is an ARID family member with non-sequence-specific DNA binding activity. Mol Cell Biol 20:3137–3146
149. Nagl NG Jr, Zweitzig DR, Thimmapaya B, Beck GR Jr, Moran E (2006) The c-myc gene is a direct target of mammalian SWI/SNF-related complexes during differentiation-associated cell cycle arrest. Cancer Res 66:1289–1293
150. Nagl NG Jr, Wang X, Patsialou A, Van Scoy M, Moran E (2007) Distinct mammalian SWI/SNF chromatin remodeling complexes with opposing roles in cell-cycle control. EMBO J 26:752–763
151. Nagl NG Jr et al (2005) The p270 (ARID1A/SMARCF1) subunit of mammalian SWI/SNF-related complexes is essential for normal cell cycle arrest. Cancer Res 65:9236–9244
152. Jones S et al (2010) Frequent mutations of chromatin remodeling gene ARID1A in ovarian clear cell carcinoma. Science 330:228–231
153. Wiegand KC et al (2010) ARID1A mutations in endometriosis-associated ovarian carcinomas. N Engl J Med 363:1532–1543
154. Maeda D et al (2010) Clinicopathological significance of loss of ARID1A immunoreactivity in ovarian clear cell carcinoma. Int J Mol Sci 11:5120–5128

155. Wiegand KC et al (2011) Loss of BAF250a (ARID1A) is frequent in high-grade endometrial carcinomas. J Pathol 224:328–333
156. Guan B et al (2011) Mutation and loss of expression of ARID1A in uterine low-grade endometrioid carcinoma. Am J Surg Pathol 35:625–632
157. Birnbaum DJ et al (2011) Genome profiling of pancreatic adenocarcinoma. Genes Chromosomes Cancer 50:456–465
158. Zhang X et al (2012) Frequent low expression of chromatin remodeling gene ARID1A in breast cancer and its clinical significance. Cancer Epidemiol 36(3):288–293
159. Wang X et al (2004) Expression of p270 (ARID1A), a component of human SWI/SNF complexes, in human tumors. Int J Cancer 112:636
160. Li XS, Trojer P, Matsumura T, Treisman JE, Tanese N (2010) Mammalian SWI/SNF–a subunit BAF250/ARID1 is an E3 ubiquitin ligase that targets histone H2B. Mol Cell Biol 30:1673–1688
161. Li M et al (2011) Inactivating mutations of the chromatin remodeling gene ARID2 in hepatocellular carcinoma. Nat Genet 43:828–829
162. Burrows AE, Smogorzewska A, Elledge SJ (2010) Polybromo-associated BRG1-associated factor components BRD7 and BAF180 are critical regulators of p53 required for induction of replicative senescence. Proc Natl Acad Sci USA 107:14280–14285
163. Drost J et al (2010) BRD7 is a candidate tumour suppressor gene required for p53 function. Nat Cell Biol 12:380–389
164. Kim S, Lee J, Park J, Chung J (2003) BP75, bromodomain-containing M(r) 75,000 protein, binds dishevelled-1 and enhances Wnt signaling by inactivating glycogen synthase kinase-3 beta. Cancer Res 63:4792–4795
165. Kikuchi M et al (2009) TRIM24 mediates ligand-dependent activation of androgen receptor and is repressed by a bromodomain-containing protein, BRD7, in prostate cancer cells. Biochim Biophys Acta 1793:1828–1836
166. Mantovani F, Drost J, Voorhoeve PM, Del Sal G, Agami R (2010) Gene regulation and tumor suppression by the bromodomain-containing protein BRD7. Cell Cycle 9:2777–2781
167. Liu H et al (2008) Promoter methylation inhibits BRD7 expression in human nasopharyngeal carcinoma cells. BMC Cancer 8:253
168. Varela I et al (2011) Exome sequencing identifies frequent mutation of the SWI/SNF complex gene PBRM1 in renal carcinoma. Nature 469:539–542
169. Xia W et al (2008) BAF180 is a critical regulator of p21 induction and a tumor suppressor mutated in breast cancer. Cancer Res 68:1667–1674
170. Hah N et al (2010) A role for BAF57 in cell cycle-dependent transcriptional regulation by the SWI/SNF chromatin remodeling complex. Cancer Res 70:4402–4411
171. Decristofaro MF et al (2001) Characterization of SWI/SNF protein expression in human breast cancer cell lines and other malignancies. J Cell Physiol 186:136–145
172. Gorrini C et al (2007) Tip60 is a haplo-insufficient tumour suppressor required for an oncogene-induced DNA damage response. Nature 448:1063–1067
173. Mattera L et al (2009) The p400/Tip60 ratio is critical for colorectal cancer cell proliferation through DNA damage response pathways. Oncogene 28:1506–1517
174. Notaridou M et al (2011) Common alleles in candidate susceptibility genes associated with risk and development of epithelial ovarian cancer. Int J Cancer 128:2063–2074
175. Wang CL et al (2009) Discovery of retinoblastoma-associated binding protein 46 as a novel prognostic marker for distant metastasis in nonsmall cell lung cancer by combined analysis of cancer cell secretome and pleural effusion proteome. J Proteome Res 8:4428–4440
176. Buganim Y et al (2010) A novel translocation breakpoint within the BPTF gene is associated with a pre-malignant phenotype. PLoS One 5:e9657
177. Thakur A et al (2007) Aberrant expression of X-linked genes RbAp46, Rsk4, and Cldn2 in breast cancer. Mol Cancer Res 5:171–181
178. Sakhinia E et al (2005) Routine expression profiling of microarray gene signatures in acute leukaemia by real-time PCR of human bone marrow. Br J Haematol 130:233–248
179. Kim MS, Chung NG, Kang MR, Yoo NJ, Lee SH (2011) Genetic and expressional alterations of CHD genes in gastric and colorectal cancers. Histopathology 58:660–668

180. Gorringe KL et al (2008) Mutation and methylation analysis of the chromodomain-helicase-DNA binding 5 gene in ovarian cancer. Neoplasia 10:1253–1258
181. Robbins CM et al (2011) Copy number and targeted mutational analysis reveals novel somatic events in metastatic prostate tumors. Genome Res 21:47–55
182. Fujita T et al (2008) CHD5, a tumor suppressor gene deleted from 1p36.31 in neuroblastomas. J Natl Cancer Inst 100:940–949
183. Bagchi A et al (2007) CHD5 is a tumor suppressor at human 1p36. Cell 128:459–475
184. Mulero-Navarro S, Esteller M (2008) Chromatin remodeling factor CHD5 is silenced by promoter CpG island hypermethylation in human cancer. Epigenetics 3:210–215
185. Wang J et al (2011) The involvement of CHD5 hypermethylation in laryngeal squamous cell carcinoma. Oral Oncol 47:601–608
186. Mokarram P et al (2009) Distinct high-profile methylated genes in colorectal cancer. PLoS One 4:e7012
187. Wang X, Lau KK, So LK, Lam YW (2009) CHD5 is down-regulated through promoter hypermethylation in gastric cancer. J Biomed Sci 16:95
188. Vissers LE et al (2004) Mutations in a new member of the chromodomain gene family cause CHARGE syndrome. Nat Genet 36:955–957
189. Wessels K et al (2010) Novel CHD7 mutations contributing to the mutation spectrum in patients with CHARGE syndrome. Eur J Med Genet 53:280–285
190. Schlesinger Y et al (2007) Polycomb-mediated methylation on Lys27 of histone H3 premarks genes for de novo methylation in cancer. Nat Genet 39:232–236
191. Widschwendter M et al (2007) Epigenetic stem cell signature in cancer. Nat Genet 39:157–158
192. Ohm JE et al (2007) A stem cell-like chromatin pattern may predispose tumor suppressor genes to DNA hypermethylation and heritable silencing. Nat Genet 39:237–242
193. Constantinides PG, Jones PA, Gevers W (1977) Functional striated muscle cells from non-myoblast precursors following 5-azacytidine treatment. Nature 267:364–366
194. Plimack ER, Kantarjian HM, Issa JP (2007) Decitabine and its role in the treatment of hematopoietic malignancies. Leuk Lymphoma 48:1472–1481
195. Matei DE, Nephew KP (2010) Epigenetic therapies for chemoresensitization of epithelial ovarian cancer. Gynecol Oncol 116:195–201
196. Cheng JC et al (2004) Preferential response of cancer cells to zebularine. Cancer Cell 6:151–158
197. Rodriguez-Paredes M, Esteller M (2011) Cancer epigenetics reaches mainstream oncology. Nat Med 17:330–339

Part II
The Impact of Epigenetic Alterations on Cancer Biology

Chapter 6
Epigenetic Regulation of miRNAs in Cancer

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Abstract MicroRNAs (miRNAs) are short noncoding RNAs with gene regulatory functions. It has been demonstrated that the genes encoding for miRNAs undergo the same regulatory epigenetic processes of protein coding genes. In turn, a specific subgroup of miRNAs, called epi-miRNAs, is able to directly target key enzymatic effectors of the epigenetic machinery (such as DNA methyltransferases, histone deacetylases, and polycomb genes), therefore indirectly affecting the expression of epigenetically regulated oncogenes and tumor suppressor genes. Also, several of the epigenetic drugs currently approved as anticancer agents affect the expression of miRNAs and this might explain part of their mechanism of action. This chapter focuses on the tight relationship between epigenetics and miRNAs and provides some insights on the translational implications of these findings, leading to the upcoming introduction of epigenetically related miRNAs in the treatment of cancer.

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6.1 Introduction

MicroRNAs (miRNAs) are small noncoding RNAs (ncRNAs), 19–25 nucleotides (nt) in length, which regulate gene expression. MiRNAs are involved in many biological processes ranging from development, differentiation, and cell cycle regulation to cell senescence and metabolism [1–5]. Mature miRNAs derive from much longer (hundreds nt long) primary transcripts, transcribed by RNA polymerase II as long, capped, polyadenylated precursor-miRNAs (pri-miRNAs) [1]. Then, the double-stranded RNA-specific ribonuclease Drosha, in conjunction with its binding partner DiGeorge syndrome critical region gene 8 (DGCR8, or Pasha), process pri-miRNAs into hairpin RNAs of 60–110 nt known as pre-miRNAs. Translocated from the cell nucleus to the cytoplasm by means of Exportin 5, the pre-miRNA is processed by a ribonuclease III (Dicer) and transactivating response RNA-binding protein (TRBP, which binds human immunodeficiency virus 1) into an 18- to 24-nt duplex. Finally, the duplex interacts with a large protein, RNA-induced silencing complex (RISC), which includes argonaute proteins (AGO1-4 in humans). One strand of the miRNA duplex remains stably associated with RISC and becomes the mature miRNA, which guides the RISC complex mainly (but not exclusively) to the 3′-untranslated region (UTR) of the target messenger RNAs (mRNAs) [1]. Consequently, the translation and/or stability of targeted mRNAs is impaired, causing a reduction in protein expression levels [6]. In addition to this "conventional" mechanism of action, miRNA regulatory effects on gene expression may be more varied than initially proposed. For example, miRNAs can also activate rather than suppress target mRNA expression in particular cell-cycle conditions [7], they can bind also to the coding and the 5′-UTR region of the target mRNAs [8, 9], and they can directly interact with proteins and function as gene promoter regulators [10]. Figure 6.1 summarizes the biogenesis and physiology of miRNAs.

Each miRNA has hundreds or thousands of target genes. We have demonstrated that a specific cluster of two miRNAs (namely, the miR-15a/16-1 cluster) is able to regulate, directly and indirectly, about 14% of the whole genome in a leukemic cell model [11]. Therefore, it is likely that the full coding genome is under the control of miRNAs. The full spectrum of miRNAs expressed in a specific cell type (the miRNome) is different between normal and pathologic tissues [12], and specific signatures of dys-regulated miRNAs harbor diagnostic and prognostic implications [13]. The first link between miRNAs and cancer came from the discovery that these ncRNAs are frequently located in cancer-associated genomic regions, which include minimal regions of amplification, loss of heterozygosity, and common breakpoints in or near oncogenes or tumor suppressor genes (TSGs) and fragile sites (preferential sites of chromatide exchange, deletion, translocation, amplification, or integration of plasmid DNA and tumor-associated viruses) [14]. Since then, myriad studies have investigated aberrations in the miRNome in most types of human cancer (for reviews, see [15–21]). In particular, while some miRNAs act mainly as TSGs, others are frequently overexpressed in human tumors and target TSGs, thereby exerting

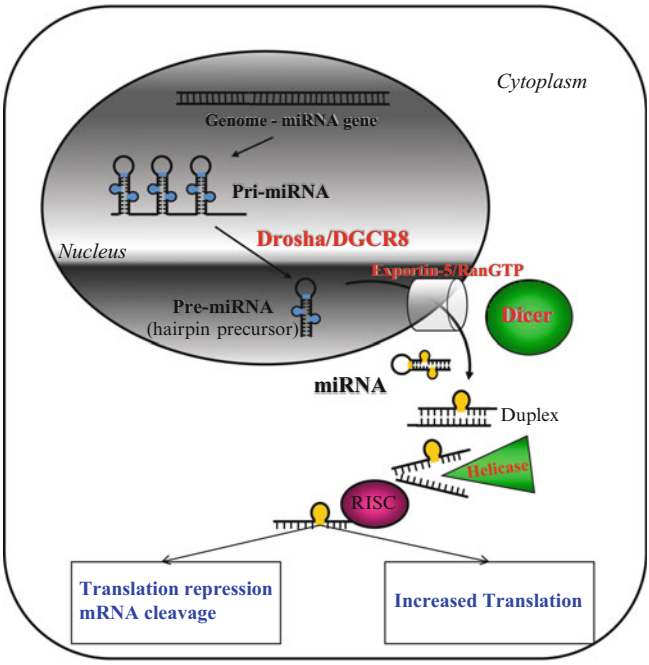

Fig. 6.1 Biogenesis and physiology of miRNAs. MiRNAs are transcribed as pri-miRNAs (in some cases as a cluster of multiple miRs, such as miR-15a and miR-16-1 on the long arm of chromosome 13) and then processed in a hairpin shaped pre-miRNA precursor in the nucleus of the cell. The precursor is then transported in the cytoplasm by means of Exportin 5 and processed until it becomes a single-stranded mature miRNA that eventually binds to a ribonucleoproteic complex (RISC) which directs the miRNA to its target mRNAs. As a result, both translational repression (or mRNA cleavage) and increased target translation can occur (see text for more explanation)

a tumorigenic function. MiRNAs with well-established roles as oncogenes, for instance, include the miR-17-92 cluster, which is transactivated by the *c-MYC* oncogene and dramatically accelerates lymphomagenesis in murine models [22, 23]; miR-155, which induces leukemia in transgenic murine models [24] and has an important function as a regulator of inflammation and the immune response [25–27], and miR-21, which targets important TSGs, such as *PTEN1* [28] and *PDCD4*, in several types of cancer [29–31]. Conversely, the miR-15a/16-1 cluster acts as a TSG in chronic lymphocytic leukemia (CLL) by targeting the antiapoptotic gene *BCL2* [32]. Interestingly, the same miR-15a-16-1 cluster also acts as an oncogene (OG), in CLL, by directly targeting the pro-apoptotic gene *p53* [33], leading to the conclusion that each miRNA should not be labeled exclusively as an OG or as a TSG, since it may have a dual nature (both as OG and TSG) [34], in which the overall effect depends on the specific conditions (tumor type, species specificity, concentration, etc.) in which it operates.

It has been demonstrated that miRNAs, similar to protein coding genes, (PCG), can undergo epigenetic regulation. More recently, it has been shown that a specific

group of miRNAs, called epi-miRNAs, can affect the epigenetic regulation of a given gene by targeting key enzymatic effectors of the epigenetic machinery, such as DNA methyltransferases (DNMTs), histone deacetylases (HDACs), and polycomb genes.

This chapter focuses on the interactions between epigenetics and miRNAs and presents how this intertwined relationship harbors fundamental implications for human carcinogenesis.

6.2 MiRNAs Are Epigenetically Regulated in Cancer

The expression of miRNAs undergoes epigenetic regulation, similarly to PCG. This regulation involves both chromatin modifications and miRNA gene promoter methylation. By treating a breast cancer cell line with the HDAC inhibitor LAQ824, Scott et al. demonstrated that the expression levels of 27 miRNAs are rapidly modified [35], indicating that HDAC and chromatin conformation affects the miRNome in human cancer. Similarly, Saito et al. showed that by treating bladder cancer cells with both a DNA demethylating agent (5-aza-2′-deoxycytidine, 5-AZA) and an HDAC inhibitor (4-phenylbutyric acid, 4-PBA) the expression levels of about 5% of all human miRNAs increased [36]. Among the most strictly epigenetically regulated miRNAs, there is miR-127, an ncRNA embedded in a CpG island and kept epigenetically silenced by both promoter hypermethylation and histone modifications in cancer cells [36]. Interestingly, this miRNA (which belongs to a large cluster that includes miR-136, -431, -432, and -433) is the only member of the cluster whose re-expression was observed when cells were treated with two epigenetic drugs [36]. Moreover, when cells were treated with each drug alone, no variation in miR-127 expression was detected [36], suggesting that miR-127 epigenetic regulation occurs by combined promoter methylation and chromatin histone modifications. Since the *BCL6* oncogene is a direct target of this miRNA [36], miR-127 acts as a TSG, therefore the severe epigenetic control of its expression represents an important mechanism for bladder carcinogenesis.

Using an HCT-116 colorectal cancer cell line with a double knockout (DKO) of DNMT1 (maintenance DNMT) and DNMT3b (de novo DNMT), Lujambio et al. compared miRNA levels of the DKO and wild-type cells. About 6% of the 320 miRNAs analyzed were upregulated in the DKO cells [37]. Among the dys-regulated miRNAs, only miR-124a was embedded in a CpG island, which is densely methylated in this cancer cell line but not in normal tissue. This might suggest that DNMTs act both directly and indirectly in miRNA expression control. MiRNA-124a directly targets CDK6, and restoration of its expression reduces the levels of CDK6 and impacts the phosphorylation status of the CDK6-downstream effector Rb protein [37]. In a group of 353 acute lymphoblastic leukemia (ALL) patients, Roman-Gomez et al. identified a signature of 13 miRNAs, embedded in CpG islands, with high heterochromatic markers (such as high levels of K9H3me2 and/or low levels of K4H3me3) [38, 39]. Treatment with 5-AZA upregulated at least 1 of the 13 miRNAs in 65% of ALLs

[38]. Among these miRNAs, miR-124a was methylated in 59% of ALLs and hypermethylation of its promoter was associated with higher relapse and mortality rates than in the non-hypermethylated cases: multivariate analysis confirmed that miR-124a promoter methylation status is an independent prognostic factor for disease-free and overall survival [39]. Moreover, miR-124a directly silences *CDK6* in ALL patients [39], confirming the impact of miR-124a on the CDK6-Rb pathway. Recently, Ando et al. showed that hypermethylation of the miR-124a promoter is involved in the formation of an epigenetic field defect, a gastric cancer predisposition condition characterized by the accumulation of abnormal DNA methylation in normal-appearing gastric mucosa that is mostly induced by *Helicobacter pylori* infection [40]. These findings reveal that miR-124a promoter hypermethylation is also an early event in gastric carcinogenesis.

In addition to miR-124a, miR-107, another epigenetically controlled miRNA, targets *CDK6* and affects pancreatic carcinogenesis [41]. In HCT-116 cells deficient for *DNMT1* and *DNMT3B*, Bruckner et al. showed increased expression of let-7a-3, an miRNA normally silenced by promoter hypermethylation in the wild-type cell line [42]. In lung adenocarcinoma primary tumors the let-7a-3 promoter was found to be hypomethylated [42], whereas it was found to be hypermethylated in epithelial ovarian cancer. This hypermethylation was associated with low expression of IGF2 (insulin-like growth factor 2) and with a good prognosis [43]. Therefore, DNA methylation could act as a protective mechanism by silencing miRNAs with oncogenic functions. Also miR-1 is epigenetically regulated and frequently silenced by promoter hypermethylation in hepatocellular carcinoma [44]. However, hypomethylation and re-expression of miR-1 were observed in DNMT1-null HCT-116 cells (but not in DNMT3B-null cells) [44], revealing that the maintenance DNMT is specifically and mainly responsible for miR-1 epigenetic regulation. Overall, these studies demonstrate that epigenetic factors can control human carcinogenesis, not only by directly affecting the expression of OGs and TSGs, but also by affecting the expression of miRNAs involved in oncogenic pathways. In addition, epigenetic control of miRNAs may be tissue-specific (since no variation in miRNA expression was observed in lung cancer cells treated with demethylating agents, HDAC inhibitors, or their combination [45]), miRNA-specific (e.g., miR-127 within the cluster it belongs to [36]), and epigenetic-effector-specific (e.g., miR-1 mainly regulated by DNMT1 [44]).

Epigenetically regulated miRNAs are also affecting one of the main aspects of malignancy: the ability to metastasize. Lujambio et al. treated three lymph node–metastatic cell lines with 5-AZA and checked miRNA levels by high-throughput microarray analysis [46]. They identified four miRNAs (namely, miR-148a, miR-34b/c, and miR-9) that showed cancer-specific CpG island hypermethylation. Epigenetic silencing of these miRNAs was also significantly associated with metastasis in human malignancies [46], while the reintroduction of miR-148a and miR-34b/c into cancer cells with epigenetic inactivation inhibited both motility and metastatic potential of the cells in xenograft models. The miR-34b/c cluster is also epigenetically regulated in colorectal cancer (promoter hypermethylation in 90% of primary colorectal cancer tumors vs. normal colon mucosa) [47], whereas

epigenetic silencing of miR-9 and miR-148a (together with miR-152, -124a, and -663) has also been described in breast cancer. In breast cancer cell lines treated with 5-AZA miR-9 was reactivated, while the levels of other aberrantly methylated miRNAs were unchanged [48], further proving that different epigenetic processes can control miRNA levels in different types of cancer.

MiR-342 is located in an intron of the Ena/Vasp-like (*EVL*) gene and represents a good model to study the relationship between miRNAs and the epigenetic regulation of cognate host genes. *EVL* promoter hypermethylation occurs in 86% of colorectal cancers and is present in 67% of adenomas at diagnosis, suggesting that it is an early event in colon carcinogenesis [49]. Treatment with 5-AZA and the HDAC inhibitor trichostatin A restores the synchronized expression of EVL and miR-342 [49]. Another gene, the *EGFL7* gene, which is frequently downregulated in several cancer cell lines and in primary bladder and prostate tumors, hosts miR-126 in one intron. The mature miR-126 can be encoded by three different transcripts of the cognate host gene, each of them with its own promoter. However, miR-126 is concomitantly upregulated with one of the EGFL7 transcripts that has a CpG-island promoter when cancer cell lines are treated with DNA methylation and histone deacetylation inhibitors, indicating that the silencing of intronic miRNAs in cancer may occur by means of epigenetic changes in cognate host genes [50].

Fazi et al. showed that transcription factors can recruit epigenetic effectors to miRNA promoter regions to regulate their expression. The AML1/ETO fusion oncoprotein, the aberrant product of the t(8;21) translocation in acute myeloid leukemia, can bind to the pre-miR-223 region. The oncoprotein recruits epigenetic effectors (i.e., DNMTs, HDAC1, and MeCP2), leading to aberrant hypermethylation of the CpG site near the AML1/ETO binding site and H3-H4 deacetylation of the same chromatin region [51].

In summary, several studies have addressed how epigenetics regulates miRNA expression in human cancer. It has emerged that epigenetic factors account for several of the miRNome aberrancies observed in human cancer, ultimately implicated in both carcinogenesis and in metastasis formation. Therefore, a better understanding of miRNA epigenetic regulation will lead to a better comprehension of the mechanisms responsible for abnormal miRNA levels in cancer and to the development of strategies able to revert these anomalies. Interestingly, miRNAs can also affect the expression of epigenetically regulated PCGs, revealing a further layer of complexity between miRNome and epigenome.

6.3 Epi-miRNAs Affect the Expression of Epigenetically Regulated Genes in Cancer

In addition to being epigenetically regulated, like PCG, miRNAs can also affect the expression of epigenetically regulated genes by targeting key enzymes responsible for epigenetic reactions. We call this group of miRNAs, epi-miRNAs. Some epi-miRNAs are also epigenetically regulated themselves. Our group provided the first

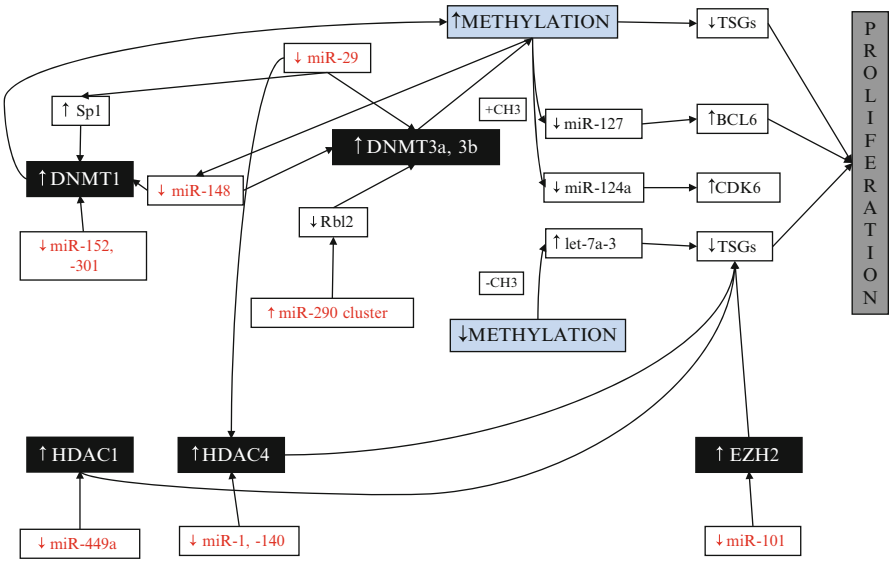

Fig. 6.2 Epi-miRNAs and cancer. Epi-miRNAs (in *red*) directly target effectors of the epigenetic machinery (in *black boxes*) and indirectly affect the expression of epigenetically regulated miRNAs and protein coding genes, ultimately affecting carcinogenesis. *TSGs* tumor suppressor genes; *DNMT* DNA methyltransferase; *HDAC* histone deacetylase; *EZH2* enhancer of zeste homolog 2; *BCL6* B-cell CLL/lymphoma 6; *CDK6* cyclin-dependent kinase 6; *SP1* Sp1 transcription factor; *RBL2* retinoblastoma-like 2 (p130); CH_3 methyl group

evidence that miRNAs can regulate the expression of members of the epigenetic machinery in humans [52]. We demonstrated in both lung cancer cell lines and primary tumors that a family of miRNAs (namely the miR-29 family, composed of miR-29a, -29b, and -29c) directly targets both DNMT3a and DNMT3b, the two key de novo DNMTs. We observed that miR-29 restoration reduces global DNA methylation, induces re-expression of TSGs (such as *WWOX* and *FHIT*, whose expression is silenced by promoter hypermethylation in lung cancer), and exerts an overall antitumoral effect both in vitro and in vivo [52]. The global hypomethylating effect observed in tumor cells upon miR-29 re-expression is the result of a direct targeting effect of these miRNAs on DNMT3a and DNMT3b, and of an indirect silencing effect on DNMT1, occurring through the direct targeting of the DNMT1 transactivating factor SP1 [53]. Figure 6.2 summarizes the relationship between epi-miRNAs and cancer. Duursma et al. [54] have shown that miR-148 also directly targets DNMT3b by binding to a conserved target sequence located in the coding region of the mRNA. Intriguingly, the authors concluded that the targeting of the coding region may play a role in determining the relative abundance of different splice variants of DNMT3b. Furthermore, miRNAs can affect the expression of DNMTs also through an indirect mechanism. Sinkkonen et al. showed that in mouse embryonic stem (ES) cells, members of the miR-290 cluster directly target Rbl2, a factor contributing to the suppression of *DNMT3* genes [55]. By restoring the expression of

the miR-290 cluster, de novo methylation, which had been disrupted in ES Dicer$^{-/-}$ cells, was reestablished, suggesting that DNMTs are indirectly regulated by the miR-290 cluster. These results were confirmed by Benetti et al. [56], who also observed that the aberrant DNA methylation occurring after miR-290 cluster silencing in ES Dicer$^{-/-}$ cells is responsible for increased telomere recombination and aberrant telomere elongation. Notably, the miR-290 Rbl2-mediated regulation of *DNMT3a* and *DNMT3b* was not observed in HEK293 cells with knockdown of Dicer [55], revealing that the described regulatory mechanism might be restricted to ES cells. Moreover, neither of the above-mentioned studies identified the miR-29 family as direct regulators of de novo DNMTs, suggesting that this interaction could also be species-, tumor-, or even histotype-specific.

Epi-miRNAs can also target *DNMT1*. In a study by Braconi et al., it was shown that miR-148a, miR-152, and miR-301 directly target *DNMT1* in cholangiocarcinoma cells [57], resulting in the re-expression of the *RASSF1A* and *p16INK4a* genes, two well-known TSGs that are epigenetically silenced in several malignancies. As previously reported, miR-29b indirectly targets *DNMT1*, by directly silencing its activator SP1 in hematological malignancies [53]. These studies suggest that miR-29b plays a key role in the epigenetic control of human genome.

Epi-miRNAs also regulate the expression of HDACs and PRC genes. *HDAC4* is a direct target of both miR-1, miR-140, and miR-29b [58–60], whereas miR-449a binds to the 3′-UTR region of *HDAC1* [61]. *HDAC1* is upregulated in several types of cancer, and miR-449a re-expression in prostate cancer cells induces cell-cycle arrest, apoptosis, and a senescent-like phenotype by reducing the levels of HDAC1 [61]. EZH2 is the catalytic subunit of PRC2 and is responsible for heterochromatin formation by trimethylating histone H3 on lysine 27 (H3K27me3), leading to the silencing of several TSGs. Varambally et al. showed in prostate cancer cell lines and primary tumors that the level of EZH2 is inversely correlated with the expression of miR-101, which decreases during cancer progression. These findings suggest a role for miR-101 as an epi-miRNA, a hypothesis that was tested and confirmed by showing that miR-101 directly targets EZH2 both in prostate and in bladder cancer models [62, 63]. Moreover, the miR-101-mediated suppression of EZH2 inhibits cancer cell proliferation and colony formation, revealing a role for miR-101 as a TSG that is mediated by its modulatory effects on the cancer epigenome [63].

In summary, an increasing number of epi-miRNAs is being identified and will clarify which epigenetic effectors are involved in the regulation of OGs and TSGs. This knowledge will lead to the development of new strategies to prevent and cure human carcinogenesis by selective modulation of the epi-miRNome.

6.4 Epigenetics and miRNAs: Clinical Implications and Final Remarks

The epigenetics–miRNA relationship harbors several clinical implications. First, some of the demethylating agents (such as 5-AZA or Vidaza) used to show that miRNAs are re-expressed upon demethylation and therefore undergo epigenetic

regulations are drugs, currently approved for the treatment of myelodysplastic syndromes (MDS) [64]. Therefore, part of the observed therapeutic effects of 5-AZA or decitabine might be mediated by their effect on the miRNome. Also, currently available anticancer drugs (such as Bortezomib) induce the expression of miR-29b [65], a key epi-miRNA targeting both DNMTs and HDACs. Moreover, SAHA (suberoylanilide hydroxamic acid), also known as Vorinostat is an HDAC inhibitor currently approved in the treatment of cutaneous T cell lyphomas, may exert an anticancer effect by re-expressing epigenetically regulated miRNAs [66, 67]. Valproic acid (VPA) is also an HDAC inhibitor currently in phase III studies for the treatment of cervical and ovarian cancer, which is able to modulate the expression of miRNAs in human cord blood-derived multipotent stem cells [68].

Overall, while basic research scientists are trying to improve their understanding of the relationship existing between epigenetics and miRNAs, clinicians have started interpreting some of the effects of epigenetic drugs in terms of their effects on the miRNome. This interaction represents an ideal translational setting, capable of bringing novel insights deriving from basic science to the patients. In addition to better understanding the implications and function of currently available epigenetic drugs on the miRNome, it is likely that in the near future this knowledge will assist in the development of miRNA- and epi-miRNA-based therapies. These therapies will be tailored to the specific set of genes that need to be reverted to a physiological expression, in order to achieve an anticancer effect. Therefore, their effect will specifically affect tumor cells, without introducing any major epigenetic perturbation in noncancerous cells, therefore leading to less side effects. These days are not far to come and will provide a new powerful therapeutic tool in the war against cancer.

References

1. Bartel DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116:281–297
2. Pasquinelli AE, Hunter S, Bracht J (2005) MicroRNAs: a developing story. Curr Opin Genet Dev 15:200–205
3. Harfe BD (2005) MicroRNAs: in vertebrate development. Curr Opin Genet Dev 15:410–415
4. Carleton M, Cleary MA, Linsley PS (2007) MicroRNAs and cell cycle regulation. Cell Cycle 6:2127–2132
5. Boehm M, Slack FJ (2006) MicroRNA control of lifespan and metabolism. Cell Cycle 5:837–840
6. Filipowicz W, Bhattacharyya SN, Sonenberg N (2008) Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? Nature Reviews 9:102–114
7. Vasudevan S, Tong Y, Steitz JA (2007) Switching from repression to activation: microRNAs can up-regulate translation. Science 318:1931–1934
8. Tay Y, Zhang J, Thomson AM, Lim B, Rigoutsos I (2008) MicroRNAs to Nanog, Oct4 and Sox2 coding regions modulate embryonic stem cell differentiation. Nature 455:1124–1128
9. Vatolin S, Navaratne K, Weil RJ (2006) A novel method to detect functional microRNA targets. J Mol Biol 358:983–996
10. Eiring AM et al (2010) MiR-328 functions as an RNA decoy to modulate hnRNP E2 regulation of mRNA translation in leukemic blasts. Cell 140:652–665

11. Calin GA et al (2008) MiR-15a and miR-16-1 cluster functions in human leukemia. Proc Natl Acad Sci USA 105:5166–5171
12. Sevignani C, Calin GA, Siracusa LD, Croce CM (2006) Mammalian microRNAs: a small world for fine-tuning gene expression. Mamm Genome 17:189–202
13. Calin GA et al (2005) A microRNA signature associated with prognosis and progression in chronic lymphocytic leukemia. N Engl J Med 353:1793–1801
14. Calin GA et al (2004) Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. Proc Natl Acad Sci USA 101:2999–3004
15. Nelson KM, Weiss GJ (2008) MicroRNAs and cancer: past, present, and potential future. Mol Cancer Ther 7:3655–3660
16. Fabbri M, Croce CM, Calin GA (2009) MicroRNAs in the ontogeny of leukemias and lymphomas. Leuk Lymphoma 50:160–170
17. Fabbri M, Croce CM, Calin GA (2008) MicroRNAs. Cancer J 14:1–6
18. Fabbri M et al (2008) MicroRNAs and noncoding RNAs in hematological malignancies: molecular, clinical and therapeutic implications. Leukemia 22:1095–1105
19. Deng S, Calin GA, Croce CM, Coukos G, Zhang L (2008) Mechanisms of microRNA deregulation in human cancer. Cell Cycle 7:2643–2646
20. Garzon R, Croce CM (2008) MicroRNAs in normal and malignant hematopoiesis. Curr Opin Hematol 15:352–358
21. Croce CM (2009) Causes, and consequences of microRNA dysregulation in cancer. Nat Rev Genet 10:704–714
22. He L et al (2005) A microRNA polycistron as a potential human oncogene. Nature 435: 828–833
23. Mendell JT (2008) miRiad roles for the miR-17-92 cluster in development and disease. Cell 133:217–222
24. Costinean S et al (2006) Pre-B cell proliferation and lymphoblastic leukemia/high-grade lymphoma in E(mu)-miR155 transgenic mice. Proc Natl Acad Sci USA 103:7024–7029
25. Thai TH et al (2007) Regulation of the germinal center response by microRNA-155. Science 316:604–608
26. Rodriguez A et al (2007) Requirement of bic/microRNA-155 for normal immune function. Science 316:608–611
27. Tili E et al (2007) Modulation of miR-155 and miR-125b levels following lipopolysaccharide/TNF-alpha stimulation and their possible roles in regulating the response to endotoxin shock. J Immunol 179:5082–5089
28. Cully M, You H, Levine AJ, Mak TW (2006) Beyond PTEN mutations: the PI3K pathway as an integrator of multiple inputs during tumorigenesis. Nat Rev Cancer 6:184–192
29. Meng F et al (2007) MicroRNA-21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer. Gastroenterology 133:647–658
30. Asangani IA et al (2008) MicroRNA-21 (miR-21) post-transcriptionally downregulates tumor suppressor Pdcd4 and stimulates invasion, intravasation and metastasis in colorectal cancer. Oncogene 27:2128–2136
31. Frankel LB et al (2008) Programmed cell death 4 (PDCD4) is an important functional target of the microRNA miR-21 in breast cancer cells. J Biol Chem 283:1026–1033
32. Cimmino A et al (2005) miR-15 and miR-16 induce apoptosis by targeting BCL2. Proc Natl Acad Sci USA 102:13944–13949
33. Fabbri M et al (2011) Association of a microRNA/TP53 feedback circuitry with pathogenesis and outcome of B-cell chronic lymphocytic leukemia. JAMA 305:59–67
34. Fabbri M, Ivan M, Cimmino A, Negrini M, Calin GA (2007) Regulatory mechanisms of microRNAs involvement in cancer. Expert Opin Biol Ther 7:1009–1019
35. Scott GK, Mattie MD, Berger CE, Benz SC, Benz CC (2006) Rapid alteration of microRNA levels by histone deacetylase inhibition. Cancer Res 66:1277–1281
36. Saito Y et al (2006) Specific activation of microRNA-127 with downregulation of the proto-oncogene BCL6 by chromatin-modifying drugs in human cancer cells. Cancer Cell 9: 435–443

37. Lujambio A et al (2007) Genetic unmasking of an epigenetically silenced microRNA in human cancer cells. Cancer Res 67:1424–1429
38. Roman-Gomez J et al (2009) Epigenetic regulation of microRNAs in acute lymphoblastic leukemia. J Clin Oncol 27:1316–1322
39. Agirre X et al (2009) Epigenetic silencing of the tumor suppressor microRNA Hsa-miR-124a regulates CDK6 expression and confers a poor prognosis in acute lymphoblastic leukemia. Cancer Res 69(10):4443–4453
40. Ando T et al (2009) DNA methylation of microRNA genes in gastric mucosae of gastric cancer patients: its possible involvement in the formation of epigenetic field defect. Int J Cancer 124:2367–2374
41. Lee KH et al (2009) Epigenetic silencing of microRNA miR-107 regulates cyclin-dependent kinase 6 expression in pancreatic cancer. Pancreatology 9:293–301
42. Brueckner B et al (2007) The human let-7a-3 locus contains an epigenetically regulated microRNA gene with oncogenic function. Cancer Res 67:1419–1423
43. Lu L, Katsaros D, de la Longrais IA, Sochirca O, Yu H (2007) Hypermethylation of let-7a-3 in epithelial ovarian cancer is associated with low insulin-like growth factor-II expression and favorable prognosis. Cancer Res 67:10117–10122
44. Datta J et al (2008) Methylation mediated silencing of MicroRNA-1 gene and its role in hepatocellular carcinogenesis. Cancer Res 68:5049–5058
45. Yanaihara N et al (2006) Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. Cancer Cell 9:189–198
46. Lujambio A et al (2008) A microRNA DNA methylation signature for human cancer metastasis. Proc Natl Acad Sci USA 105:13556–13561
47. Toyota M et al (2008) Epigenetic silencing of microRNA-34b/c and B-cell translocation gene 4 is associated with CpG island methylation in colorectal cancer. Cancer Res 68:4123–4132
48. Lehmann U et al (2008) Epigenetic inactivation of microRNA gene hsa-mir-9-1 in human breast cancer. J Pathol 214:17–24
49. Grady WM et al (2008) Epigenetic silencing of the intronic microRNA hsa-miR-342 and its host gene EVL in colorectal cancer. Oncogene 27:3880–3888
50. Saito Y et al (2009) Epigenetic therapy upregulates the tumor suppressor microRNA-126 and its host gene EGFL7 in human cancer cells. Biochem Biophys Res Commun 379:726–731
51. Fazi F et al (2007) Epigenetic silencing of the myelopoiesis regulator microRNA-223 by the AML1/ETO oncoprotein. Cancer Cell 12:457–466
52. Fabbri M et al (2007) MicroRNA-29 family reverts aberrant methylation in lung cancer by targeting DNA methyltransferases 3A and 3B. Proc Natl Acad Sci USA 104:15805–15810
53. Garzon R et al (2009) MicroRNA -29b induces global DNA hypomethylation and tumor suppressor gene re-expression in acute myeloid leukemia by targeting directly DNMT3A and 3B and indirectly DNMT1. Blood 113:6411–6418
54. Duursma AM, Kedde M, Schrier M, le Sage C, Agami R (2008) miR-148 targets human DNMT3b protein coding region. RNA 14:872–877
55. Sinkkonen L et al (2008) MicroRNAs control de novo DNA methylation through regulation of transcriptional repressors in mouse embryonic stem cells. Nat Struct Mol Biol 15:259–267
56. Benetti R et al (2008) A mammalian microRNA cluster controls DNA methylation and telomere recombination via Rbl2-dependent regulation of DNA methyltransferases. Nat Struct Mol Biol 15:268–279
57. Braconi C, Huang N, Patel T (2010) MicroRNA-dependent regulation of DNA methyltransferase-1 and tumor suppressor gene expression by interleukin-6 in human malignant cholangiocytes. Hepatology 51:881–890
58. Chen JF et al (2006) The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation. Nat Genet 38:228–233
59. Tuddenham L et al (2006) The cartilage specific microRNA-140 targets histone deacetylase 4 in mouse cells. FEBS Lett 580:4214–4217
60. Li Z et al (2009) Biological functions of miR-29b contribute to positive regulation of osteoblast differentiation. J Biol Chem 282:15676–15684

61. Noonan EJ et al (2009) miR-449a targets HDAC-1 and induces growth arrest in prostate cancer. Oncogene 28:1714–1724
62. Varambally S et al (2008) Genomic loss of microRNA-101 leads to overexpression of histone methyltransferase EZH2 in cancer. Science 322:1695–1699
63. Friedman JM et al (2009) The putative tumor suppressor microRNA-101 modulates the cancer epigenome by repressing the polycomb group protein EZH2. Cancer Res 69:2623–2629
64. Kaminskas E, Farrell AT, Wang YC, Sridhara R, Pazdur R (2005) FDA drug approval summary: azacitidine (5-azacytidine, Vidaza) for injectable suspension. Oncologist 10:176–182
65. Liu S et al (2010) Sp1/NFkappaB/HDAC/miR-29b regulatory network in KIT-driven myeloid leukemia. Cancer Cell 17:333–347
66. Shin S et al (2009) MicroRNAs that respond to histone deacetylase inhibitor SAHA and p53 in HCT116 human colon carcinoma cells. Int J Oncol 35:1343–1352
67. Kretzner L et al (2011) Combining histone deacetylase inhibitor vorinostat with aurora kinase inhibitors enhances lymphoma cell killing with repression of c-Myc, hTERT, and microRNA levels. Cancer Res 71:3912–3920
68. Lee S et al (2011) Histone deacetylase regulates high mobility group A2-targeting microRNAs in human cord blood-derived multipotent stem cell aging. Cell Mol Life Sci 68:325–336

Chapter 7
DNA Hypomethylation and Activation of Germline-Specific Genes in Cancer

Charles De Smet and Axelle Loriot

Abstract DNA methylation, occurring at cytosines in CpG dinucleotides, is a potent mechanism of transcriptional repression. Proper genomic methylation patterns become profoundly altered in cancer cells: both gains (hypermethylation) and losses (hypomethylation) of methylated sites are observed. Although DNA hypomethylation is detected in a vast majority of human tumors and affects many genomic regions, its role in tumor biology remains elusive. Surprisingly, DNA hypomethylation in cancer was found to cause the aberrant activation of only a limited group of genes. Most of these are normally expressed exclusively in germline cells and were grouped under the term "cancer-germline" (CG) genes. CG genes represent unique examples of genes that rely primarily on DNA methylation for their tissue-specific expression. They are also being exploited to uncover the mechanisms that lead to DNA hypomethylation in tumors. Moreover, as CG genes encode tumor-specific antigens, their activation in cancer highlights a direct link between epigenetic alterations and tumor immunity. As a result, clinical trials combining epigenetic drugs with anti-CG antigen vaccines are being considered.

7.1 Introduction

Although DNA hypomethylation was the first epigenetic alteration to be described in human cancers, its effect on gene expression programs and tumor biology has remained enigmatic. Initial examination of cancer genomes identified most losses of DNA methylation in repeated elements [29]. This is not surprising, since these DNA elements are highly abundant and comprise most of the CpG sites that are normally methylated in healthy somatic tissues. A crucial question was whether

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DNA hypomethylation also affected protein-encoding genes, leading to their aberrant expression in tumor cells. It appeared, however, that genome hypomethylation in tumors is not generally associated with the ectopic activation of a multitude of genes [5]. A plausible explanation for this is that most tissue-specific genes use other regulatory mechanisms, including histone modifications, and that DNA methylation, if present, serves merely as secondary layer of repression. Losses of DNA methylation within such genes would therefore not be sufficient to trigger transcriptional activation.

Later work, aiming at isolating genes that code for tumor-specific antigens, led to the identification of a particular group of genes, which are normally expressed exclusively in germline cells but become aberrantly activated in a wide variety of tumors [86]. Given this expression profile, these genes were termed "cancer-germline" (CG) genes. Interestingly, CG genes were found to rely primarily on DNA methylation for repression in normal somatic tissues, and their activation in tumors was shown to be a direct consequence of genome hypomethylation [22]. These observations highlighted an unexpected link between epigenetic alterations in tumors and cancer immunity. They also provided clear examples of genes that owe their tissue-specific expression to DNA methylation. Moreover, CG genes are being exploited to try to uncover the molecular mechanisms underlying genome hypomethylation in tumors, as this epigenetic process remains largely unexplained.

7.2 Characterization of CG Genes

Human tumors express specific antigens, as evidenced by the existence in the blood of cancer patients of cytolytic T lymphocytes (CTL) that recognize antigens present on their tumor cells but not on normal cells [10]. Using a gene library transfection approach and a CTL clone isolated from a melanoma patient, Boon and colleagues identified the first human tumor antigen-encoding gene [85]. The gene was named melanoma antigen 1 or *MAGE-1* (later renamed *MAGEA1*). *MAGEA1* expression was not found in normal tissues except for testis, but was instead detected in a significant fraction of melanoma samples, as well as in various other tumor types [20, 23]. The same genetic approach led to the identification of other melanoma antigen genes, namely *BAGE*, *GAGE*, and *MAGEA3*, a gene closely related to *MAGEA1* [9, 34, 84]. For these genes too, expression among normal tissues was restricted to testis, and activation in tumors was detected among various cancer types. Additional tumor antigen genes were subsequently identified, using an alternative cloning approach, called SEREX (serological analysis of recombinant tumor cDNA expression libraries), and based on the presence of high titers of antitumor IgGs in the blood of tumor-bearing patients [73]. Again, several of the identified genes, including *SSX2* and *NY-ESO-1*, had their normal expression restricted to testis and were activated in a percentage of different tumor types. Later studies indicated that the normal expression of most isolated genes was confined to the germ cells in both testis and fetal ovary [44, 52, 82].

Together, these findings led to the important notion that specific antigens in tumors arise from the aberrant activation of genes that are normally transcribed exclusively in the germline. From an immunological point of view, this dual expression pattern is understandable. Unlike most somatic cells, germ cells lack MHC class I molecules, which are required to present antigenic peptides at the cell surface [37]. Activation of germline-specific genes in tumor cells therefore leads to the expression of truly tumor-specific antigens, which can be recognized as nonself by the immune system.

Further studies using cDNA subtraction procedures or database mining have permitted the identification of additional genes expressed in germ cells and cancer but not in normal somatic tissues [56, 60, 63, 75]. Some genes identified in this way were subsequently shown to encode tumor-specific antigens recognized by CTLs [86]. Altogether about 50 human genes or gene families were identified, which displayed specific expression in the germline and activation in a significant proportion of cancers [2]. These genes appear to exert a variety of cellular functions, but on the basis of their common expression pattern they were grouped under the term cancer-germline (CG) genes. CG genes are dispersed on several chromosomes, with a marked preference for the X chromosome. In human cancers, CG genes are expressed more frequently in specific tumor types, like for instance lung cancer, head and neck cancer, bladder cancer, and melanoma [76]. Other tumor types like colon cancer, renal cancer, and leukemia only rarely show activation of CG genes. An important feature of CG genes is their frequent co-activation in tumors [74]. It was observed indeed that positive tumors often express several CG genes. Clearly, the widespread and concerted expression of CG genes in tumors indicates that their activation in cancer results from a global gene activation process, rather than stochastic individual events.

7.3 DNA Demethylation in the Activation of CG Genes in Tumors

The marked tendency of CG genes to become co-expressed in tumors suggested that these genes share, at least in part, a common mechanism of transcriptional activation. Initial studies were performed with the *MAGEA1* gene in order to identify essential promoter elements and corresponding transcription factors that may contribute to the cell-type-specific expression of the gene. Surprisingly, however, transfection experiments revealed that all cells, including those that do not express *MAGEA1*, contain transcription factors capable of inducing significant *MAGEA1* promoter activity [24]. Transfection experiments with other CG gene promoter constructs led to similar results [17, 89]. This implied that nonexpressing cells have a repression mechanism, probably operating at the chromatin level that protects CG gene promoters against spurious activation.

The initial observation by Weber and colleagues that *MAGEA1* could be induced in nonexpressing melanoma cell lines following treatment with the DNA

methylation inhibitor 5-aza-2′-deoxycytidine provided a first hint that DNA methylation may contribute to the transcriptional regulation of this gene [91]. This was confirmed by studies showing that the promoter of *MAGEA1* is invariably methylated in all normal somatic tissues and instead unmethylated in germ cells [26]. Likewise, activation of the *MAGEA1* gene in tumors was strictly correlated with demethylation of its promoter [26]. Further studies showed that DNA methylation was similarly involved in the regulation of other CG genes [17, 26, 52, 56, 89]. Altogether, these observations indicated that CG genes rely on DNA methylation for repression in somatic tissues, and that aberrant activation of these genes in tumors results from demethylation of their promoter.

Interestingly, demethylation and activation of CG genes in tumors was found to correlate with global genome hypomethylation [14, 25, 45]. This association was further confirmed by a study on microdissected tumor samples, revealing that intratumor heterogeneity of CG gene expression also correlates with global genome hypomethylation levels [96]. These observations provided therefore the first clear evidence that the process of genome-wide demethylation, common to many cancers, not only affects repeated sequences but also single copy genes, and can lead to aberrant gene activation. The frequent co-activation of CG genes in tumors likely reflects the global process of DNA demethylation, which can simultaneously affect many loci across the cancer genome.

7.4 DNA Methylation in the Regulation of Germline Genes

Considering the potent effect of DNA methylation on transcriptional repression, it was originally proposed that this DNA modification might serve as a general mechanism to control the programmed expression of tissue-specific genes [39, 72]. Evidence, however, indicates that most tissue-specific genes rely on mechanisms other than DNA methylation for repression in nonexpressing cells [8, 88]. This may be ascribed to the distribution of CpG sequences, where cytosine methylation can occur. Vertebrate genomes show a general depletion of CpG dinucleotides, which was attributed to the high mutability of methylated cytosines, and hence the progressive disappearance of this sequence during evolution [7]. Discrete genomic regions however, which appear generally free of CpG methylation, maintained a high density of CpG sites. These so-called CpG islands often overlap gene promoters [19]. Many tissue-specific genes contain a methylation-free CpG island within their promoter and can therefore not rely on DNA methylation for repression in nonexpressing tissues. On the other hand, genes with few CpG sites within their promoter are only little affected by DNA methylation, and often show an inconstant relationship between promoter methylation and transcriptional silencing [12]. It was therefore proposed that DNA methylation in vertebrates is solely involved in the control of retrotransposable elements, monoallelically expressed imprinted genes, and X chromosome inactivation, the only cases where consistent methylation of CpG-rich regions appeared to exist [101].

This view was challenged by the discovery of CG genes, which were found to be characterized by the presence of a high density of CpG sites within their promoter [26]. Yet, unlike classical CpG islands, CpG-rich promoters of CG genes are methylated in all normal somatic tissues. CG gene promoters appear therefore favorably disposed to DNA methylation-mediated regulation. Consistently, transfection experiments with in vitro methylated CG gene constructs indicated that DNA methylation was sufficient to repress transcription, even in cells that express the corresponding endogenous CG gene, and therefore obviously contain appropriate transcriptional activators [17, 26, 27, 78, 89]. This and the above-mentioned observation that unmethylated CG gene promoters are transcriptionally active in nonexpressing cells provided strong evidence that DNA methylation is an essential component of the repression of this group of germline-specific genes in somatic cells.

More recently, genome-wide studies were conducted in order to identify the distribution of differentially methylated CpG sites across the genome of distinct types of human cells [77, 93]. These studies revealed the existence of novel sets of genes with a CpG-rich promoter that was densely methylated in somatic tissues (in addition to the previously characterized CG genes). Remarkably, most of these genes were specifically demethylated and expressed in testis. It appears therefore that DNA methylation has a particular role in the regulation of germline-specific genes.

Why would DNA methylation be particularly suitable for the regulation of genes with specific expression in germline cells rather than in other cell types? A plausible explanation may be that methylation-dependent germline genes have the advantage of being little exposed to the evolutionary loss of methylated CpGs, because they are unmethylated precisely in the cells that transmit their genome to the offspring. As a result, such genes maintain a high density of CpG sites within their promoter and remain therefore fully responsive to DNA methylation.

7.5 Mechanisms Leading to Hypomethylation of CG Genes in Cancer

CG genes have served as model sequences to investigate the distribution and dynamics of methylation losses in tumor genomes. Detailed analysis of the *MAGEA1* locus revealed preferential hypomethylation of a restricted region surrounding the transcription start site of the gene in expressing tumor cells, suggesting that hypomethylated CpG sites are unevenly distributed across cancer genomes [27]. Consistently, recent genome-wide DNA methylation studies confirmed that DNA hypomethylation in tumors adopts mosaic patterns, with defined hypomethylated domains (between one kilobase and several megabases in size) surrounded by normally methylated regions [66, 71, 92]. These observations indicate that certain genomic regions, including CG promoters, are particularly susceptible to DNA hypomethylation in tumors.

The possibility that *MAGEA1*-expressing tumor cells possess a DNA demethylation activity targeted towards the 5′-region of the gene was investigated [27, 58].

Thus, a large genomic fragment comprising the *MAGEA1* gene was methylated in vitro and then stably transfected into several human tumor cell lines, where the endogenous *MAGEA1* gene is hypomethylated and active. The newly integrated *MAGEA1* transgenes did not undergo demethylation, indicating that the process that once led to demethylation of the endogenous *MAGEA1* gene was not preserved in these cells. Remarkably, when unmethylated *MAGEA1* constructs were introduced into such cells, de novo methylation of the transgenes occurred except in a region overlapping the *MAGEA1* promoter [27]. This mechanism of protection against de novo DNA methylation was lost when mutations that impair the *MAGEA1* promoter activity were introduced into the transgene, or when the transgene was transfected into tumor cells that induce only little *MAGEA1* promoter activity. Altogether, these data suggest that site-specific hypomethylation of *MAGEA1* in tumors results from a past event of transient DNA demethylation and is maintained locally by the presence of potent transcriptional activators that prevent remethylation.

In vivo studies, evaluating global genome methylation levels in colon and breast cancers, demonstrated that DNA hypomethylation is present in the early stages of the disease, and does not progress towards later stages, adding support the transient nature of the DNA demethylation process [30, 41]. Other studies, however, reported a higher prevalence of genome hypomethylation and an increased frequency of CG gene activation in more advanced tumor stages [53, 100]. This was interpreted as an indication that DNA demethylation might instead be a continuous process leading to progressive methylation losses with tumor development. Other interpretations for the increased hypomethylation in advanced tumor genomes, which implicate a transient DNA demethylation process, are however possible: (1) transient demethylation would initially produce a mixed population of precancerous cells with varying levels of DNA hypomethylation, and cells with the most hypomethylated genome would later be selected to contribute to the more advanced stages of the disease; or (2) the transient demethylation process could occur at varying time points during tumor progression and would therefore be more likely to have already occurred in late stage tumor samples [22]. Additional support for a transient DNA demethylation process comes from the observation that tumor cell lines with a hypomethylated genome do not show further CpG methylation losses during culturing [32, 55, 94]. Of note, many tumor cells display instead de novo methylation activities [3, 43].

Considering the suggested dynamics of DNA demethylation in tumors, it is reasonable to propose that hypomethylation of CG genes in tumors is mediated by two groups of factors: those that contribute to the transient DNA demethylation process and those that are required to protect the CG gene promoter region against subsequent remethylation.

7.5.1 Process of DNA Demethylation

Factors contributing to the DNA demethylation process during cancer development remain unknown. The apparent transient nature of this process suggests that activation of such demethylation-inducing factors might occur in association with

one (or several) of the multiple steps through which precancerous cells are progressing before acquiring full malignancy. Interestingly, a recent study evaluating genome methylation levels in an isogenic series of human mammary epithelial cell cultures transitioning from normal to malignantly transformed revealed that most losses of DNA methylation occurred at the stage of acquisition of indefinite lifespan [67]. Another study reported that genome hypomethylation and CG gene activation is more prevalent in tumors displaying the alternative telomere (ALT) maintenance phenotype rather than telomerase activation, the two possible mechanisms by which cancer cells stabilize their telomeres and acquire immortality [83]. These observations establish therefore a possible link between DNA demethylation and cellular immortalization. Underlying molecular mechanisms remain, however, to be identified.

Theoretically, DNA demethylation in tumor cells could possibly occur through two distinct processes commonly referred to as active demethylation and passive demethylation [16]. Active demethylation would involve the activation of demethylating enzymes, which can remove methylation marks from the DNA in a replication-independent manner. Enzymes contributing to active DNA demethylation in animal cells are beginning to be characterized [16], but their potential involvement in cancer genome demethylation has not yet been reported. Passive demethylation on the other hand, would rely on the inhibition of DNA methyltransferases, which normally preserve the DNA methylation marks through the successive replication cycles. Three DNA methyltransferases exist in mammals: DNMT1, DNMT3A, and DNMT3B [6]. DNMT1 is primarily involved in DNA methylation maintenance, as it appears to be specialized in copying preexisting methylation sites onto the newly synthesized strand during replication. DNMT3A and DNMT3B instead have de novo DNA methylation activity and are responsible for the establishment of new DNA methylation marks in the developing embryo. For CG genes in particular, studies based on targeted depletion of the distinct DNMTs indicate that DNMT1 is the principal enzyme for methylation maintenance [42, 57]. It is therefore likely that passive DNA demethylation of CG genes in tumors would necessarily involve factors that decrease the amount or impair proper functioning of DNMT1. In certain tumor cells, however, combined depletion of DNMT1 and DNMT3 enzymes was required to obtain efficient demethylation and activation of CG genes [42, 95]. This indicates that de novo methyltransferases can be targeted to these genes, where they might restore lost methylation sites, and underscores the importance of acquiring mechanisms of protection against remethylation for long-term activation.

7.5.2 Factors that Protect Against Remethylation

Studies with the *MAGEA1* promoter suggest that protection of the promoter against DNA remethylation is dependent on the level of transcriptional activation [27]. It is therefore likely that maintenance of CG gene promoter hypomethylation in tumor cells relies on the presence of appropriate transcription factors, as well as on the activation of such factors by upstream signaling pathways.

Several DNA-binding factors have been identified, which appear to induce activation of CG gene promoters. Transcriptional activation of several genes of the *MAGEA* family has been shown to depend on the binding of ETS transcription factors within their promoter [21, 24]. Interestingly, ETS-binding sequences in *MAGEA* promoters contain a CpG site, and it was shown that methylation of this site inhibits binding of the corresponding factor [25]. In the promoter of *MAGEA1*, two ETS-binding sites were shown to be essential to maintain hypomethylation of the promoter in expressing tumor cells, as evidenced by remethylation of transfected *MAGEA1* constructs containing mutations within these two essential promoter elements [27]. The ETS family of transcription factors comprises about 30 members in humans, which all bind a similar DNA motif with a central GGAA/T sequence [68]. The precise member(s) involved in the regulation of *MAGEA* genes remain(s) to be characterized.

SP1 is another transcription factor, which was shown to contribute to the activation of several *MAGEA* genes, as well as the *CTAG1* gene (also termed *NY-ESO-1*) [24, 46]. The ubiquitously expressed SP1 factor acts as a transcriptional activator and recognizes a consensus DNA sequence (GC box element), which includes a CpG site [80]. SP1-binding elements are therefore often present in CG-rich promoter sequences. Binding of SP1 to the *CTAG1* gene was shown to occur only in cells where the promoter is unmethylated [46]. Interestingly, SP1-binding elements were previously shown to be involved in preserving the methylation-free status of classical CpG-island promoters [13, 62]. It is therefore likely that, once bound to the demethylated promoter of CG genes, SP1 proteins contribute to protect the region against remethylation.

BORIS (also known as CTCFL) is a testis-specific paralog of the ubiquitously expressed DNA-binding protein CTCF, which is involved in various aspects of epigenetic regulation, including gene imprinting and X chromosome inactivation [59]. Both proteins share a highly similar central DNA-binding domain, and recognize therefore overlapping DNA sequences, but contain divergent amino- and carboxy-terminal domains. The gene-encoding BORIS belongs to the CG group of genes, as its expression is regulated by DNA methylation and becomes activated in a wide variety of tumors [38, 49, 87, 95]. Remarkably, it has been demonstrated that in expressing tumors cells, BORIS is targeted to the promoters of other CG genes, namely *MAGEA1* and *CTAG1*, where its recruitment coincides with loss of CTCF binding [40, 87]. BORIS exerts transcriptional activation of CG genes, possibly in cooperation with SP1 transcription factors [46, 87]. In one study, forced overexpression of BORIS led to demethylation (albeit only partially) and activation of various CG genes in normal human fibroblasts, suggesting that BORIS activation in tumors might represent a primary triggering event for the epigenetic de-repression of other CG genes [87]. However, similar experiments from other groups did not confirm CG gene demethylation and activation resulting from BORIS overexpression [49, 97]. Moreover, it was found that many tumors display activation of various CG genes in the absence of BORIS expression. It is therefore unlikely that BORIS is a necessary factor for the derepression of other CG genes in tumors. Its presence in certain tumor cells may, however, facilitate maintenance of the hypomethylated and active state of CG gene promoters.

Many more transcription factors involved in CG gene regulation remain to be identified, and it is likely that each particular CG gene is controlled by a distinct combination of transcription factors. Tissue-specific differences in the content of transcription factors probably account for the fact that, while CG genes tend to be co-activated in hypomethylated tumors, some of them nevertheless show preferential activation in specific tumor types [36, 56].

Cell signaling through tyrosine kinase receptors appears to represent an additional level of control of CG gene regulation. A study in mast cell lines reported that signaling through KIT, an oncogenic receptor hyper-activated in several types of cancers, increases transcription of *MAGE* genes [99]. Other studies revealed that signaling through FGFR2, an FGF receptor often down-regulated in thyroid and pituitary cancers, exerts a negative effect on *MAGEA3* and *MAGEA6* transcription [51, 102]. It is therefore possible that particular dysregulations in cancers, such as those affecting cell signaling pathways, increase the activity of transcription factors that target CG genes, and thereby facilitate long-term activation of these genes in hypomethylated tumor cells. This may partially explain the observation that experimental DNA demethylation, by the use of DNMT inhibitors, often induces CG gene activation more efficiently in tumor cells than in normal cells [47].

7.5.3 Histone Modifications

Active CG gene promoters in tumors usually display a hypomethylated region that comprises one to several kilobases [27]. It is therefore likely that the protective influence of transcription factors against DNA remethylation extends beyond their narrow-binding site. Consistently, impaired binding of ETS transcription factors to *MAGEA1* transgenes, as caused by mutations in their recognition sites, resulted in de novo methylation of CpG sites within the entire promoter region, not just those located nearby the mutated ETS-binding sites [27]. This regional, rather than site-specific effect, might be related to the presence of modifications on the chromatin, such as histone modifications, which after being initiated by specific transcription factors often propagate themselves over larger domains [31]. Histone modifications can indeed influence DNA methylation states [15]. Repressive histone marks, such as methylation of lysine 9 and 27 of histone H3 (H3K9 and H3K27), favor local DNA methylation, whereas active marks, such as histone acetylation or methylation of lysine 4 of histone H3 (H3K4), appear to exclude the DNA methylation machinery. Studies from several groups have shown that demethylation and activation of CG genes in tumor cells is always associated with gains in histone acetylation and H3K4 methylation [42, 70]. The repressed state of human CG genes instead has been associated to a certain extent with the presence of H3K27 and H3K9 methylation marks [42, 70]. The exact relationship between histone modifications changes and DNA demethylation in CG gene promoters remains unclear. A crucial question is whether the varying histone modifications in CG gene promoters are a cause or a consequence of DNA methylation alterations. Studies using inhibitors of histone-modifying enzymes showed that these were on their own unable to induce significant

demethylation and activation of CG genes. Only in combination with inhibitors of DNA methylation, did they significantly modulate the level of activation of CG genes [35, 54, 70]. These observations support the notion that DNA methylation exerts a dominant role in the epigenetic repression of CG genes. But it remains possible that histone modifications assume the responsibility of maintaining the active status of the promoter following its demethylation.

7.5.4 Multiple Factors Determining CG Gene Activation in Tumors

Considering the above, it appears that activation of a particular CG gene in a tumor cell will depend on several factors: (1) the extent of CpG methylation losses resulting from the transient DNA demethylation process; (2) the level of de novo DNA methylation activities in the cell, which might induce remethylation of the promoter; (3) the presence of transcriptional activators and histone-modifying enzymes capable of counteracting remethylation activities. The likelihood that a CG gene becomes activated in a tumor cell probably depends on a complex balance between these different factors (Fig. 7.1).

7.6 Oncogenic Function of CG Genes

Activation of CG genes in tumor cells raises the possibility that their proteins might have oncogenic activities. The biological function of most of these genes, which encode very diverse proteins, remains however poorly understood. One extreme possibility is that the main contribution of DNA hypomethylation to tumor progression resides in its repercussions on genomic instability [33], and that the accompanying activation of CG genes is merely a side effect with no impact on malignancy (other than inducing the expression of tumor antigens). Another possibility has been proposed, in which the concerted expression of CG genes in cancer would correspond to the activation of a gametogenic program, thereby bestowing tumor cells with germ cell properties, including the capacity to self-renew (a feature of spermatogonial stem cells) and increased motility (a feature of sperm cells) [79]. Activation of CG genes in tumors is however only partial, making it very unlikely that all genes necessary for inducing a gametogenic program become expressed at the same time. Nevertheless, it remains possible that some CG genes contribute to tumor progression. Several MAGE proteins were found to inhibit p53 transactivation function, thereby exerting antiapoptotic properties [28, 64, 98]. GAGE proteins were also shown to render cells resistant to apoptosis [18]. Other studies reported that MAGEA11 serves as a co-stimulator for the androgen receptor and might therefore contribute to the development of prostate tumors that have become independent of the presence of androgen for their growth [4, 48]. Moreover, it was noted that

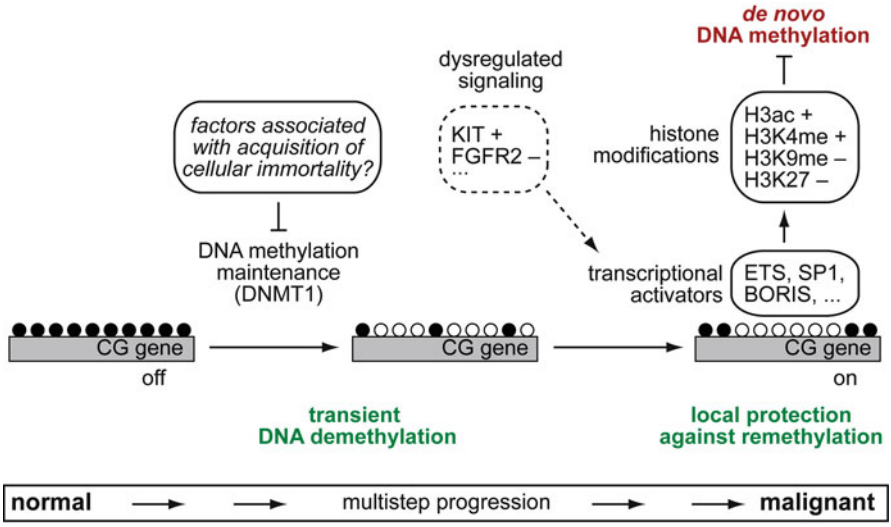

Fig. 7.1 Proposed model of demethylation and activation of CG genes during tumor development. The activation of CG genes in tumors depends on several factors: the extent of the transient DNA demethylation process, occurring at some step of tumor development; the level of counteracting de novo methylation activities in the cell; and the presence of transcriptional activators that protect the CG gene promoter against remethylation, for instance by increasing (+) or decreasing (–) distinct histone marks locally. *Filled circles* represent methylated CpG, *empty circles* unmethylated cytosines

several CG genes, including *BORIS*, *BRDT,* and *ATAD2*, encode nuclear proteins that have a potential impact on chromatin structures and might therefore be involved in the epigenetic alterations commonly affecting cancer genomes [90]. Altogether, these observations support the notion that the activation of several CG genes in tumors, resulting from DNA demethylation, might be associated with the acquisition of oncogenic properties.

Surprisingly, however, two independent studies indicate that *MAGEA4* displays instead tumor-suppressor functions. In one study, MAGEA4 was shown to interact with gankyrin and to inhibit anchorage-independent growth in vitro and tumor formation in mice [65]. In the other study, MAGEA4 was found to promote tumor cell death and to increase their sensitivity to apoptotic stimuli [69]. Clearly, more studies will be required before we can evaluate the full spectrum of consequences of CG gene activation in tumors.

7.7 DNA Hypomethylation in Cancer: An Immunological Paradox

There is now compelling evidence that the immune system is able to identify and destroy tumor cells [81]. This immune surveillance of cancer is believed to provide a barrier to cancer development, even though progressing tumors eventually escape

this obstacle by activating a variety of immune evasion strategies. Evidence for the existence of such surveillance of cancer by the immune system is provided for instance by the observation that solid tumors are often infiltrated by lymphocytes. Not surprisingly, several of these tumor-infiltrating lymphocytes were shown to be directed against antigens encoded by CG genes [50]. This suggests therefore that DNA hypomethylation and the consequent activation of CG genes has, at least at some stage of oncogenesis, a detrimental effect on tumor development. Yet, DNA hypomethylation is observed in most tumors, suggesting that it must otherwise have a strong tumor-promoting effect that outweighs this negative immunogenic effect.

7.8 Epigenetically Assisted Cancer Immunotherapy

Clinical trials of therapeutic vaccination of cancer patients using antigens encoded by CG genes are underway. Noticeable clinical responses were observed, albeit in only a fraction of the treated patients [11]. An interesting possibility to increase vaccination efficiencies would be the use of epigenetic drugs, such as the DNA methylation inhibitor decitabine, which should increase the number of expressed CG genes in the tumors, thereby rendering them more visible to the immune system. Importantly, decitabine is expected to induce reactivation of epigenetically silenced tumor-suppressor genes as well, and hence to reduce the growth rate of the tumors at the same time. Clinical trials combining decitabine and vaccination against antigens encoded by CG gene have been initiated [1].

There are, however, several points concerning the efficiency and safety of such approaches, which remain to be addressed. The first point concerns the specificity of decitabine-induced expression of CG genes in tumor cells rather than normal cells. Although studies have found that tumor cells are more sensitive to decitabine [47], it is obvious that the drug also induces CG genes in normal cell cultures, including fibroblasts and blood lymphocytes [25, 56, 61]. It will therefore be crucial to monitor decitabine/vaccine-treated patients for potential autoimmune reactions directed against their healthy tissues. Another concern relates to the duration of CG gene expression following decitabine treatment. Several studies have shown that CG gene expression in tumor cells was only transient following exposure to decitabine [26, 91]. This may be related to the absence of appropriate transcription factors, and hence lack of protection of the promoters against remethylation. The duration of CG gene expression in tumor cells may be critical to allow complete rejection by the immune cells. In this particular immune context, tumor cells that lose CG gene expression might be strongly selected. Prolonged decitabine treatment or combination with another epigenetic drug favoring protection of CG promoters against remethylation (e.g., drugs affecting histone marks) might be a solution to the problem. Finally, as genome hypomethylation is obviously associated with tumor development, there is a concern that decitabine treatment may generate strongly hypomethylated tumor cells with increased malignancy [33]. This is particularly problematic if it is confirmed that CG genes themselves exert oncogenic functions.

Clearly, a better understanding of the mechanisms of activation and of the biological functions of CG genes should help to resolve these questions, and may help to design the most efficient and safest ways to epigenetically augment tumor immunogenicity, thereby rendering cancer cells more vulnerable to vaccination.

References

1. Akers SN, Odunsi K, Karpf AR (2010) Regulation of cancer germline antigen gene expression: implications for cancer immunotherapy. Future Oncol 6(5):717–732
2. Almeida LG, Sakabe NJ, deOliveira AR, Silva MC, Mundstein AS, Cohen T, Chen YT, Chua R, Gurung S, Gnjatic S, Jungbluth AA, Caballero OL, Bairoch A, Kiesler E, White SL, Simpson AJ, Old LJ, Camargo AA, Vasconcelos AT (2009) CTdatabase: a knowledge-base of high-throughput and curated data on cancer-testis antigens. Nucleic Acids Res 37(Database issue):D816–819
3. Antequera F, Boyes J, Bird A (1990) High levels of de novo methylation and altered chromatin structure at CpG islands in cell lines. Cell 62(3):503–514
4. Bai S, He B, Wilson EM (2005) Melanoma antigen gene protein MAGE-11 regulates androgen receptor function by modulating the interdomain interaction. Mol Cell Biol 25(4):1238–1257
5. Baylin SB, Herman JG, Graff JR, Vertino PM, Issa JP (1998) Alterations in DNA methylation: a fundamental aspect of neoplasia. Adv Cancer Res 72:141–196
6. Bestor TH (2000) The DNA methyltransferases of mammals. Hum Mol Genet 9(16):2395–2402
7. Bird AP (1980) DNA methylation and the frequency of CpG in animal DNA. Nucleic Acids Res 8:1499–1504
8. Bird A (2002) DNA methylation patterns and epigenetic memory. Genes Dev 16(1):6–21
9. Boël P, Wildmann C, Sensi M-L, Brasseur R, Renauld J-C, Coulie P, Boon T, van der Bruggen P (1995) BAGE, a new gene encoding an antigen recognized on human melanomas by cytolytic T lymphocytes. Immunity 2:167–175
10. Boon T, Cerottini J-C, Van den Eynde B, van der Bruggen P, Van Pel A (1994) Tumor antigens recognized by T lymphocytes. Annu Rev Immunol 12:337–365
11. Boon T, Coulie PG, Van den Eynde BJ, van der Bruggen P (2006) Human T cell responses against melanoma. Annu Rev Immunol 24:175–208
12. Boyes J, Bird A (1992) Repression of genes by DNA methylation depends on CpG density and promoter strength: evidence for involvement of a methyl-CpG binding protein. EMBO J 11(1):327–333
13. Brandeis M, Frank D, Keshet I, Siegfried Z, Mendelsohn M, Nemes A, Temper V, Razin A, Cedar H (1994) Sp1 elements protect a CpG island from de novo methylation. Nature 371(6496):435–438
14. Cadieux B, Ching TT, VandenBerg SR, Costello JF (2006) Genome-wide hypomethylation in human glioblastomas associated with specific copy number alteration, methylenetetrahydrofolate reductase allele status, and increased proliferation. Cancer Res 66(17):8469–8476
15. Cedar H, Bergman Y (2009) Linking DNA methylation and histone modification: patterns and paradigms. Nat Rev Genet 10(5):295–304
16. Chen ZX, Riggs AD (2011) DNA methylation and demethylation in mammals. J Biol Chem 286(21):18347–18353
17. Cho B, Lee H, Jeong S, Bang YJ, Lee HJ, Hwang KS, Kim HY, Lee YS, Kang GH, Jeoung DI (2003) Promoter hypomethylation of a novel cancer/testis antigen gene CAGE is correlated with its aberrant expression and is seen in premalignant stage of gastric carcinoma. Biochem Biophys Res Commun 307(1):52–63

18. Cilensek ZM, Yehiely F, Kular RK, Deiss LP (2002) A member of the GAGE family of tumor antigens is an anti-apoptotic gene that confers resistance to Fas/CD95/APO-1, Interferon-gamma, taxol and gamma-irradiation. Cancer Biol Ther 1(4):380–387
19. Cross SH, Bird AP (1995) CpG islands and genes. Curr Opin Genet Dev 5:309–314
20. De Plaen E, Arden K, Traversari C, Gaforio JJ, Szikora J-P, De Smet C, Brasseur F, van der Bruggen P, Lethé B, Lurquin C, Brasseur R, Chomez P, De Backer O, Cavenee W, Boon T (1994) Structure, chromosomal localization and expression of twelve genes of the MAGE family. Immunogenetics 40:360–369
21. De Plaen E, Naerhuyzen B, De Smet C, Szikora J-P, Boon T (1997) Alternative promoters of gene MAGE4a. Genomics 40:305–313
22. De Smet C, Loriot A (2010) DNA hypomethylation in cancer: epigenetic scars of a neoplastic journey. Epigenetics 5(3):206–213
23. De Smet C, Lurquin C, van der Bruggen P, De Plaen E, Brasseur F, Boon T (1994) Sequence and expression pattern of the human MAGE2 gene. Immunogenetics 39:121–129
24. De Smet C, Courtois SJ, Faraoni I, Lurquin C, Szikora JP, De Backer O, Boon T (1995) Involvement of two Ets binding sites in the transcriptional activation of the MAGE1 gene. Immunogenetics 42(4):282–290
25. De Smet C, De Backer O, Faraoni I, Lurquin C, Brasseur F, Boon T (1996) The activation of human gene MAGE-1 in tumor cells is correlated with genome-wide demethylation. Proc Natl Acad Sci USA 93(14):7149–7153
26. De Smet C, Lurquin C, Lethé B, Martelange V, Boon T (1999) DNA methylation is the primary silencing mechanism for a set of germ line- and tumor-specific genes with a CpG-rich promoter. Mol Cell Biol 19:7327–7335
27. De Smet C, Loriot A, Boon T (2004) Promoter-dependent mechanism leading to selective hypomethylation within the 5′ region of gene MAGE-A1 in tumor cells. Mol Cell Biol 24(11):4781–4790
28. Doyle JM, Gao J, Wang J, Yang M, Potts PR (2010) MAGE-RING protein complexes comprise a family of E3 ubiquitin ligases. Mol Cell 39(6):963–974
29. Ehrlich M (2002) DNA methylation in cancer: too much, but also too little. Oncogene 21(35):5400–5413
30. Feinberg AP, Gehrke CW, Kuo KC, Ehrlich M (1988) Reduced genomic 5-methylcytosine content in human colonic neoplasia. Cancer Res 48(5):1159–1161
31. Felsenfeld G, Groudine M (2003) Controlling the double helix. Nature 421(6921):448–453
32. Flatau E, Gonzales FA, Michalowsky LA, Jones PA (1984) DNA methylation in 5-aza-2′-deoxycytidine-resistant variants of C3H 10T1/2C18 cells. Mol Cell Biol 4(10):2098–2102
33. Gaudet F, Hodgson JG, Eden A, Jackson-Grusby L, Dausman J, Gray JW, Leonhardt H, Jaenisch R (2003) Induction of tumors in mice by genomic hypomethylation. Science 300(5618):489–492
34. Gaugler B, Van den Eynde B, van der Bruggen P, Romero P, Gaforio JJ, De Plaen E, Lethé B, Brasseur F, Boon T (1994) Human gene MAGE-3 codes for an antigen recognized on a melanoma by autologous cytolytic T lymphocytes. J Exp Med 179:921–930
35. Goodyear O, Agathanggelou A, Novitzky-Basso I, Siddique S, McSkeane T, Ryan G, Vyas P, Cavenagh J, Stankovic T, Moss P, Craddock C (2010) Induction of a CD8+ T-cell response to the MAGE cancer testis antigen by combined treatment with azacitidine and sodium valproate in patients with acute myeloid leukemia and myelodysplasia. Blood 116(11):1908–1918
36. Grunwald C, Koslowski M, Arsiray T, Dhaene K, Praet M, Victor A, Morresi-Hauf A, Lindner M, Passlick B, Lehr HA, Schafer SC, Seitz G, Huber C, Sahin U, Tureci O (2006) Expression of multiple epigenetically regulated cancer/germline genes in nonsmall cell lung cancer. Int J Cancer 118(10):2522–2528
37. Haas GG Jr, D'Cruz OJ, De Bault LE (1988) Distribution of human leukocyte antigen-ABC and -D/DR antigens in the unfixed human testis. Am J Reprod Immunol Microbiol 18(2):47–51
38. Hoffmann MJ, Muller M, Engers R, Schulz WA (2006) Epigenetic control of CTCFL/BORIS and OCT4 expression in urogenital malignancies. Biochem Pharmacol 72(11):1577–1588

39. Holliday R, Pugh JE (1975) DNA modification mechanisms and gene activity during development. Science 186:226–232
40. Hong JA, Kang Y, Abdullaev Z, Flanagan PT, Pack SD, Fischette MR, Adnani MT, Loukinov DI, Vatolin S, Risinger JI, Custer M, Chen GA, Zhao M, Nguyen DM, Barrett JC, Lobanenkov VV, Schrump DS (2005) Reciprocal binding of CTCF and BORIS to the NY-ESO-1 promoter coincides with derepression of this cancer-testis gene in lung cancer cells. Cancer Res 65(17):7763–7774
41. Jackson K, Yu MC, Arakawa K, Fiala E, Youn B, Fiegl H, Muller-Holzner E, Widschwendter M, Ehrlich M (2004) DNA hypomethylation is prevalent even in low-grade breast cancers. Cancer Biol Ther 3(12):1225–1231
42. James SR, Link PA, Karpf AR (2006) Epigenetic regulation of X-linked cancer/germline antigen genes by DNMT1 and DNMT3b. Oncogene 25(52):6975–6985
43. Jones PA, Wolkowicz MJ, Rideout WM III, Gonzales FA, Marziasz CM, Coetzee GA, Tapscott SJ (1990) De novo methylation of the MyoD1 CpG island during the establishment of immortal cell lines. Proc Natl Acad Sci USA 87(16):6117–6121
44. Jungbluth AA, Chen YT, Stockert E, Busam KJ, Kolb D, Iversen K, Coplan K, Williamson B, Altorki N, Old LJ (2001) Immunohistochemical analysis of NY-ESO-1 antigen expression in normal and malignant human tissues. Int J Cancer 92(6):856–860
45. Kaneda A, Tsukamoto T, Takamura-Enya T, Watanabe N, Kaminishi M, Sugimura T, Tatematsu M, Ushijima T (2004) Frequent hypomethylation in multiple promoter CpG islands is associated with global hypomethylation, but not with frequent promoter hypermethylation. Cancer Sci 95(1):58–64
46. Kang Y, Hong JA, Chen GA, Nguyen DM, Schrump DS (2007) Dynamic transcriptional regulatory complexes including BORIS, CTCF and Sp1 modulate NY-ESO-1 expression in lung cancer cells. Oncogene 26(30):4394–4403
47. Karpf AR, Lasek AW, Ririe TO, Hanks AN, Grossman D, Jones DA (2004) Limited gene activation in tumor and normal epithelial cells treated with the DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine. Mol Pharmacol 65(1):18–27
48. Karpf AR, Bai S, James SR, Mohler JL, Wilson EM (2009) Increased expression of androgen receptor coregulator MAGE-11 in prostate cancer by DNA hypomethylation and cyclic AMP. Mol Cancer Res 7(4):523–535
49. Kholmanskikh O, Loriot A, Brasseur F, De Plaen E, De Smet C (2008) Expression of BORIS in melanoma: lack of association with MAGE-A1 activation. Int J Cancer 122(4):777–784
50. Khong HT, Wang QJ, Rosenberg SA (2004) Identification of multiple antigens recognized by tumor-infiltrating lymphocytes from a single patient: tumor escape by antigen loss and loss of MHC expression. J Immunother 27(3):184–190
51. Kondo T, Zhu X, Asa SL, Ezzat S (2007) The cancer/testis antigen melanoma-associated antigen-A3/A6 is a novel target of fibroblast growth factor receptor 2-IIIb through histone H3 modifications in thyroid cancer. Clin Cancer Res 13(16):4713–4720
52. Koslowski M, Bell C, Seitz G, Lehr HA, Roemer K, Muntefering H, Huber C, Sahin U, Tureci O (2004) Frequent nonrandom activation of germ-line genes in human cancer. Cancer Res 64(17):5988–5993
53. Lin CH, Hsieh SY, Sheen IS, Lee WC, Chen TC, Shyu WC, Liaw YF (2001) Genome-wide hypomethylation in hepatocellular carcinogenesis. Cancer Res 61(10):4238–4243
54. Link PA, Gangisetty O, James SR, Woloszynska-Read A, Tachibana M, Shinkai Y, Karpf AR (2009) Distinct roles for histone methyltransferases G9a and GLP in cancer germ-line antigen gene regulation in human cancer cells and murine embryonic stem cells. Mol Cancer Res 7(6):851–862
55. Lorincz MC, Schubeler D, Goeke SC, Walters M, Groudine M, Martin DI (2000) Dynamic analysis of proviral induction and de novo methylation: implications for a histone deacetylase-independent, methylation density-dependent mechanism of transcriptional repression. Mol Cell Biol 20(3):842–850
56. Loriot A, Boon T, De Smet C (2003) Five new human cancer-germline genes identified among 12 genes expressed in spermatogonia. Int J Cancer 105(3):371–376

57. Loriot A, De Plaen E, Boon T, De Smet C (2006) Transient down-regulation of DNMT1 methyltransferase leads to activation and stable hypomethylation of MAGE-A1 in melanoma cells. J Biol Chem 281(15):10118–10126
58. Loriot A, Sterpin C, De Backer O, De Smet C (2008) Mouse embryonic stem cells induce targeted DNA demethylation within human MAGE-A1 transgenes. Epigenetics 3(1):38–42
59. Loukinov DI, Pugacheva E, Vatolin S, Pack SD, Moon H, Chernukhin I, Mannan P, Larsson E, Kanduri C, Vostrov AA, Cui H, Niemitz EL, Rasko JE, Docquier FM, Kistler M, Breen JJ, Zhuang Z, Quitschke WW, Renkawitz R, Klenova EM, Feinberg AP, Ohlsson R, Morse HC III, Lobanenkov VV (2002) BORIS, a novel male germ-line-specific protein associated with epigenetic reprogramming events, shares the same 11-zinc-finger domain with CTCF, the insulator protein involved in reading imprinting marks in the soma. Proc Natl Acad Sci USA 99(10):6806–6811
60. Lucas S, De Smet C, Arden KC, Viars CS, Lethe B, Lurquin C, Boon T (1998) Identification of a new MAGE gene with tumor-specific expression by representational difference analysis. Cancer Res 58(4):743–752
61. Lurquin C, De Smet C, Brasseur F, Muscatelli F, Martelange V, De Plaen E, Brasseur R, Monaco AP, Boon T (1997) Two members of the human MAGEB gene family located in Xp21.3 are expressed in tumors of various histological origins. Genomics 46(3):397–408
62. Macleod D, Charlton J, Mullins J, Bird AP (1994) Sp1 sites in the mouse aprt gene promoter are required to prevent methylation of the CpG island. Genes Dev 8(19):2282–2292
63. Martelange V, De Smet C, De Plaen E, Lurquin C, Boon T (2000) Identification on a human sarcoma of two new genes with tumor-specific expression. Cancer Res 60(14):3848–3855
64. Monte M, Simonatto M, Peche LY, Bublik DR, Gobessi S, Pierotti MA, Rodolfo M, Schneider C (2006) MAGE-A tumor antigens target p53 transactivation function through histone deacetylase recruitment and confer resistance to chemotherapeutic agents. Proc Natl Acad Sci USA 103(30):11160–11165
65. Nagao T, Higashitsuji H, Nonoguchi K, Sakurai T, Dawson S, Mayer RJ, Itoh K, Fujita J (2003) MAGE-A4 interacts with the liver oncoprotein gankyrin and suppresses its tumorigenic activity. J Biol Chem 278(12):10668–10674
66. Novak P, Jensen T, Oshiro MM, Watts GS, Kim CJ, Futscher BW (2008) Agglomerative epigenetic aberrations are a common event in human breast cancer. Cancer Res 68(20):8616–8625
67. Novak P, Jensen TJ, Garbe JC, Stampfer MR, Futscher BW (2009) Stepwise DNA methylation changes are linked to escape from defined proliferation barriers and mammary epithelial cell immortalization. Cancer Res 69(12):5251–5258
68. Oikawa T, Yamada T (2003) Molecular biology of the Ets family of transcription factors. Gene 303:11–34
69. Peikert T, Specks U, Farver C, Erzurum SC, Comhair SA (2006) Melanoma antigen A4 is expressed in non-small cell lung cancers and promotes apoptosis. Cancer Res 66(9): 4693–4700
70. Rao M, Chinnasamy N, Hong JA, Zhang Y, Zhang M, Xi S, Liu F, Marquez VE, Morgan RA, Schrump DS (2011) Inhibition of histone lysine methylation enhances cancer-testis antigen expression in lung cancer cells: implications for adoptive immunotherapy of cancer. Cancer Res 71(12):4192–4204
71. Rauch TA, Zhong X, Wu X, Wang M, Kernstine KH, Wang Z, Riggs AD, Pfeifer GP (2008) High-resolution mapping of DNA hypermethylation and hypomethylation in lung cancer. Proc Natl Acad Sci USA 105(1):252–257
72. Riggs AD (1989) DNA methylation and cell memory. Cell Biophys 15(1–2):1–13
73. Sahin U, Tureci O, Schmitt H, Cochlovius B, Johannes T, Schmits R, Stenner F, Luo G, Schobert I, Pfreundschuh M (1995) Human neoplasms elicit multiple specific immune responses in the autologous host. Proc Natl Acad Sci USA 92:11810–11813
74. Sahin U, Tureci O, Chen YT, Seitz G, Villena-Heinsen C, Old LJ, Pfreundschuh M (1998) Expression of multiple cancer/testis (CT) antigens in breast cancer and melanoma: basis for polyvalent CT vaccine strategies. Int J Cancer 78(3):387–389

75. Scanlan MJ, Gordon CM, Williamson B, Lee SY, Chen YT, Stockert E, Jungbluth A, Ritter G, Jager D, Jager E, Knuth A, Old LJ (2002) Identification of cancer/testis genes by database mining and mRNA expression analysis. Int J Cancer 98(4):485–492
76. Scanlan MJ, Simpson AJ, Old LJ (2004) The cancer/testis genes: review, standardization, and commentary. Cancer Immun 4:1
77. Shen L, Kondo Y, Guo Y, Zhang J, Zhang L, Ahmed S, Shu J, Chen X, Waterland RA, Issa JP (2007) Genome-wide profiling of DNA methylation reveals a class of normally methylated CpG island promoters. PLoS Genet 3(10):2023–2036
78. Sigalotti L, Coral S, Nardi G, Spessotto A, Cortini E, Cattarossi I, Colizzi F, Altomonte M, Maio M (2002) Promoter methylation controls the expression of MAGE2, 3 and 4 genes in human cutaneous melanoma. J Immunother 25(1):16–26
79. Simpson AJ, Caballero OL, Jungbluth A, Chen YT, Old LJ (2005) Cancer/testis antigens, gametogenesis and cancer. Nat Rev Cancer 5(8):615–625
80. Suske G (1999) The Sp-family of transcription factors. Gene 238(2):291–300
81. Swann JB, Smyth MJ (2007) Immune surveillance of tumors. J Clin Invest 117(5):1137–1146
82. Takahashi K, Shichijo S, Noguchi M, Hirohata M, Itoh K (1995) Identification of MAGE-1 and MAGE-4 proteins in spermatogonia and primary spermatocytes of testis. Cancer Res 55(16):3478–3482
83. Tilman G, Loriot A, Van Beneden A, Arnoult N, Londono-Vallejo JA, De Smet C, Decottignies A (2009) Subtelomeric DNA hypomethylation is not required for telomeric sister chromatid exchanges in ALT cells. Oncogene 28(14):1682–1693
84. Van den Eynde B, Peeters O, De Backer O, Gaugler B, Lucas S, Boon T (1995) A new family of genes coding for an antigen recognized by autologous cytolytic T lymphocytes on a human melanoma. J Exp Med 182:689–698
85. van der Bruggen P, Traversari C, Chomez P, Lurquin C, De Plaen E, Van den Eynde B, Knuth A, Boon T (1991) A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. Science 254(5038):1643–1647
86. Van Der Bruggen P, Zhang Y, Chaux P, Stroobant V, Panichelli C, Schultz ES, Chapiro J, Van Den Eynde BJ, Brasseur F, Boon T (2002) Tumor-specific shared antigenic peptides recognized by human T cells. Immunol Rev 188(1):51–64
87. Vatolin S, Abdullaev Z, Pack SD, Flanagan PT, Custer M, Loukinov DI, Pugacheva E, Hong JA, Morse H III, Schrump DS, Risinger JI, Barrett JC, Lobanenkov VV (2005) Conditional expression of the CTCF-paralogous transcriptional factor BORIS in normal cells results in demethylation and derepression of MAGE-A1 and reactivation of other cancer-testis genes. Cancer Res 65(17):7751–7762
88. Walsh CP, Bestor TH (1999) Cytosine methylation and mammalian development. Genes Dev 13(1):26–34
89. Wang Z, Zhang J, Zhang Y, Lim SH (2006) SPAN-Xb expression in myeloma cells is dependent on promoter hypomethylation and can be upregulated pharmacologically. Int J Cancer 118(6):1436–1444
90. Wang J, Emadali A, Le Bescont A, Callanan M, Rousseaux S, Khochbin S (2011) Induced malignant genome reprogramming in somatic cells by testis-specific factors. Biochim Biophys Acta 1809(4–6):221–225
91. Weber J, Salgaller M, Samid D, Johnson B, Herlyn M, Lassam N, Treisman J, Rosenberg SA (1994) Expression of the MAGE-1 tumor antigen is up-regulated by the demethylating agent 5-aza-2′-deoxycytidine. Cancer Res 54(7):1766–1771
92. Weber M, Davies JJ, Wittig D, Oakeley EJ, Haase M, Lam WL, Schubeler D (2005) Chromosome-wide and promoter-specific analyses identify sites of differential DNA methylation in normal and transformed human cells. Nat Genet 37(8):853–862
93. Weber M, Hellmann I, Stadler MB, Ramos L, Paabo S, Rebhan M, Schubeler D (2007) Distribution, silencing potential and evolutionary impact of promoter DNA methylation in the human genome. Nat Genet 39(4):457–466
94. Wilson VL, Jones PA (1983) DNA methylation decreases in aging but not in immortal cells. Science 220(4601):1055–1057

95. Woloszynska-Read A, James SR, Link PA, Yu J, Odunsi K, Karpf AR (2007) DNA methylation-dependent regulation of BORIS/CTCFL expression in ovarian cancer. Cancer Immun 7:21
96. Woloszynska-Read A, Mhawech-Fauceglia P, Yu J, Odunsi K, Karpf AR (2008) Intertumor and intratumor NY-ESO-1 expression heterogeneity is associated with promoter-specific and global DNA methylation status in ovarian cancer. Clin Cancer Res 14(11):3283–3290
97. Woloszynska-Read A, James SR, Song C, Jin B, Odunsi K, Karpf AR (2010) BORIS/CTCFL expression is insufficient for cancer-germline antigen gene expression and DNA hypomethylation in ovarian cell lines. Cancer Immun 10:6
98. Yang B, O'Herrin SM, Wu J, Reagan-Shaw S, Ma Y, Bhat KM, Gravekamp C, Setaluri V, Peters N, Hoffmann FM, Peng H, Ivanov AV, Simpson AJ, Longley BJ (2007) MAGE-A, mMage-b, and MAGE-C proteins form complexes with KAP1 and suppress p53-dependent apoptosis in MAGE-positive cell lines. Cancer Res 67(20):9954–9962
99. Yang B, Wu J, Maddodi N, Ma Y, Setaluri V, Longley BJ (2007) Epigenetic control of MAGE gene expression by the KIT tyrosine kinase. J Invest Dermatol 127(9):2123–2128
100. Yegnasubramanian S, Haffner MC, Zhang Y, Gurel B, Cornish TC, Wu Z, Irizarry RA, Morgan J, Hicks J, DeWeese TL, Isaacs WB, Bova GS, De Marzo AM, Nelson WG (2008) DNA hypomethylation arises later in prostate cancer progression than CpG island hypermethylation and contributes to metastatic tumor heterogeneity. Cancer Res 68(21):8954–8967
101. Yoder JA, Walsh CP, Bestor TH (1997) Cytosine methylation and the ecology of intragenomic parasites. Trends Genet 13(8):335–340
102. Zhu X, Asa SL, Ezzat S (2008) Fibroblast growth factor 2 and estrogen control the balance of histone 3 modifications targeting MAGE-A3 in pituitary neoplasia. Clin Cancer Res 14(7):1984–1996

Chapter 8
APC and DNA Demethylation in Cell Fate Specification and Intestinal Cancer

Angela Andersen and David A. Jones

Abstract Most cases of colon cancer are initiated by mutation or loss of the tumor suppressor gene adenomatous polyposis coli (*APC*). APC controls many cellular functions including intestinal cell proliferation, differentiation, migration, and polarity. This chapter focuses on the role of APC in regulating a recently identified DNA demethylase system, consisting of a cytidine deaminase and a DNA glycosylase. A global decrease in DNA methylation is known to occur soon after loss of APC; however, how this occurs and its contribution to tumorigenesis has been unclear. In the absence of wild-type *APC*, ectopic expression of the DNA demethylase system leads to the hypomethylation of specific loci, including intestinal cell fating genes, and stabilizes intestinal cells in an undifferentiated state. Further, misregulation of this system may influence the acquisition of subsequent genetic mutations that drive tumorigenesis.

Colon cancer is the second leading cause of cancer-related death in the western world [1]. Truncating mutations in the tumor suppressor gene (TSG) adenomatous polyposis coli (*APC*) underlie 70–80% of sporadic colon cancers, and germ line mutations in *APC* cause familial adenomatous polyposis (FAP) syndrome, which inevitably leads to colon cancer unless the colon is removed [2, 3]. Mutations in *APC* are observed in early intestinal lesions including aberrant crypt foci, and their frequency is similar in benign adenomas and advanced stage carcinomas, suggesting that the loss of *APC* function initiates tumorigenesis [4]. Additional genetic and epigenetic events affect the rate of tumor progression. Changes in DNA methylation are detected in early stage adenomas, and can be classified as drivers or passengers of tumor progression, analogous to genetic mutations [5–8]. Mutations that activate

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the oncogene *KRAS* are infrequent in small polyps but are common in larger, less differentiated adenomas [9]. Loss of p53 function appears to arise even later in tumor progression and is observed mostly in carcinomas [10]. Technological advances in genome and epigenome analyses should facilitate extensive characterization of the spectrum, sequence, and interdependence of the molecular events that promote colon cancer and should also enable the development of more precise, personalized diagnoses and treatments.

8.1 Tumor Suppressor Functions of APC

A well-appreciated role for *APC* in tumor suppression is as a negative regulator of Wnt signaling [11]. In the absence of Wnt signaling, *APC* forms a destruction complex with Axin and two kinases, casein kinase 1 and glycogen syntase kinase 3β, that phosphorylate the transcriptional co-activator β-catenin. Phosphorylated β-catenin is then ubiquitinated and targeted for proteasomal degradation. Wnt signaling inhibits the formation of the destruction complex, thereby stabilizing β-catenin, which subsequently translocates to the nucleus, binds to the transcription factor TCF4, and activates target genes such as *c-myc* and *cyclin D1*. Deleterious mutations in *APC* stabilize β-catenin and are thus thought to trigger ectopic Wnt signaling. This, in turn, affects multiple cellular functions including adhesion, migration, apoptosis, and proliferation. Consistent with this model, stabilizing mutations within the gene encoding β-catenin are sufficient to initiate adenoma formation in transgenic mice and are associated with about 7% of sporadic colon cancers [12–14].

At the same time, a number of studies have suggested that loss of APC function is not sufficient to induce Wnt signaling. For example, tissues lacking functional APC do not always exhibit the predicted nuclear localization of β-catenin associated with activated Wnt signaling [15]. Blaker et al. showed that early adenomas with mild dysplasia displayed elevated levels of β-catenin in the cytoplasm but not the nucleus, whereas β-catenin was nuclear only in late stage adenomas. In addition, Anderson et al. examined grossly uninvolved and adenoma tissues taken from FAP patients and were unable to identify unambiguous staining for nuclear β-catenin in over 90% of the adenomas [16]. Recent advances suggest that Wnt signaling induces posttranslational modifications of β-catenin that regulate its subcellular localization and function as a transcriptional co-activator with TCF4. For instance, β-catenin is upregulated but confined to the cytoplasm in the intestines of homozygous *apc* mutant zebrafish (*apc*^{mcr}) embryos [17]. These mutant zebrafish display a decrease in the number of intestinal epithelial cells, consistent with reduced Wnt signaling and cell proliferation. This study showed that activation of EGF signaling was required to cooperate with loss of APC in order to stimulate nuclear translocation of β-catenin, activate Wnt signaling, and induce proliferation in *apc*^{mcr} mutant fish. The nuclear accumulation of β-catenin depended on Rac1 and Jnk2 activity, extend-

ing previous observations that these kinases are required for canonical Wnt signaling during mouse development [18]. Similarly, the detection of nuclear β-catenin in advanced human colon adenomas is coincident with increased levels of phospho-cJun, an indicator of JNK activity. Thus, loss of APC appears to stabilize β-catenin without necessarily inducing nuclear translocation and activation of target genes. In this model, aberrant Wnt/β-catenin signaling is a distinct event that contributes to tumor progression after loss of APC.

Indeed, the mechanism of tumor initiation following loss of APC activity may involve functions that are independent of β-catenin. For instance, APC binds to microtubules and regulates mitotic spindle dynamics, which in turn may influence many cellular functions, including chromosome segregation, genomic stability, and cell polarity [19–21]. APC was recently shown to promote asymmetric division of intestinal stem cells, possibly by affecting cell shape [22]. In addition, APC also acts as a positive regulator of retinoic acid (RA) biosynthesis, and, as a result, intestinal cell fate specification [23–26]. Retinoic acid is known to play important roles in controlling cell patterning, fate, and differentiation through the binding and activation of specific RA receptors, retinoid A receptors (RARα, RARβ, and RARγ), or retinoid X receptors (RXRα, RXRβ, and RXRγ) [27]. These receptors belong to the nuclear hormone receptor superfamily and are thought to act, following ligand binding, as direct activators or repressors of gene transcription [28]. A number of studies have implicated retinoids in normal colonocyte function and in the development of colon neoplasms. Compelling evidence for retinoic acid in intestinal development comes from previous studies demonstrating that retinol dehydrogenases Rdh1 and Rdh1l were essential for normal development and in intestinal differentiation in zebrafish [25, 26]. Specifically, knockdown of either Rdh1 or Rdh1l function resulted in well-known RA-deficient phenotypes including loss of pectoral fin formation, lack of jaw development, small eyes, absence of differentiated exocrine pancreas, and aberrant intestinal development. Further studies demonstrated a clear genetic connection between loss of APC and impaired retinoic acid biosynthesis. *apc*^{mcr} zebrafish lack rdhs expression and share a number of developmental phenotypes present in rdh-deficient fish. In addition, exogenous retinoic acid can improve developmental abnormalities in APC-deficient zebrafish, including failed intestinal cell differentiation. Despite the data implicating retinoic acid in intestinal cell functions, the direct functions of retinoic acid in this context remained unexplained.

8.2 Aberrant DNA Methylation Is Associated with Colon Cancer Progression

Retinoic acid induces cell differentiation of different cell types in vitro and in vivo and is thus associated with changes in DNA methylation [28–30]. About 4% of cytosines in a vertebrate genome are methylated by the action of DNA methyltransferases (Dnmt) [31]. Methylcytosine can further be converted to hydroxymethylcytosine,

formylmethylcytosine, and carboxymethylcytosine [32–34]. Methylated cytosine usually occurs at CpG dinucleotides, although significant cytosine methylation outside the CpG context is observed in embryonic stem cells and induced pluripotent cells [35]. Methylated CpG sites are enriched within repetitive sequences such as long interspersed nuclear elements (LINEs) and satellites. Dense methylation of these regions contributes to genomic stability by silencing retrotransposons and suppressing recombination. In contrast, CpG islands, which are short CpG-rich regions frequently found within promoters, tend to be unmethylated in normal tissue [36]. CpG island shores, which are regions located outside of gene promoters but within 2 kb of CpG islands, are differentially methylated in pluripotent cells, different tissues and tumors [37, 38]. Methylation of CpG islands and CpG island shores is associated with gene silencing; however, DNA methylation within gene bodies and intergenic regions has been shown to promote transcription [39]. In addition, DNA methylation was recently shown to influence alternative splicing [40]. Thus, specific patterns of DNA methylation throughout the genome regulate genomic stability and cell-type-specific gene expression.

Aberrant DNA methylation occurs soon after loss of APC, and evidence suggests that it promotes cancer progression. Widespread DNA hypomethylation, inferred from a decrease in LINE-1 methylation, is observed in small adenomas as well as late-stage carcinomas. It was recently shown that most of this hypomethylation corresponds to large, discrete blocks encompassing half the genome and consisting of repetitive sequences as well as genes [41]. Genes within these hypomethylated blocks displayed increased expression variability in different cancer samples, but were not expressed in normal samples, and it was postulated that this stochastic gene expression may contribute to tumor heterogeneity and facilitate the survival of cancer cells in different environments. Demethylation is thought to induce genomic instability by activating retrotransposons and by increasing the frequency of recombination events within repetitive heterochromatin. In addition, hypomethylation could contribute to the chromatin restructuring and nuclear disorganization associated with cancer cells. Smaller regions outside of these blocks were also differentially methylated relative to normal tissue. Hypomethylation was typically observed at CpG island shores and correlated with increased gene expression. In contrast, hypermethylation was associated with CpG islands and gene silencing. The genes that were identified as differentially methylated in colon cancer are enriched for those that are normally differentially methylated between tissues and appear to function in pluripotency, differentiation, and cell fate specification.

8.3 APC Regulates DNA Demethylation and Cell Fate Through Retinoic Acid

DNA methylation may be lost passively or actively removed. Passive demethylation occurs when unmethylated cytosine is incorporated into DNA during replication in the absence of maintenance Dnmt activity. In contrast, during active demethylation methylated cytosines are replaced with unmethylated ones by an enzymatic process

independent of DNA replication. Both mechanisms of demethylation likely contribute to the DNA hypomethylation observed in tumors. An age-dependent decrease of methylation has been observed both in normal tissues and in tumors, consistent with errors in methylated cytosine replication fidelity [42]. This passive, gradual loss of DNA methylation could facilitate tumor initiation or progression by triggering genomic instability and changes in gene expression.

Genetic mutations may also lead to aberrant DNA demethylation. Recently, it was shown that homozygous *apc*^{mcr} zebrafish embryos have reduced DNA methylation at the promoters of genes implicated in intestinal cell fate specification and colorectal cancer, such as *hoxd13a* and *pitx2* [43]. Moreover, these APC-deficient embryos had upregulated the components of a DNA demethylase system, including the cytidine deaminases Aid and Apobec2a, the thymine glycosylase Mbd4, and the DNA repair protein Gadd45α [44, 45]. Knockdown of Mbd4 or of the cytosine deaminases in *apc*^{mcr} zebrafish embryos restored methylation levels. In addition, human colon adenoma samples harboring germ line *APC* mutations also showed reduced DNA methylation at the corresponding loci and upregulation of Aid, Mbd4, and Gadd45α. Thus, APC prevents hypomethylation of key intestinal fating and colorectal cancer genes by repressing the demethylase system.

The upregulation of the demethylase system upon loss of APC was shown to be a consequence of loss of RA production, not misregulated Wnt signaling. Treatment of mutant zebrafish embryos with all-trans retinoic acid, which restores RA levels, but not a pharmacological inhibitor of Cox2, which reduces β-catenin levels downstream of activated Wnt signaling, precluded the upregulation of Aid, Mbd4, and Gadd45α. Further, pharmacological inhibition of RA production in wild-type, adult zebrafish also increased the expression of the demethylase genes and reduced cytosine methylation. Together these observations indicated that DNA demethylation and the expression of the demethylase system are regulated by RA production downstream of APC [43].

Genetic or epigenetic deregulation of genes controlling cell fate decisions can lead to tumorigenesis by precluding the differentiation of progenitor cells [43]. Indeed, DNA hypomethylation of *apc*^{mcr} zebrafish embryos is associated with an expansion of intestinal progenitor cells, revealed by the promoter demethylation and increased expression of intestinal cell fating genes and of *aldh1a2*, a marker of colon crypt progenitor cells, and by the decreased expression of a marker for intestinal differentiation, *fabp2*. Knockdown of the demethylase system components induced intestinal differentiation, indicating that hypomethylation is required to stabilize intestinal cells in a progenitor-like state. In addition, increased cell proliferation was observed in the brain of *apc*^{mcr} zebrafish embryos, and this also depended on the demethylase system. Patterning defects were excluded since the mutant embryos expressed primordial brain and intestinal markers. These data support a role for APC in cell fate specification and differentiation through the regulation of RA production and, in turn, DNA methylation. Thus, loss of APC may initiate tumorigenesis in part by hypomethylating and deregulating cell fate genes, resulting in the expansion of proliferative, progenitor-like cells.

The proposed mechanism of demethylation by this system couples enzyme-mediated deamination of methylated cytosine (me-dC), to produce thymine (dT), with

glycosylase-mediated base excision repair to replace the dG:dT mismatch with a dG:dC base pair [45]. Aid, Mbd4, and Gadd45α were shown promote demethylation of a methylated, double-stranded DNA fragment injected into wild-type zebrafish embryos, and also of bulk genomic DNA. The injected DNA fragment is not replicated, excluding a passive mechanism of demethylation arising from rounds of DNA replication without subsequent cytosine methylation of the newly synthesized strand. Further, co-expression of Aid with a catalytic mutant of Mbd4 in zebrafish embryos stabilized the dG:dT mismatches that would be generated by deamination. Indeed, Aid and a related cytosine deaminase Apobec1 have been shown to deaminate me-dC to dT within single-stranded DNA in vitro [46]. Nevertheless, the field awaits biochemical support for the proposed mechanism and insight into how Aid accesses me-dC within duplex DNA. Given that Mbd4 can recognize and extrude me-dC from duplex DNA, this component of the demethylase system could both target the deaminase to me-dC and promote substrate accessibility [47, 48]. Consistent with this model, Mbd4 was required not only for repair of the dG:dT mismatch, but also for Aid-mediated deamination of me-dC in zebrafish embryos. Moreover, Gadd45α appears to stabilize the physical interaction of Mbd4 with Aid [45]. The stable association of a deaminase with a glycosylase may be important not only for targeting demethylation but also for mediating the repair of the dG:dT intermediate.

That APC may suppress tumor formation partly through negative regulation of DNA demethylase components is consistent with previous observations. Mice carrying the APC multiple intestinal neoplasia (Apc^{min}) mutant allele, which produces truncated APC, develop intestinal lesions similar to human FAP and are frequently employed as a mouse model for colon carcinogenesis. Interestingly, genetic deletion of the cytidine deaminase Apobec1 reduced adenoma formation in $Apc^{min/+}$ mice [49]. Apobec1 is highly expressed in the small intestine and targets a number of mRNAs for C to U editing [50]. It had previously been shown that Apobec1 binds and stabilizes cyclooxygenase 2 (Cox2) mRNA in vitro [51]. Adenomas from $Apc^{min/+}$ *Apobec-1*$^{-/-}$ mice displayed decreased expression of Cox2 and it was suggested that this could account for the reduced tumor burden. This model is consistent with previous reports that Cox2 expression is increased in adenomas, and that genetic or pharmacological inhibition of Cox2 also decreases polyp formation in APC mutant mice [52]. However, Apobec1 can also deaminate DNA, and this activity may also promote tumor progression. Deamination of dC or me-dC results in transitions to dT, and Apobec-1 knockout mice would be predicted to have a reduced frequency of these mutations. This in turn could decrease polyp initiation by preventing second-hit mutations. In addition, given that components of the DNA demethylase system are ectopically expressed in the absence of APC, Apobec1 may also cooperate with a thymine glycosylase to promote DNA demethylation, altered gene expression, and the expansion of intestinal progenitor cells in $Apc^{min/+}$ mice. Thus, $Apc^{min/+}$ *Apobec-1*$^{-/-}$ mice may display reduced adenoma formation in part due to reduced transition mutations and to restored DNA methylation patterns and differentiation of intestinal progenitor cells.

In considering the development of APC loss-dependent colorectal cancer, it is plausible to envision a role for DNA demethylation given its role in reprogramming

in other systems. Genome-wide demethylation of the paternal genome in the mammalian zygote occurs within hours after fertilization [53–55]. Later in embryogenesis, during specification of mouse primordial germ cells, the cytosine methylation that underlies parental imprints is erased and pluripotency is reestablished [56, 57]. Interestingly, genome-wide bisulphite sequencing analysis revealed an increase in global DNA methylation levels in PGCs derived from Aid-null embryos relative to wild-type embryos [58]. However, significant demethylation occurred even in the absence of Aid, suggesting that this process may involve other deaminases like Apobec1 [46] or another mechanism. Similarly, reduced levels of DNA demethylation in zebrafish required simultaneous knockdown of Aid and Apobec2 [45], suggesting redundancy among members of the Aid/Apobec family. DNA demethylation is also a rate-limiting step for reprogramming somatic cells to a pluripotent state [59–61]. Indeed, Aid was required for the demethylation and induction of pluripotency genes in heterokaryons generated by fusing mouse embryonic stem (ES) cells with human fibroblasts. Importantly, Aid-mediated DNA demethylation did not require cell proliferation or DNA replication, providing further support for a role for Aid in active DNA demethylation. Prior to cell fusion, Aid is bound to distinct, methylated promoters in each cell type. For instance, Aid associates with the methylated promoters of Oct4 and Nanog in fibroblasts, but not with their unmethylated promoters in ES cells [61]. These observations suggest that cell-type-specific factors stimulate Aid's deaminase activity at methylated target loci. Thus, active DNA demethylation mechanisms employing deaminases stabilize a pluripotent state in different biological contexts.

The misregulation of the demethlyase system in APC-deficient animals may also reconcile some apparent contradictions arising from previous studies. Adenoma formation in $Apc^{min/+}$ mice is suppressed either by pharmacologic inhibition of Dnmt activity with 5-aza-deoxycytidine or by genetic loss of the DNA methyltransferase *Dnmt1* or *Dnmt3b* [62–65]. However, 5-aza-deoxycytidine did not preclude microadenoma formation, nor did it preclude adenoma progression once a polyp had formed, suggesting an irreversible event occurs prior to, and is required for, the transition to a macroadenoma. Microadenomas have lost the wild-type allele of *APC*, indicating that this step is not rate limiting for macroadenoma formation. One explanation for these findings could be that hypermethylation and silencing of TSGs is required for tumor growth, and that reducing Dnmt activity inhibits this step [66, 67]. It has been shown that the CpG islands upstream of some TSGs are methylated in some cells within the normal intestinal mucosa of $Apc^{min/+}$ mice, and that their methylation increases in polyps [62]. Genetic loss of *Dnmt1* reduced the extent of methylation at these sites in both normal mucosa and polyps, and reduced polyp formation, extending the correlation between localized methylation and tumor growth. Although these observations are consistent with a reduction in TSG expression promoting tumor progression, DNA methylation could also contribute to tumorigenesis by affecting the rate and spectrum of genetic mutations [68, 69]. Spontaneous or enzymatic deamination of me-dC yields dT, resulting in a dC to dT transition mutation if it is not repaired prior to replication. Transition mutations at CpG dinucleotides, the target for DNA methylation, contribute significantly to tumorigenesis despite the under-representation of CpG in the genome [70, 71]. Loss of *APC* could increase

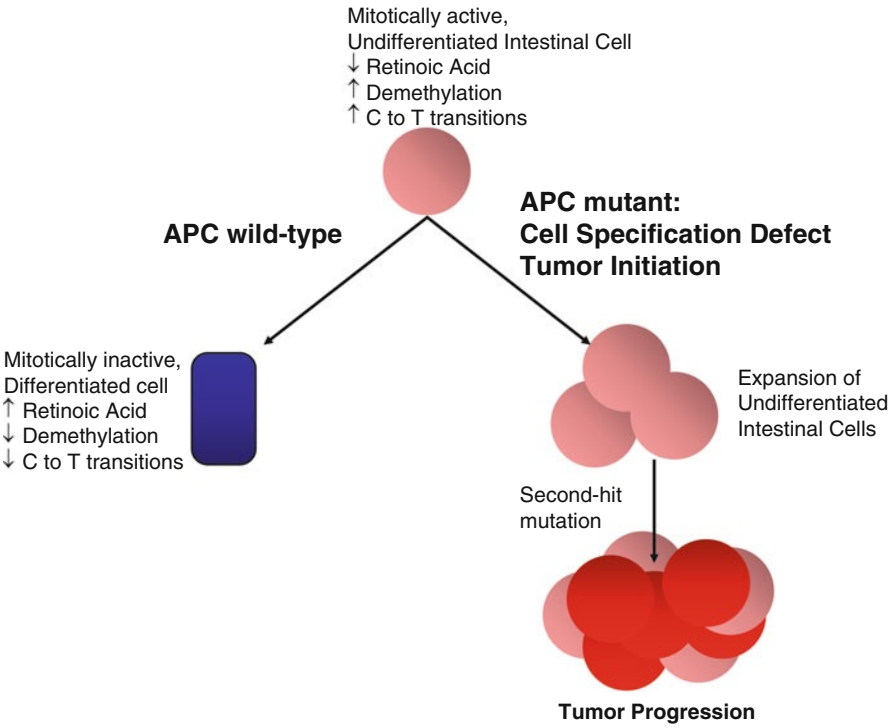

Fig. 8.1 In the intestine, APC promotes differentiation through the production of retinoic acid and the negative regulation of DNA demethylase components. In APC mutants, there is decreased retinoic acid production, maintaining cells in an undifferentiated state due to the continued expression of the demethylase system and of genes controlling cell fate and proliferation. In addition, expression of the demethylase system may promote C to T transition mutations. Both the cell specification defect and accumulation of second-hit mutations upon loss of APC may contribute to tumorigenesis

the rate of dC to dT transitions due to the upregulation of deaminases such as Aid and Apobec2 [43]. Thus, in addition to stabilizing a progenitor-like state, loss of *APC* and deregulation of the DNA demethylase system may separately contribute to tumorigenesis by increasing the likelihood of second-hit transition mutations. In this model, inhibition of Dnmt activity would suppress adenoma formation upon loss of *APC* by reducing the levels of me-dC, a substrate for deamination, which ultimately decreases the frequency of tumor-promoting dC to dT transitions. Similarly, genetic loss of Mbd4, which can repair the dT generated by deamination of me-dC, increased the rate of dC to dT transitions at CpG dinucleotides and accelerated intestinal tumorigenesis in APC mutant mice [72, 73].

The above findings support a new model linking loss of APC, impaired intestinal differentiation, and tumor initiation to RA-mediated control of DNA methylation dynamics. APC serves a critical role in cell fate specification by positive regulation of RA production and, in turn, inhibition of the DNA demethylase system (Fig. 8.1).

In the absence of APC function, there is an expansion of intestinal progenitor cells. Further, the misregulation of deaminases downstream of loss of APC may lead to an increased frequency of second-hit mutations. In this way, loss of APC may both directly and indirectly affect tumor initiation and progression.

References

1. Markowitz SD (2007) Aspirin and colon cancer–targeting prevention? N Engl J Med 356(21):2195–2198
2. Bienz M, Clevers H (2000) Linking colorectal cancer to Wnt signaling. Cell 103(2): 311–320
3. Fearon ER (2011) Molecular genetics of colorectal cancer. Annu Rev Pathol 6:479–507
4. Kinzler KW, Vogelstein B (1996) Lessons from hereditary colorectal cancer. Cell 87(2):159–170
5. Sunami E et al (2011) LINE-1 hypomethylation during primary colon cancer progression. PLoS One 6(4):e18884
6. Feinberg AP et al (1988) Reduced genomic 5-methylcytosine content in human colonic neoplasia. Cancer Res 48(5):1159–1161
7. Cravo M et al (1996) Global DNA hypomethylation occurs in the early stages of intestinal type gastric carcinoma. Gut 39(3):434–438
8. Goelz SE et al (1985) Hypomethylation of DNA from benign and malignant human colon neoplasms. Science 228(4696):187–190
9. Fearon ER, Vogelstein B (1990) A genetic model for colorectal tumorigenesis. Cell 61(5):759–767
10. Baker SJ et al (1990) p53 gene mutations occur in combination with 17p allelic deletions as late events in colorectal tumorigenesis. Cancer Res 50(23):7717–7722
11. Clevers H (2006) Wnt/beta-catenin signaling in development and disease. Cell 127(3):469–480
12. Sparks AB et al (1998) Mutational analysis of the APC/beta-catenin/Tcf pathway in colorectal cancer. Cancer Res 58(6):1130–1134
13. Morin PJ et al (1997) Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC. Science 275(5307):1787–1790
14. Romagnolo B et al (1999) Intestinal dysplasia and adenoma in transgenic mice after overexpression of an activated beta-catenin. Cancer Res 59(16):3875–3879
15. Blaker H et al (2003) Somatic mutations in familial adenomatous polyps. Nuclear translocation of beta-catenin requires more than biallelic APC inactivation. Am J Clin Pathol 120(3):418–423
16. Anderson CB, Neufeld KL, White RL (2002) Subcellular distribution of Wnt pathway proteins in normal and neoplastic colon. Proc Natl Acad Sci USA 99(13):8683–8688
17. Phelps RA et al (2009) A two-step model for colon adenoma initiation and progression caused by APC loss. Cell 137(4):623–634
18. Wu X et al (2008) Rac1 activation controls nuclear localization of beta-catenin during canonical Wnt signaling. Cell 133(2):340–353
19. Caldwell CM, Green RA, Kaplan KB (2007) APC mutations lead to cytokinetic failures in vitro and tetraploid genotypes in Min mice. J Cell Biol 178(7):1109–1120
20. Caldwell CM, Kaplan KB (2009) The role of APC in mitosis and in chromosome instability. Adv Exp Med Biol 656:51–64
21. Green RA, Wollman R, Kaplan KB (2005) APC and EB1 function together in mitosis to regulate spindle dynamics and chromosome alignment. Mol Biol Cell 16(10):4609–4622
22. Quyn AJ et al (2010) Spindle orientation bias in gut epithelial stem cell compartments is lost in precancerous tissue. Cell Stem Cell 6(2):175–181
23. Jette C et al (2004) The tumor suppressor adenomatous polyposis coli and caudal related homeodomain protein regulate expression of retinol dehydrogenase L. J Biol Chem 279(33):34397–34405

24. Nadauld LD et al (2006) Adenomatous polyposis coli control of C-terminal binding protein-1 stability regulates expression of intestinal retinol dehydrogenases. J Biol Chem 281(49): 37828–37835
25. Nadauld LD et al (2004) Adenomatous polyposis coli control of retinoic acid biosynthesis is critical for zebrafish intestinal development and differentiation. J Biol Chem 279(49): 51581–51589
26. Nadauld LD et al (2005) The zebrafish retinol dehydrogenase, rdh1l, is essential for intestinal development and is regulated by the tumor suppressor adenomatous polyposis coli. J Biol Chem 280(34):30490–30495
27. Mark M, Ghyselinck NB, Chambon P (2009) Function of retinoic acid receptors during embryonic development. Nucl Recept Signal 7:e002
28. Duester G (2008) Retinoic acid synthesis and signaling during early organogenesis. Cell 134(6):921–931
29. Deb-Rinker P et al (2005) Sequential DNA methylation of the Nanog and Oct-4 upstream regions in human NT2 cells during neuronal differentiation. J Biol Chem 280(8):6257–6260
30. Fisher CL, Fisher AG (2011) Chromatin states in pluripotent, differentiated, and reprogrammed cells. Curr Opin Genet Dev 21(2):140–146
31. Wild L, Flanagan JM (2010) Genome-wide hypomethylation in cancer may be a passive consequence of transformation. Biochim Biophys Acta 1806(1):50–57
32. Ito S et al (2011) Tet proteins can convert 5-methylcytosine to 5-formylcytosine and 5-carboxylcytosine. Science 333(6047):1300–1303
33. Kriaucionis S, Heintz N (2009) The nuclear DNA base 5-hydroxymethylcytosine is present in Purkinje neurons and the brain. Science 324(5929):929–930
34. Tahiliani M et al (2009) Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. Science 324(5929):930–935
35. Lister R et al (2009) Human DNA methylomes at base resolution show widespread epigenomic differences. Nature 462(7271):315–322
36. Deaton AM, Bird A (2011) CpG islands and the regulation of transcription. Genes Dev 25(10):1010–1022
37. Doi A et al (2009) Differential methylation of tissue- and cancer-specific CpG island shores distinguishes human induced pluripotent stem cells, embryonic stem cells and fibroblasts. Nat Genet 41(12):1350–1353
38. Irizarry RA et al (2009) The human colon cancer methylome shows similar hypo- and hypermethylation at conserved tissue-specific CpG island shores. Nat Genet 41(2):178–186
39. Wu H et al (2010) Dnmt3a-dependent nonpromoter DNA methylation facilitates transcription of neurogenic genes. Science 329(5990):444–448
40. Shukla S et al (2011) CTCF-promoted RNA polymerase II pausing links DNA methylation to splicing. Nature 479(7371):74–79
41. Hansen KD et al (2011) Increased methylation variation in epigenetic domains across cancer types. Nat Genet 43(8):768–775
42. Suzuki K et al (2006) Global DNA demethylation in gastrointestinal cancer is age dependent and precedes genomic damage. Cancer Cell 9(3):199–207
43. Rai K et al (2010) DNA demethylase activity maintains intestinal cells in an undifferentiated state following loss of APC. Cell 142(6):930–942
44. Barreto G et al (2007) Gadd45a promotes epigenetic gene activation by repair-mediated DNA demethylation. Nature 445(7128):671–675
45. Rai K et al (2008) DNA demethylation in zebrafish involves the coupling of a deaminase, a glycosylase, and gadd45. Cell 135(7):1201–1212
46. Morgan HD et al (2004) Activation-induced cytidine deaminase deaminates 5-methylcytosine in DNA and is expressed in pluripotent tissues: implications for epigenetic reprogramming. J Biol Chem 279(50):52353–52360
47. Hendrich B et al (1999) Genomic structure and chromosomal mapping of the murine and human Mbd1, Mbd2, Mbd3, and Mbd4 genes. Mamm Genome 10(9):906–912

48. Wu P et al (2003) Mismatch repair in methylated DNA. Structure and activity of the mismatch-specific thymine glycosylase domain of methyl-CpG-binding protein MBD4. J Biol Chem 278(7):5285–5291
49. Blanc V et al (2007) Deletion of the AU-rich RNA binding protein Apobec-1 reduces intestinal tumor burden in Apc(min) mice. Cancer Res 67(18):8565–8573
50. Rosenberg BR et al (2011) Transcriptome-wide sequencing reveals numerous APOBEC1 mRNA-editing targets in transcript 3′ UTRs. Nat Struct Mol Biol 18(2):230–236
51. Anant S et al (2004) Apobec-1 protects intestine from radiation injury through posttranscriptional regulation of cyclooxygenase-2 expression. Gastroenterology 127(4):1139–1149
52. Oshima M et al (1996) Suppression of intestinal polyposis in Apc delta716 knockout mice by inhibition of cyclooxygenase 2 (COX-2). Cell 87(5):803–809
53. Mayer W et al (2000) Demethylation of the zygotic paternal genome. Nature 403(6769): 501–502
54. Oswald J et al (2000) Active demethylation of the paternal genome in the mouse zygote. Curr Biol 10(8):475–478
55. Santos F et al (2002) Dynamic reprogramming of DNA methylation in the early mouse embryo. Dev Biol 241(1):172–182
56. Hajkova P et al (2002) Epigenetic reprogramming in mouse primordial germ cells. Mech Dev 117(1–2):15–23
57. Feng S, Jacobsen SE, Reik W (2010) Epigenetic reprogramming in plant and animal development. Science 330(6004):622–627
58. Popp C et al (2010) Genome-wide erasure of DNA methylation in mouse primordial germ cells is affected by AID deficiency. Nature 463(7284):1101–1105
59. Simonsson S, Gurdon J (2004) DNA demethylation is necessary for the epigenetic reprogramming of somatic cell nuclei. Nat Cell Biol 6(10):984–990
60. Mikkelsen TS et al (2008) Dissecting direct reprogramming through integrative genomic analysis. Nature 454(7200):49–55
61. Bhutani N et al (2010) Reprogramming towards pluripotency requires AID-dependent DNA demethylation. Nature 463(7284):1042–1047
62. Eads CA, Nickel AE, Laird PW (2002) Complete genetic suppression of polyp formation and reduction of CpG-island hypermethylation in Apc(Min/+) Dnmt1-hypomorphic Mice. Cancer Res 62(5):1296–1299
63. Yamada Y et al (2005) Opposing effects of DNA hypomethylation on intestinal and liver carcinogenesis. Proc Natl Acad Sci USA 102(38):13580–13585
64. Lin H et al (2006) Suppression of intestinal neoplasia by deletion of Dnmt3b. Mol Cell Biol 26(8):2976–2983
65. Laird PW et al (1995) Suppression of intestinal neoplasia by DNA hypomethylation. Cell 81(2):197–205
66. Linhart HG et al (2007) Dnmt3b promotes tumorigenesis in vivo by gene-specific de novo methylation and transcriptional silencing. Genes Dev 21(23):3110–3122
67. Eads CA et al (2000) Fields of aberrant CpG island hypermethylation in Barrett's esophagus and associated adenocarcinoma. Cancer Res 60(18):5021–5026
68. Jones PA et al (1992) Methylation, mutation and cancer. Bioessays 14(1):33–36
69. Laird PW, Jaenisch R (1994) DNA methylation and cancer. Hum Mol Genet 3 Spec No:1487–1495
70. Greenblatt MS et al (1994) Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. Cancer Res 54(18):4855–4878
71. Holliday R, Grigg GW (1993) DNA methylation and mutation. Mutat Res 285(1):61–67
72. Wong E et al (2002) Mbd4 inactivation increases Cright-arrowT transition mutations and promotes gastrointestinal tumor formation. Proc Natl Acad Sci USA 99(23):14937–14942
73. Millar CB et al (2002) Enhanced CpG mutability and tumorigenesis in MBD4-deficient mice. Science 297(5580):403–405

Chapter 9
Epigenetic Changes During Cell Transformation

Bernard W. Futscher

Abstract Malignant cancer emerges from normal healthy cells in a multistep process that involves both genetic and epigenetic lesions. Both genetic and environmental inputs participate in driving the epigenetic changes that occur during human carcinogenesis. The pathologic changes seen in DNA methylation and histone posttranslational modifications are complex, deeply intertwined, and act in concert to produce malignant transformation. To better understand the causes and consequences of the pathoepigenetic changes in cancer formation, a variety of experimentally tractable human cell line model systems that accurately reflect the molecular alterations seen in the clinical disease have been developed. Results from studies using these cell line model systems suggest that early critical epigenetic events occur in a stepwise fashion prior to cell immortalization. These epigenetic steps coincide with the cell's transition through well-defined cell proliferation barriers of stasis and telomere dysfunction. Following cell immortalization, stressors, such as environmental toxicants, can induce malignant transformation in a process in which the epigenetic changes occur in a smoother progressive fashion, in contrast to the stark stepwise epigenetic changes seen prior to cell immortalization. It is hoped that developing a clearer understanding of the identity, timing, and consequences of these epigenetic lesions will prove useful in future clinical applications that range from early disease detection to therapeutic intervention in malignant cancer.

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9.1 Introduction

Malignant cancer cells arise from normal cells via a multistep process that involves both genetic and epigenetic change. Similar to genetic lesions, epigenetic lesions can be diverse in nature, serving to alter the structure and function of the genome thereby participating in a cell's acquisition of limitless uncontrolled growth and the phenotypic hallmarks of the malignant cancer cell. In general, the degree of epigenetic difference between cancer cells and normal cells greatly exceeds the epigenetic differences that are seen between normal cells of different phenotypes and even different germ layers (e.g., fibroblasts and epithelial cells). Since epigenetic mechanisms are a primary determinant governing normal cell identity, this comparison underscores how epigenetically different cancer cells are from normal cells. Mutation and altered expression of proteins involved in the writing or reading of the epigenetic code are two mechanisms that help produce aberrant epigenetic changes seen in not only cancer, but other human diseases as well. The complexity and the frequency of the epigenetic changes seen in cancer cells, however, seem to defy explanations that rely on a single event. Instead, it appears that pathologic epigenetic change during carcinogenesis results from myriad genetic mutations and environmental inputs which perturb the manifold nodes of epigenetic regulation.

Environmental inputs acting on the epigenetic nodes are highly variable and can include contributions from both physiologic and xenobiotic sources such as hormonal status; microenvironmental milieu; nutritional, metabolic, or oxidative state; and toxicant and therapeutic drug exposures. Since the epigenetic state is important in governing cell identity, cellular nodes of epigenetic control acted upon by stimuli will show some variation between different cell types, suggesting that environmental inputs may show cell type selectivity, as well as display activity towards a broad array of cell types. Once these epigenetic changes are "fixed" into the chromatin, they can be vertically transmitted through cell generations. The inherent plasticity of the epigenetic control systems coupled to the cancer cell's limitless replicative potential provides the ability to generate extraordinary phenotypic diversity and rapidly respond to changing environmental stimuli and stresses.

Chromatin is rich in epigenetic marks, and these marks participate in the regulation and control of likely most or all genomic functions. The primary epigenetic mark found on DNA, 5-methylcytosine, is produced via the enzymatic methylation of the C5 position of cytosine through the action of multiple specialized DNA methyltransferases. The patterns and levels of DNA methylation across the genome have been mapped for a variety of normal and cancer cells, with cancer cells showing complex and extensive patterns of DNA methylation derangements. These DNA methylation derangements either participate in or reflect a number of different genomic processes, with its role in the regulation of gene expression being the best understood. Other C5 cytosine modifications have been identified recently, such as 5-hydroxymethylcytosine. It appears that these newly identified modifications are a result of an active DNA demethylation process and it is likely that these DNA epigenetic marks will prove biologically important; however, it has not yet been elucidated how these marks change and participate in the process of malignant transformation.

Posttranslational histone modifications are an additional layer of epigenetic control altered during human carcinogenesis. These posttranslational modifications include acetylation, methylation, phosphorylation, ubiquitylation, and sumoylation, and over 40 different amino acid residues in histones are currently known to undergo one or more of these modifications, especially in histones H3 and H4. Similar to DNA methylation, the histone posttranslational marks participate in a number of different genomic processes. Some histone marks are highly predictive of gene promoter location and transcriptional activity, such as histone H3K4 trimethylation and histone H3 and H4 lysine acetylation, and these modifications show strong negative correlations with DNA methylation levels in a typical genomic region. Other posttranslational histone modifications are linked to a transcriptionally repressed state and display positive correlations with DNA methylation levels, such as H3K9 methylation repressive marks. Still other histone marks, such as H3K27 trimethylation, are closely linked to transcriptional repression, preferentially target developmentally regulated genes and largely appears to be a repressive epigenetic control system that operates independently of the repressive DNA methylation system. Overall, a number of in vitro studies have provided clear mechanistic links between DNA methylation and histone modification state indicating that the control of the DNA methylation and histone modification patterns are deeply intertwined. As such, it is not surprising that, similar to DNA methylation, the normal levels and patterns of histone posttranslational modifications become compromised in human cancer cells.

In a clinical setting, the multistep nature of epithelial cell malignant transformation manifests as hyperplasia, dysplasia, benign tumor, carcinoma in situ, and finally frank malignancy and metastases; analogous pathologic progressions can be seen in some hematologic pathologies, as well, and may very well exist for most or all human cancers. Analysis of clinical specimens has shown that epigenetic aberrations are seen in the earliest stages of this multistep process, although obtaining quantitative information-rich epigenetic data from minute clinical specimens creates unique technical challenges that have slowed the ability to identify pathoepigenetic events that directly translate to clinical impact with respect to the detection, prognostication, treatment, and management of human cancer. For example, technical limitations such as specimen size and quality have hindered success in analyzing the posttranslational modification state of histones in clinical specimens. With respect to DNA methylation analysis, quantitative high resolution approaches for the analysis of the minute clinical cancer specimens typically available have been available for over 20 years in the form of bisulfite sequencing [1, 2], and today comprehensive DNA methylome sequencing approaches have emerged and should attain wide availability over the next few years [3, 4]. In the translational science arena, there are a few early applications where the results indicate DNA methylation analysis may be a useful tool in predicting response to cancer therapy [5, 6]. Results such as these should provide significant optimism and encouragement to investigators that epigenetic analysis will prove useful in the areas of prediction, detection, prognostication, as well as treatment of cancer. While significant progress has been made in understanding the causes, consequences, and temporal sequence of pathologic epigenetic events in cancer, their utility on the clinical management of cancer is largely a promissory note with their potential not yet fully realized.

9.2 Laboratory Model Systems of Cell Transformation

To better discover and understand the pathoepigenetic events that mechanistically participate in the conversion of a normal cell to a malignant cell, there is value in using experimentally tractable models systems that faithfully reflect the in vivo process. To this end, a variety of useful and complementary in vitro human cell line and animal model systems have been developed that recapitulate aspects of clinical multistep carcinogenesis and that allow for detailed analysis of epigenetic/epigenomic events as they unfold during the transformation from the normal to the malignant phenotype. These models have a number of advantages as laboratory tools—certainly the most important being that the genetic and epigenetic changes present in them accurately reflect the known (epi)genetic etiology of the clinical form of the disease, thereby providing a solid platform for the discovery and dissection of new epigenetic events relevant to clinical cancer. These cell line systems also allow for the production of pure and reproducible populations of cells that can be fairly easily generated in large number and at relatively low costs. In our experience, the epigenetic state of the cell line models we have employed does not vary to a significant extent when grown under appropriate and consistent conditions. We routinely verify cell line identity using STR profiling using 13 CODIS markers; reference DNA fingerprinting data for most of the widely used cell lines are available from cell line collections such as the ATCC or from the investigators who developed the models [7, 8].

A majority of the human cell culture model systems that have been developed perhaps best address the final step(s) of malignant human cancer, specifically the steps that follow cell immortalization. Since immortalization through telomerase activation may be a rate limiting step in human carcinogenesis, these models may not be best suited for the identification of the earliest epigenetic events in carcinogenesis. Cell model systems that adequately address the earliest steps in human carcinogenesis, prior to cell immortalization, are more limited. These are discussed later in the chapter. As is always the case, each model system used to evaluate the steps from normal finite life span cell to immortal malignant cancer cell has distinct qualities and limitations. Together, these laboratory models allow for the molecular dissection of epigenetic dysfunction during the pathologic process and help provide new insights that can be used to develop approaches to better detect, prognosticate, treat, and manage the myriad human cancers.

9.3 Immortalization to Malignant Transformation

Cell line systems that model the epigenetic events that occur following epithelial cell immortalization are widespread and provide useful tools to study malignant transformation (meant here as the in vitro assessments of anchorage independent growth and tumor forming ability in immunocompromised mice). These immortal-

ized cell line model systems have generally overcome normal cell proliferation barriers either by (1) direct immortalization of primary cell strains through overexpression of hTERT, (2) selective genetic strategies that inactivate the p16/Rb and p53 pathways, frequently via viral approaches, or (3) establishing cell lines from cultured pathologic specimens that are already immortal, but not fully malignant. A variety of immortalized variants of different epithelial cell models have been generated and examples include, but are not limited to, prostate epithelial cells immortalized by HPV18 (RWPE), bronchial epithelial cells immortalized with SV40 (HBE16, BEAS-2B), keratinocytes that arose spontaneously in culture from primary cells (HaCAT), breast epithelial cells derived from diseased tissue (MCF10A) or non-diseased healthy tissue (HMEC), and urinary bladder cells immortalized with hTERT or SV40 (UROtsa) [9–18]. Although some approaches used to immortalize cells are not themselves etiologic agents involved in clinical human carcinogenesis (e.g., viral inactivation of p53 or the genetic introduction of hTERT), they do provide reproducible approaches that target proteins and pathways known to be critical to the human tumor cell phenotype.

These immortalized cell line systems should not be considered normal cells; however, since they have had perhaps the most dramatic phenotypic shift possible—acquisition of limitless replicative potential. In addition, these cells have often also acquired genetic abnormalities (e.g., deletions, translocations, aneuploidy). It is highly likely that these immortalized cells have undergone changes in the epigenetic state, if compared to its normal finite life span counterpart, although detailed studies to this end are limited. Indeed, the p53 inactivation strategies used in immortalization strategies may instigate epigenetic change itself. Following a cellular stress, activated p53 binds to DNA in a sequence-specific manner while also recruiting coactivators or corepressors to participate in transcriptional regulation. Thus, loss of p53 binding and coactivator/corepressor recruitment may produce long-term epigenetic changes at p53 target loci disrupting their normal transcriptional regulation and altering attendant cellular phenotypes [19–21]. As such, these immortalized models likely provide more limited information regarding the nature of the epigenetic changes that may occur early in multistep carcinogenesis and prior to immortalization. Overall, these models have proven useful in identifying novel epigenetic changes, the molecular mechanisms responsible for these epigenetic changes, and the genetic and/or environmental events that provoke the epigenetic changes.

9.4 Epigenetic Remodeling by Environmental Arsenicals

Our laboratory has been interested in the effect that environmental arsenicals has on the epigenetic state. Arsenic is a widespread environmental toxicant that exists as a number of different molecular species and ranks as the 20th most common element in the earth's crust. Humans may be exposed to arsenicals to varying degrees through water, air, soil, and food. Arsenic may also be the world's most well recognized poison. Acute high dose exposure to arsenic has been used repeatedly throughout

history for murder by intentional poisoning and has earned the moniker, “Poison of Kings and King of Poisons [22].” In contrast, various forms of arsenic have also been used for centuries to treat a wide range of illnesses, including syphilis, malaria, asthma, chorea, eczema, psoriasis, and cancer [23]. Today, one molecular species of arsenic, arsenic trioxide (As_2O_3) is an FDA-approved therapy to treat acute promyelocytic leukemia and also shows promising anticancer activity in laboratory models of other human cancers [24–26]. In the most common setting, however, that of chronic low dose, environmental exposures, arsenicals are associated with a number of human maladies, among them cancer, neurologic disorders, cardiovascular disease, developmental abnormalities, and diabetes [27–30].

Of all the pathologic effects associated with long-term arsenic exposure, cancer is the most widely studied. A number of epidemiological studies have convincingly linked human arsenic exposure with various cancers, especially cancers of the lung, urinary tract, and skin [31]. Arsenicals are classified as a group 1 carcinogen by the International Agency for Research on Cancer (IARC); however, a precise mechanism of arsenical action remains wanting. A few observations suggest that epigenetic remodeling may be important in arsenical-associated cancers. Arsenicals do not appear to cause point mutations and on their own are unable to cause cancer in standard animal assays or immortalize primary human epithelial cells [32, 33]. However, earlier studies showed arsenicals can change DNA methylation levels [34], and long-term nontoxic exposure to arsenicals has been sufficient to reproducibly induce malignant transformation in a variety of immortalized nonmalignant human epithelial cells derived from tissues with known arsenical sensitivity. Examples of cell line models that have been malignantly transformed by arsenicals include HaCaT, BEAS-2B, RWPE, and UROtsa [35–39].

Human transitional carcinoma of the bladder arises from the transformation of urinary bladder epithelial cells, and those tumors that progress clinically to a malignant phenotype generally demonstrate genetic inactivation of the p16/Rb and p53 pathways [40]. In vitro, benign immortalized urothelial cell lines that resemble the earlier stages of clinical bladder cancer can be reproducibly generated from finite life span urothelial cell strains via genetic manipulations that target these pathways for inactivation. In our studies of epigenetic changes that occur during the transition from a benign immortal cell to a malignant cancer cell, we have used the immortalized, non-tumorigenic human urothelial cell line, UROtsa, generated from the urothelial cells of a young female donor and immortalized using a temperature sensitive SV40 large-T antigen construct [14]. Further evaluation of these cells has revealed hypodiploidy, genetic deletion of a small region of chromosome 9 that contains p16, and hTERT expression (unpublished observations).

Malignant transformation of UROtsa cells using long-term nontoxic exposures to environmental toxicants such as arsenic has been successfully performed by multiple independent laboratories [36, 39]. The phenotypic manifestations of the malignant conversion process can first be detected in these cells at approximately 12 weeks of exposure at a faster growth rate. With increased exposure time, the ability to form colonies in an anchorage independent fashion occurs, and finally arsenic-exposed

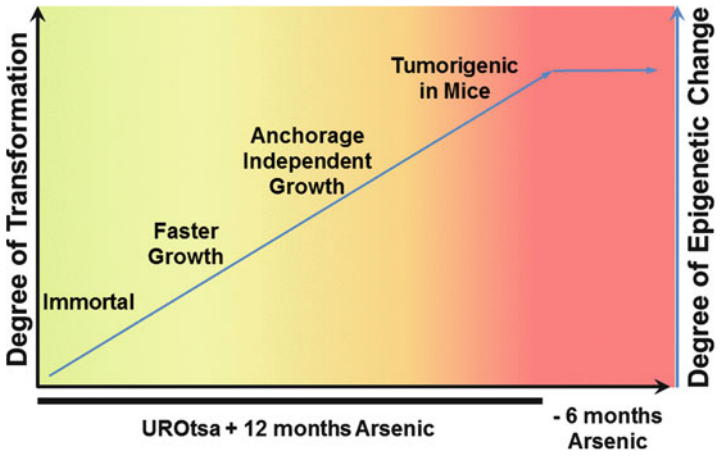

Fig. 9.1 UROtsa cell line model of malignant transformation. The immortalized urothelial cell line UROtsa was exposed to arsenicals for periods of up to a year. Arsenical exposed cells were probed at various time points for markers of malignant transformation. After 3 months there was a significant increase in proliferation rate, after 6 months a significant increase in anchorage independent growth, and after 12 months, arsenic exposed cells formed tumors in immune compromised mice [36, 39]. Progressive epigenetic changes occur during this transition from a benign immortal to malignant phenotype

UROtsa cells acquire the ability to form tumors in immunocompromised mice. Interestingly, the arsenical-induced malignant phenotype is stable, as removal of the toxicant for at least 6 months has not led to the reversion to a more benign phenotype (Fig. 9.1).

Broad epigenetic changes begin to rise in UROtsa cells during exposure to arsenic at concentrations seen in real-world situations, such as can be found in drinking water from wells (5–10 ppb). We examined epigenetic changes in a genome-wide and temporal manner using histone modification-specific chromatin and 5-methylcytosine-specific immunoprecipitations coupled to two-color DNA microarray analysis. We found global changes emerging around 12 weeks after initial exposure. These epigenetic changes appear progressive—the degree of epigenetic change increases at the individual targets with time. The epigenetic changes also are stable—after malignant transformation, the toxicant can be removed, but the malignant phenotype as well as the epigenetic changes remains. Some of the epigenetic changes identified were in genes overtly relevant to the malignant phenotype and have functional roles in cancer in general, and bladder cancer in particular [41], while the roles for most of the changes seen remain enigmatic. It appears unlikely that the observed epigenetic changes seen in UROtsa following arsenical exposure are simply due to the outgrowth or simple selection of a preexisting clone, since the arsenical-transformed cells grow significantly faster (~35%) than the non-malignant parental UROtsa cell line. Rather, it seems possible that (epi)genetic alterations may arise during and as a result of arsenic exposure, and given enough time (cell divisions), which is provided by the cell immortality, and optimal growth

conditions, a faster growing, more malignant population of cells emerges, which are then selected for based on their growth characteristics.

Probing the DNA methylation profile of the arsenical transformed UROtsa cells and comparing them to the non-transformed immortal parental cells revealed that ~3% of the assessed regions were hypermethylated, while ~1% were hypomethylated. The hypermethylation events occurred mostly within gene promoters, whereas the hypomethylation events were more prevalent in repetitive elements spread throughout the genome [42], consistent with what is well established for human cancers. We attempted to assess whether the DNA methylation changes acquired during malignant transformation were specifically or randomly distributed in the genome by analyzing two different arsenical-transformed UROtsa cell lines, created in two different laboratories using two different arsenicals (i.e., sodium arsenite and monomethyl arsenous acid). A statistical analysis of the numerical size of the overlap of aberrantly DNA methylated promoters between these two cell lines indicates that the DNA methylation changes seen are nonrandom and suggest that common epigenetic changes occur in association with arsenical malignant transformation.

The types of DNA methylation changes observed during the arsenical-mediated malignant transformation can be roughly divided into two groups, focal and long range. Focal DNA methylation events refer to DNA differentially methylated regions that cover a single gene promoter and are typically ≤1 kb in size. These types of aberrant DNA hypermethylation events seem to predominate and are closely linked to the silencing of a large number of tumor suppressor genes. In the UROtsa malignant transformation model, several potential tumor suppressor genes were found to be hypermethylated such as DBCCR1 (deleted in bladder cancer chromosome region candidate1); its relevance to bladder cancer having been previously ascertained [41]. Overall, the DNA hypermethylation changes were correlated to corresponding losses in the permissive histone modification marks of histone acetylation and H3K4 methylation and loss of gene expression, although as is often the case, apparent exceptions to the general rules could also be detected.

The DNA differentially methylated regions that cover much larger contiguous regions, along with corresponding changes in histone modifications, are linked to chromatin remodeling of more extended regions of the genome in a process termed long-range epigenetic silencing [43]. This type of epigenetic lesion has been found in a number of human cancer cell lines as well as clinical tumor specimens, suggesting that this type of coordinate epigenetic regulation over large regions may be a common and important event in cancer [43–46]. Interestingly, it appears that the gain of aberrant agglomerative DNA methylation changes and associated long-range epigenetic silencing can be observed over the time course of arsenical-mediated transformation of UROtsa from a benign to a malignant phenotype. Recent studies in the laboratory indicate that the PCDH and HOXC gene clusters undergo extensive aberrant DNA and that these epigenetic lesions are also found in malignant human bladder cancer specimens. Overall, these results suggest that the UROtsa malignant transformation model may be a laboratory tool to discern the molecular underpinnings responsible for long-range epigenetic silencing and identifies a

significant environmental toxicant as a possible etiologic agent of this pathologic epigenetic lesion.

In an initial measure evaluating the commonality of the epigenetic change in arsenical-induced malignant transformation, we sought other human epithelial cell line models of arsenical-mediated malignant transformation. The immortalized human prostate epithelial cell line RWPE-1 was shown to undergo genomic hypomethylation after chronic exposure to AsIII [47, 48], and we have made preliminary comparisons between this model and the UROtsa model. We have found a significant overlap in gene promoters targeted for aberrant DNA methylation in both the UROtsa and RWPE models of arsenical-mediated malignant transformation that is beyond what is expected by random chance. These results suggest that a common ground of epigenetic change occurs in these laboratory models of arsenical exposure and suggests that they may be useful to help identify new epigenetically targeted genes important to malignant transformation and the cellular processes responsible for these epigenetic changes.

Epigenetic regulation resides at a nexus of gene–environment interactions. Together these results suggest that environmental arsenicals may exert their carcinogenic activity by eliciting epigenetic change thereby acting as an epimutagen, an agent whose exposure induces stable and heritable changes to the epigenetic state. The epigenetic changes seen are linked to gene expression changes and coincide with the advent of an increasingly malignant phenotype. Furthermore, results from epigenome-wide analysis suggest that common regions are epigenetically targeted during arsenical-mediated malignant transformation. Importantly, the DNA methylation changes seen in the laboratory models are consistent with what is seen in the relevant in vivo correlates—clinical cancer specimens. These experimentally tractable systems provide a unique opportunity to better discern the causes and consequences of epigenetic change in arsenical-associated cancers.

9.5 Epigenetic Models of Finite Life span to Immortalization (and Beyond)

A cell model we have found particularly useful to study the epigenetics of cell transformation is the human mammary epithelial cell (HMEC) model system developed by Dr. Martha Stampfer during the past 30 years [9, 44, 49, 50]. The utility of this model system for the examination of the early molecular events in human breast carcinogenesis has been demonstrated in a number of studies, both with respect to genetic and epigenetic events [49–53]. In our estimation this isogenic cell model system offers a number of benefits and allows for the temporal analysis of molecular events that occur during the transitions from finite life span through immortalization and on to malignant transformation. This model also allows one to study the effects that directed genetic changes and environmental stressors can have on the epigenetic state.

In this model system, cultured finite life span HMEC must overcome two distinct proliferation barriers in order to achieve immortality and ultimately acquire a malignant phenotype. The first proliferation barrier is termed stasis or stress-induced senescence and is mediated by the Rb protein, characterized by elevated levels of p16INK4A. This first barrier, stasis, has been overcome or bypassed in cultured HMEC by various means, such as exposure to benzo(a)pyrene. The resultant post-stasis cells commonly show p16 inactivation by gene mutation or promoter hypermethylation [50, 54]. Loss of p16 expression due to silencing or mutation is also a common event during in vivo human breast cell transformation [55]. When grown in a serum-free medium, rare HMEC will "spontaneously" silence p16, generating a type of post-stasis HMEC population that has been called post-selection [9, 54]. HMEC that escape the stasis barrier can continue to proliferate for dozens of additional population doublings before encountering a second more stringent proliferation barrier resulting from critically shortened telomeres [49, 56]. When approaching the telomere dysfunction barrier, HMEC exhibit increased chromosomal instability and a DNA damage response. Rare cells that gain telomerase expression may escape this barrier and become immortal, whereby HMEC activates telomerase by as yet undefined, and potentially novel, epigenetic mechanisms. In addition, HMEC systems can acquire immortality through genetic perturbations. For example, under appropriate circumstances direct genetic introduction of constructs that express CMYC, or ZNF217, hTERT can promote HMEC immortalization [57, 58]. Nondirected mutagenesis can also promote HMEC immortalization, as evidenced by the effects of the complete carcinogen benzo(a)pyrene on HMEC. This limitless replicative potential allows for the acquisition and accumulation of additional epigenetic and genetic events that promote the development of additional malignant properties [50, 59–61].

We have used this HMEC model system to begin to develop a timeline of the DNA methylation changes that occurs over the course of multistep breast carcinogenesis, with a particular interest on the earliest stages of the process. Figure 9.2 shows a generalized view of cells we have analyzed, their temporal position in relation to the cellular proliferation barriers, the approximate clinical correlates, and the timing of DNA methylation changes. This figure is an example and not an exhaustive or detailed review of the HMEC strains and cell lines or the multiple treatments and exposures used to create them, and for a more detailed view one can see [62] or visit http://hmec.lbl.gov/mindex.html. In our initial studies using this model system, DNA methylation state was determined using 5-methylcytosine antibody immunopreciptations (MeDIP) coupled to two-color hybridization on a custom 13,500 element human gene promoter microarray and verified using the orthogonal technology of mass spectrometric analysis using Sequenom MassArray [63].

Overall, in this model we observed a stepwise progression of DNA methylation changes with each step coinciding with overcoming a cellular proliferation barrier [62]. In HMEC that overcame stasis produced by stress-inducing serum-free medium, we found, in addition to p16 methylation, hundreds of other differentially methylated regions in the post-stasis cells when compared to pre-stasis cells, representing approximately 2% of all gene promoters on the microarray. These DNA

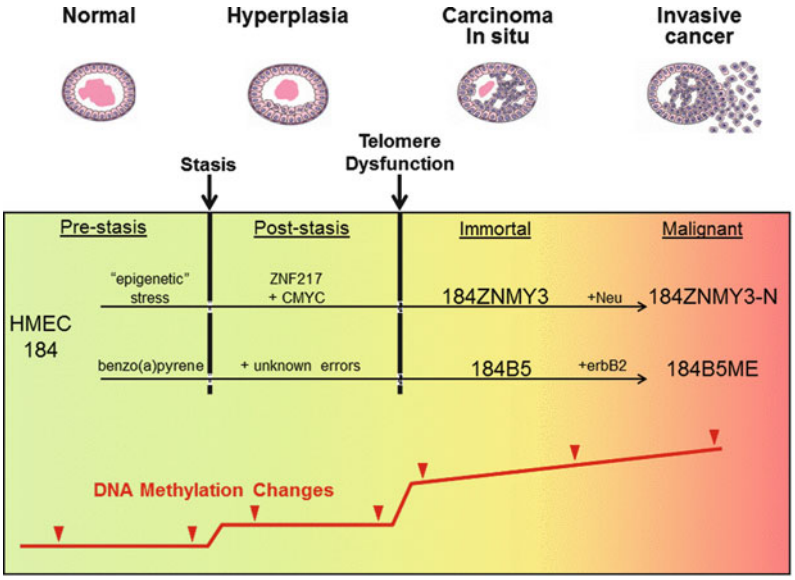

Fig. 9.2 Schematic representation of breast cancer progression and the timing of the underlying DNA methylation changes, with connections between the in vitro HMEC model system and clinical progression based on earlier work [51, 56, 65]. *Top*, the clinical correlates of the HMEC system in relation to the temporal position of the two epithelial cell proliferation barriers of stasis and telomere dysfunction that divides the timeline into pre-stasis, post-stasis, immortal, and malignant epithelial cells. *Middle*, a very simplified view and two examples of HMEC culture models, and the treatment or genetic manipulations used to generate these models. *Bottom*, the timeline of DNA methylation changes identified during the passage of finite life span HMEC through stasis, telomere dysfunction, and culminating in a malignant phenotype. *Arrows* on the DNA methylation changes *curve* show the time points analyzed for DNA methylation state

methylation events were both of the focal and long-range variety. Considering that probably 5–10% of gene promoters in malignant cancer cells show aberrant DNA methylation, a considerable number of DNA methylation changes may occur very early in multistep breast carcinogenesis, and these changes are coincident with overcoming the critical Rb/p16 cell proliferation barrier. Since a majority of the DNA methylation changes seen in the transition of HMEC from pre-stasis to post-stasis in this setting are also seen in malignant breast cancer cell lines and tumor specimens, this transition through the stasis proliferation barrier may represent a critical early event in some pathways of human breast carcinogenesis.

It is worth noting here that current commercial sources of HMEC appear to be of this post-stasis (or post-selection or variant) stage, since these HMEC are produced via the process described above—post-stasis cells that emerge from serum-free media induced stress. As such, the commercially available HMEC may have not only undergone p16 DNA methylation, but are likely to have also acquired hundreds of additional aberrant DNA methylation events [62]. As such, caution should be exercised when evaluating the epigenetic state of primary epithelial cells and considering what is epigenetically "normal."

HMEC that become post-stasis following exposure to the genotoxin and complete carcinogen benzo(a)pyrene showed more than an order of magnitude reduction in DNA differentially methylated regions when compared to the DNA methylation changes induced by stressful serum-free growth conditions. Similarly, HMEC that became post-stasis following genetic knockout of p16 using p16-targeted shRNA have very few DNA methylation changes, underscoring the functional importance of p16 in the first growth barrier. The few DNA methylation changes seen in the benzo(a)pyrene and p16 shRNA-treated cell lines suggest that different pathways through the stasis barrier will have distinct effects on the epigenetic state.

A second step of epigenetic change occurs when telomere dysfunction is overcome and cells acquire immortality. Regardless of the mechanism by which cells pass through telomere dysfunction, hundreds of DNA methylation changes occur. Similar to the DNA methylation changes acquired during the pre-stasis to post-stasis transition, changes that occur during the transition from finite life span to immortal can be focal (≤1 kb) and limited to a single gene or the changes can represent examples of long-range epigenetic silencing and cover extended regions of the genome [64].

These changes seen in the premalignant stages represented by the HMEC model show significant overlap to the DNA methylation changes seen in other human breast cancer cell lines and clinical tumor specimens. Overall, results from the studies using the HMEC model indicate that epigenetic changes occur in a stepwise fashion at critical junctions in the path to cell immortality. These results are consistent with an epigenetic progenitor model where epigenetic changes may occur early, in a stepwise fashion, can precede genetic mutation and allow for an expansion of epigenetically compromised population of cells. The large number of genes affected by epigenetic changes during the transitions through proliferation barriers can provide a foundation for the phenotypic variability and biologic heterogeneity often seen in clinical disease. The DNA methylation changes identified can potentially provide a bank of epigenetic biomarkers for assessing breast cancer risk in premalignant lesions and provide targets for therapeutic interventions.

9.6 Conclusion

In summary, complex and intertwined epigenetic changes occur during multistep carcinogenesis. These changes may be viewed as epigenetic lesions and exist in the genome in a number of forms, from focal to long range. The scope of the epigenetic lesions is likely due to multiple distinct inputs: genetic, such as mutations to chromatin modifier genes; physiologic, such as hormonal and nutritional state; and environmental, such as toxicant exposures. Experimentally tractable laboratory model systems that accurately reflect clinical cancer have been developed and allow for investigations into the causes and consequences of epigenetic change during cell transformation. Results from these systems suggest that early critical epigenetic

events occur prior to cell immortalization and coincide with the transition through well-defined barriers of cell proliferation. Following immortalization, laboratory models suggest that cells can be induced towards malignancy by a variety of stimuli, and that the epigenetic changes arise in a seemingly more progressive smoother fashion, as opposed to the stark stepwise events prior to immortalization. It is hoped that developing a clearer understanding of the identity, timing, and consequences of these epigenetic lesions will prove useful in future clinical applications that range from early disease detection to therapeutic intervention in malignant cancer.

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References

1. Clark SJ et al (1994) High sensitivity mapping of methylated cytosines. Nucleic Acids Res 22(15):2990–2997
2. Frommer M et al (1992) A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. Proc Natl Acad Sci USA 89(5):1827–1831
3. Lister R et al (2009) Human DNA methylomes at base resolution show widespread epigenomic differences. Nature 462(7271):315–322
4. Maunakea AK et al (2010) Conserved role of intragenic DNA methylation in regulating alternative promoters. Nature 466(7303):253–257
5. Drew Y et al (2011) Therapeutic potential of poly(ADP-ribose) polymerase inhibitor AG014699 in human cancers with mutated or methylated BRCA1 or BRCA2. J Natl Cancer Inst 103(4):334–346
6. Hegi ME et al (2005) MGMT gene silencing and benefit from temozolomide in glioblastoma. N Engl J Med 352(10):997–1003
7. Lorenzi PL et al (2009) DNA fingerprinting of the NCI-60 cell line panel. Mol Cancer Ther 8(4):713–724
8. Nims RW et al (2010) Short tandem repeat profiling: part of an overall strategy for reducing the frequency of cell misidentification. In Vitro Cell Dev Biol Anim 46(10):811–819
9. Hammond SL, Ham RG, Stampfer MR (1984) Serum-free growth of human mammary epithelial cells: rapid clonal growth in defined medium and extended serial passage with pituitary extract. Proc Natl Acad Sci USA 81(17):5435–5439
10. Amstad P et al (1988) Neoplastic transformation of a human bronchial epithelial cell line by a recombinant retrovirus encoding viral Harvey ras. Mol Carcinog 1(3):151–160
11. Boukamp P et al (1988) Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. J Cell Biol 106(3):761–771
12. Ke Y et al (1988) Human bronchial epithelial cells with integrated SV40 virus T antigen genes retain the ability to undergo squamous differentiation. Differentiation; research in biological diversity 38(1):60–66
13. Miller FR et al (1993) Xenograft model of progressive human proliferative breast disease. J Natl Cancer Inst 85(21):1725–1732

14. Petzoldt JL et al (1995) Immortalisation of human urothelial cells. Urol Res 23(6):377–380
15. Bello D et al (1997) Androgen responsive adult human prostatic epithelial cell lines immortalized by human papillomavirus 18. Carcinogenesis 18(6):1215–1223
16. Kiyono T et al (1998) Both Rb/p16INK4a inactivation and telomerase activity are required to immortalize human epithelial cells. Nature 396(6706):84–88
17. Dickson MA et al (2000) Human keratinocytes that express hTERT and also bypass a p16(INK4a)-enforced mechanism that limits life span become immortal yet retain normal growth and differentiation characteristics. Mol Cell Biol 20(4):1436–1447
18. Chapman E et al (2006) Expression of hTERT immortalises normal human urothelial cells without inactivation of the p16/Rb pathway. Oncogene 25(36):5037–5045
19. Chang CJ et al (2011) p53 regulates epithelial-mesenchymal transition and stem cell properties through modulating miRNAs. Nat Cell Biol 13(3):317–323
20. Junk DJ et al (2008) Different mutant/wild-type p53 combinations cause a spectrum of increased invasive potential in nonmalignant immortalized human mammary epithelial cells. Neoplasia 10(5):450–461
21. Vrba L et al (2008) p53 induces distinct epigenetic states at its direct target promoters. BMC Genomics 9:486
22. Vahidnia A, van der Voet G, de Wolff F (2007) Arsenic neurotoxicity—a review. Hum Exp Toxicol 10:823–832
23. Rohe G (1896) Arsenic. In: Foster F (ed) Reference-book of practical therapeutics. D. Appleton, New York, p 142
24. Emadi A, Gore SD (2010) Arsenic trioxide—an old drug rediscovered. Blood Rev 24(4–5):191–199
25. Wu D et al (2010) Antitumor effect and mechanisms of arsenic trioxide on subcutaneously implanted human gastric cancer in nude mice. Cancer Genet Cytogenet 2:90–96
26. Yeh K et al (2011) Tumor growth inhibition of metastatic nasopharyngeal carcinoma cell lines by low dose of arsenic trioxide via alteration of cell cycle progression and induction of apoptosis. Head Neck 5:734–742
27. Chen C et al (2007) Arsenic and diabetes and hypertension in human populations: a review. Toxicol Appl Pharmacol 3:298–304
28. Grandjean P, Murata K (2007) Developmental arsenic neurotoxicity in retrospect. Epidemiology 1:25–26
29. Smith A et al (1998) Marked increase in bladder and lung cancer mortality in a region of northern Chile due to arsenic in drinking water. Am J Epidemiol 7:660–669
30. Vahter M (2008) Health effects of early life exposure to arsenic. Basic Clin Pharmacol Toxicol 2:204–211
31. Chen C et al (1992) Cancer potential in liver, lung, bladder and kidney due to ingested inorganic arsenic in drinking water. Br J Cancer 5:888–892
32. IARC Working Group on the Evaluation of Carcinogenic Risks to Humans (2004) Some drinking-water disinfectants and contaminants, including arsenic. IARC Monogr Eval Carcinog Risks Hum 84:1–477
33. Rossman TG et al (1980) Absence of arsenite mutagenicity in *E. coli* and Chinese hamster cells. Environ Mutagen 2(3):371–379
34. Zhao C et al (1997) Association of arsenic-induced malignant transformation with DNA hypomethylation and aberrant gene expression. Proc Natl Acad Sci USA 20:10907–10912
35. Achanzar W et al (2002) Inorganic arsenite-induced malignant transformation of human prostate epithelial cells. J Natl Cancer Inst 24:1888–1891
36. Bredfeldt T et al (2006) Monomethylarsonous acid induces transformation of human bladder cells. Toxicol Appl Pharmacol 1:69–79
37. Chang Q et al (2010) Reduced reactive oxygen species-generating capacity contributes to the enhanced cell growth of arsenic-transformed epithelial cells. Cancer Res 70(12):5127–5135
38. Pi J et al (2008) Arsenic-induced malignant transformation of human keratinocytes: involvement of Nrf2. Free Radic Biol Med 45(5):651–658

39. Sens D et al (2004) Inorganic cadmium- and arsenite-induced malignant transformation of human bladder urothelial cells. Toxicol Sc 1:56–63
40. Dinney CP et al (2004) Focus on bladder cancer. Cancer Cell 6(2):111–116
41. Nishiyama H et al (2001) Negative regulation of G(1)/S transition by the candidate bladder tumour suppressor gene DBCCR1. Oncogene 23:2956–2964
42. Jensen TJ et al (2009) Arsenicals produce stable progressive changes in DNA methylation patterns that are linked to malignant transformation of immortalized urothelial cells. Toxicol Appl Pharmacol 241(2):221–229
43. Frigola J et al (2006) Epigenetic remodeling in colorectal cancer results in coordinate gene suppression across an entire chromosome band. Nat Genet 38(5):540–549
44. Novak P et al (2006) Epigenetic inactivation of the HOXA gene cluster in breast cancer. Cancer Res 66(22):10664–10670
45. Rauch T et al (2007) Homeobox gene methylation in lung cancer studied by genome-wide analysis with a microarray-based methylated CpG island recovery assay. Proc Natl Acad Sci USA 104(13):5527–5532
46. Stransky N et al (2006) Regional copy number-independent deregulation of transcription in cancer. Nat Genet 38(12):1386–1396
47. Benbrahim-Tallaa L et al (2005) Molecular events associated with arsenic-induced malignant transformation of human prostatic epithelial cells: aberrant genomic DNA methylation and K-ras oncogene activation. Toxicol Appl Pharmacol 3:288–298
48. Coppin J, Qu W, Waalkes M (2008) Interplay between cellular methyl metabolism and adaptive efflux during oncogenic transformation from chronic arsenic exposure in human cells. J Biol Chem 28:19342–19350
49. Garbe JC et al (2007) Inactivation of p53 function in cultured human mammary epithelial cells turns the telomere-length dependent senescence barrier from agonescence into crisis. Cell Cycle 6(15):1927–1936
50. Stampfer MR, Bartley JC (1985) Induction of transformation and continuous cell lines from normal human mammary epithelial cells after exposure to benzo[a]pyrene. Proc Natl Acad Sci USA 82(8):2394–2398
51. Chin K et al (2004) In situ analyses of genome instability in breast cancer. Nat Genet 36(9):984–988
52. Holst CR et al (2003) Methylation of p16(INK4a) promoters occurs in vivo in histologically normal human mammary epithelia. Cancer Res 63(7):1596–1601
53. Li Y et al (2007) Transcriptional changes associated with breast cancer occur as normal human mammary epithelial cells overcome senescence barriers and become immortalized. Mol Cancer 6:7
54. Brenner AJ, Stampfer MR, Aldaz CM (1998) Increased p16 expression with first senescence arrest in human mammary epithelial cells and extended growth capacity with p16 inactivation. Oncogene 17(2):199–205
55. Geradts J, Wilson PA (1996) High frequency of aberrant p16(INK4A) expression in human breast cancer. Am J Pathol 149(1):15–20
56. Romanov SR et al (2001) Normal human mammary epithelial cells spontaneously escape senescence and acquire genomic changes. Nature 409(6820):633–637
57. Nonet GH et al (2001) The ZNF217 gene amplified in breast cancers promotes immortalization of human mammary epithelial cells. Cancer Res 61(4):1250–1254
58. Stampfer MR et al (2001) Expression of the telomerase catalytic subunit, hTERT, induces resistance to transforming growth factor beta growth inhibition in p16INK4A(−) human mammary epithelial cells. Proc Natl Acad Sci USA 98(8):4498–4503
59. Clark R et al (1988) Transformation of human mammary epithelial cells by oncogenic retroviruses. Cancer Res 48(16):4689–4694
60. Olsen CL et al (2002) Raf-1-induced growth arrest in human mammary epithelial cells is p16-independent and is overcome in immortal cells during conversion. Oncogene 21(41): 6328–6339

61. Stampfer MR, Yaswen P (2003) Human epithelial cell immortalization as a step in carcinogenesis. Cancer Lett 194(2):199–208
62. Novak P et al (2009) Stepwise DNA methylation changes are linked to escape from defined proliferation barriers and mammary epithelial cell immortalization. Cancer Res 69(12):5251–5258
63. Ehrich M et al (2005) Quantitative high-throughput analysis of DNA methylation patterns by base-specific cleavage and mass spectrometry. Proc Natl Acad Sci USA 102(44): 15785–15790
64. Coolen MW et al (2010) Consolidation of the cancer genome into domains of repressive chromatin by long-range epigenetic silencing (LRES) reduces transcriptional plasticity. Nat Cell Biol 12(3):235–246
65. Walen KH, Stampfer MR (1989) Chromosome analyses of human mammary epithelial cells at stages of chemical-induced transformation progression to immortality. Cancer Genet Cytogenet 37(2):249–261

Chapter 10
Epigenetic Reprogramming of Mesenchymal Stem Cells

Yu-Wei Leu, Tim H.-M. Huang, and Shu-Huei Hsiao

Abstract Mesenchymal stem cells (MSCs) are multipotent stem cells of mesodermal origin that can be isolated from various sources and induced into different cell types. Although MSCs possess immune privilege and are more easily obtained than embryonic stem cells, their propensity to tumorigenesis has not been fully explored. Epigenomic changes in DNA methylation and chromatin structure have been hypothesized to be critical in the determination of lineage-specific differentiation and tumorigenesis of MSCs, but this has not been formally proven. We applied a targeted DNA methylation method to methylate a Polycomb group protein-governed gene, *Trip10*, in MSCs, which accelerated the cell fate determination of MSCs. In addition, targeted methylation of *HIC1* and *RassF1A*, both tumor suppressor genes, transformed MSCs into tumor stem cell-like cells. This new method will allow better control of the differentiation of MSCs and their use in downstream applications.

10.1 Introduction

Mesenchymal stem cells (MSCs) are somatic stem cells that can be isolated from various sources including bone marrow and fat tissue [80, 99]. Although MSCs possess more restricted pluripotency than embryonic stem (ES) cells, MSCs can still be induced to adipocytes, muscles, liver, bones, and neurons in vitro [55, 72, 73], making them a candidate for future cell therapy. From a safety consideration, there are

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debates about whether the MSCs could be transformed in vivo and whether they might be supportive or suppressive to tumoral growth [39, 88, 139]. Therefore, if the application and safety of MSCs could be monitored and well controlled, the application of MSCs will be broadened further.

Epigenetic regulation, including DNA methylation, histone modifications, and microRNAs (miRNAs), possesses the power to maintain the self-renewal or control the differentiation of stem cells [16, 32, 37, 69, 131]. Studies of ES cells have revealed the critical role of epigenetic regulation in controlling cell fate [44, 76, 107, 142]. Because there are almost no coding sequence differences between the ES cells and their derived cells, the differences between these cells are likely to come from differential gene expression [32, 47, 123]. The same rationale has prompted the use of epigenomic modifications as molecular codes to distinguish ES cells, MSCs, and their derived somatic cells. If the rationale were valid and the differences among different cell types originated from the epigenomic modifications, these distinct epigenetic states could represent the "stemness" in MSCs and ES cells, and changes of these epigenetic states might direct/interfere with the MSC differentiation.

Polycomb group proteins such as EZH2 and YY1 regulate part of the bivalent marks that represent the stemness in stem cells [119, 122]. There are loci in ES cells and MSCs associated with both active histone marks like histone 3 lysine 4 trimethylation (H3K4me3) [24, 42, 92] and repressive marks like histone 3 lysine 27 trimethylation (H3K27me3), and these are designated as bivalent loci [43, 114, 133]. These bivalent loci are often silenced [66] but are hypomethylated [134]. Among the histone marks, H3K27me3 is the substrate of Polycomb group proteins and loss of the maintenance of this histone mark is associated with the differentiation of stem cells [1, 21, 53, 70, 86]. These loci can be further activated by the association of active transcription factors and histone modifications like acetylation [61, 71, 94, 111], while their silencing could be further enhanced by DNA methylation in other lineage [5, 31, 48, 79, 87, 108, 109, 111, 118, 128, 138]. The identification of the epigenomic modifications within the bivalent loci could then reveal the ultimate fate of lineage-determining genes.

DNA methylation is one of the most dominant gene silencing mechanisms in cells and changes of methylation states correlate with the switch in cell lineages [58, 110]. It is known that changes in methylation states are inversely correlated with the expressions of corresponding genes, but the changed methylation status may not change cell fate directly. Therefore, a method that can methylate target genes and subsequently change cell fate would be an important demonstration that DNA methylation changes are sufficient to regulate cell fate decisions.

For instance, *Trip10* locus was identified as the target of Polycomb group protein and modified by DNA methylation during MSC differentiation [55]. Methylation of *Trip10* appears to be cell type specific in normal tissues as well as in cancers [55, 57]. This information suggests that *Trip10* methylation might be lineage specific and the targeted methylation of *Trip10* might then be able to direct MSC differentiation. When *Trip10* was methylated in MSCs, the MSC differentiation lineages were limited [55]. The success of the forward evaluation of the cell fate determination by

DNA methylation also opens the gateway to finetune MSC differentiation. On the other hand, the tumor suppressor genes might not have bivalent marks and methylation of these loci may lead to cell transformation. As demonstrated in a recent report, *hypermethylated in cancer* (*HIC1*) and *RassF1A* are two tumor suppressor genes that are not associated with bivalent histone marks and their methylation could transform MSCs [125].

10.2 Mesenchymal Stem Cells

MSCs can be isolated from various sources including bone marrow, adipose tissue, liver, muscle, amniotic fluid, dental pulp, placenta, and umbilical cord blood; the properties among these MSCs seem to vary accordingly [9, 49, 82, 100, 105, 113, 120, 143]. Different cell surface markers identified from different MSCs are reflective of their propensity to differentiate into different cell lineages [19, 34, 98]. Because MSCs can differentiate into different cell types in vitro, it is believed that there are common gene expression repertoires among these MSCs to maintain their stemness, but there are also different gene expression signatures that define the identities and differentiation potentials of different MSCs. Thus understanding the molecular mechanisms underlying the maintenance of cellular identities and determination of cell lineages is critical for the future clinical use of MSCs.

Unregulated differentiation is another reason to decipher the molecular codes that characterize MSCs. Different routes of transplantation make isolated clones of MSCs possess varied degrees of differentiation capacities, and dysregulation of these processes might consequently lead to disease. For example, MSCs together with or without hematopoietic stem cells (HSCs) isolated from bone marrow can be transplanted and differentiated into lung, gut, skin [68], liver and biliary epithelium [68, 97, 126, 127], skeletal myoblast [41, 46], neuroectodermal cells [18, 106], and endothelium [4, 77, 144]. These co-transplantation results imply that there are molecular machineries that might be used to define the MSCs and their derived lineages. These molecular codes also respond to neighboring cells and/or microenvironment of MSCs to maintain or differentiate cell fates. The importance of interplay with the environment is also evident by the reports that MSCs can either inhibit or support tumor growth in a cell setting-specific manner [67, 116]. The other controversy is that MSCs are proposed to both boost the immune system and suppress it [105, 129]; thus, the clinical safety of MSCs remains to be clarified.

10.3 Epigenetic Regulation and the Maintenance of MSC

Stemness needs to be maintained when the stem cells are self-renewing [50, 109, 121]. Since the coding sequences are all the same within ES cells, MSCs, and the differentiated somatic cells, there ought to be other somatic inheritable marks that

could represent the maintenance of stemness. Epigenetic marks are somatically inheritable modifications that regulate gene expression but do not change the associated gene sequence. These cellular epigenetic marks, while they can be reshaped by the environmental factors like diet and growth factors, in general are faithfully passed on to the descended lineage of cells. These properties make the epigenomic marks good candidates for the control of cellular stemness.

Bivalent loci in the stem cells are associated with both active and repressive epigenetic marks and are critical for cellular differentiation [6, 16, 32, 91]. Interactions between different epigenetic modifications can lead the governed genes to become permanently silenced or activated. The Polycomb group proteins, and associated histone modifications like H3K27me3, are one of the representative markers that are associated with stemness [20, 28, 45, 101]. Polycomb group proteins are reported to mediate the transition between the transcriptional silencing and active states of the associated gene [95] and their transitional regulatory role is evidenced by the co-existence of enhancer and suppressor genetic modifier phenotypes when the Polycomb group proteins lost their functions [85]. H3K27me3-associated loci can be further silenced by other epigenetic modifications including DNA methylation and the formation of heterochromatin [6, 62, 84, 137, 141]. On the other hand, the repressive trimethylation can be demethylated and the associated genes can then be reactivated. Loss of maintenance of these trimethylation states leads to differentiation of stem cells, which strongly suggests that maintaining these bivalent marks is critical for the maintenance of stemness [1, 21, 53, 70, 86].

Bivalent loci have been profiled in ES cells, tumors, differentiated cells, and MSCs [6]. Because the identified bivalent loci are different among these cells, the data support the hypothesis that these bivalent loci represent the unique stemness state in different cell types. From a direct comparison, there are more shared bivalent marks between ES cells and tumors than between the differentiated tissues and tumors, suggesting that tumors might be evolved from cells with more stem-like marks, and inappropriate maintenance of these marks could cause devious cell fate changes [23, 96, 132].

The bivalent loci in MSCs also mark developmentally important genes and can be further modified epigenetically [55]. The epigenetic marks on the MSC bivalent loci are distinct from those in the ES cells and differentiated cells. The bivalent loci that reside within the MSCs are often low in DNA methylation (hypomethylated) and can be further methylated or activated. The number and function of these bivalent genes might limit the lineages into which the MSCs can differentiate. It has been reported that undifferentiated MSCs contain both repressive and active chromatin marks on β-catenin-bound *c-myc* and *cyclin D* promoters [15, 35, 36]. When these MSCs became lineage committed, e.g., osteogenic, H3K4me3 was lost. This example indicates that epigenetic modifications regulate the Wnt signaling pathway in MSC, and similar epigenetic modifications are found in ES cells as well. We identified the H3K27me3-associated loci in MSCs that are differentially methylated when the MSCs are differentially induced into hepatocytes or adipocytes [55]. Loci that are not associated with DNA methylation association protein, MeCP2, were

considered hypomethylated. We found more than 383 of these bivalent loci are further associated with MeCP2 and proved to be methylated in either MSC-derived hepatocytes or adipocytes [55]. Therefore, these bivalent loci in MSCs might mark the lineages into which the MSCs are differentiated, and the later-added DNA methylation might further strengthen the cell fate evolution.

10.4 DNA Methylation and the Differentiation of MSC

DNA methylation is one of the most dominant silencing epigenetic modifications and occurs at the CpG dinucleotide in the human genome. A high frequency of CpG dinucleotides is often found at the promoter and/or first exon of genes and are named CpG islands [10, 12, 33]. Up to now, almost all the identified DNA methylation at the CpG islands silence the associated genes [11, 13, 124]. DNA methylation is a reversible event [8, 29, 60], and the removal of the silencing mark is critical for the activation of the associated genes [74, 75]. Compared with histone deacetylation inhibitors that cause less significant gene activation, demethylation induced by 5-aza-2′-deoxycytidine (5-Aza), a DNA methylation inhibitor, often causes a greater extent of restoration of gene expression [22]. Our previous results also indicated that when the estrogen receptor (ER)-targeted genes were silenced long term by DNA methylation, adding estrogen and/or overexpression of ER was insufficient to reactivate the ER target genes. Only after the DNA methylation was removed, could the expression of ER target genes be restored by the stimuli of estrogen [75]. Also, global demethylation results in global reactivation of the expression of these genes [74]. These observations all indicate that DNA methylation is a dominant silencing mark; its appearance leads to the silenced locus and the changes in methylation states reflect the changes in cellular physiology.

Altered DNA methylation status often correlates with the normal differentiation or the onset of diseases like cancer. DNA methylation is now considered a reliable biomarker and the profiling of methylation changes can be used to probe cellular or pathological events. Environmental factors relay their influence into the cells through specific signaling pathways. These influences are then recorded as epigenetic marks like DNA methylation during cell passages and are further selected in the descended population of cells. For example, when ER was knocked down by siRNA in a breast cancer cell line that once expressed ER, the downstream ER target/regulated genes were silenced gradually by various epigenetic marks, and later by DNA methylation [75]. DNA methylation also was accumulated within the ER target loci when the ER-expressing breast cancer cells were cultured long term in an estrogen-deprived environment. The recruitment and accumulation of DNA methylation within the estrogen signaling pathway left specific marks for us to track cell lineage which previously encountered the changed cellular environment. Evidence from genetic models also indicates that the environmental factors work through different signaling pathways and leave different but traceable patterns of DNA methylation. When signals like MYC or P53 were genetically manipulated, specific sets

of genes were methylated in the descended mice [93]. Therefore, the accumulated DNA methylation does not appear to occur at random.

Methylation changes caused by environmental changes like diet can be inherited and may influence cellular physiology as well as the onset of disease. The cofactor for DNA methylation reactions, *S*-adenosyl-methionine (SAM), is produced from dietary folate, and this provides the opportunity for diet to influence DNA methylation [25, 65, 104, 115, 117]. Mammals go through two genomic methylation revolutions during their development: one is during their formation of gametes, the other is directly after the fertilization is complete [64, 102, 112]. DNA methylation is erased during these two stages and re-established according to their paternal or maternal origins [136]. An elegant experiment in which pregnant mice were fed with various concentrations of food that could be converted into corresponding concentrations of SAM caused varied degrees of methylation. The newborn mice showed different degrees of fur color according to the concentration of methyl-supplemented diet consumed by the mothers, and these patterns of color lasted throughout their lives [38, 83, 89, 135]. In this example, environmental factors influenced methylation memories and changed the phenotype of the individuals in a somatically heritable way.

There is evidence indicating that changes in DNA methylation might be involved with the cell fate changes in MSCs as well. The methylation states within somatic stem/progenitor cells are different from the ones in ES cells and differentiated cells. For example, the promoter regions of *OCT4*, *NANOG,* and *SOX2* in adipose-derived MSCs display a greater extent of DNA methylation than in ES cells [6]. This methylation difference also provides an explanation for the fact that MSCs have lower differentiation capacity than the ES cells. Also, there are methylation differences within the promoters of tissue-specific genes between the bone- and adipose-derived MSCs; they correlate with their differences in lineage differentiation potential [63]. Osteoblast-specific genes such as *RUNX2* and *BGLAP* are hypermethylated in adipose-derived MSCs as compared to the bone-derived MSCs, whereas *PPARγ2*, the adipocyte-specific gene, is hypomethylated in adipose-derived MSCs [63]. Our previous data also identified a panel of genes that are differentially methylated within the differentiated hepatocytes or adipocytes when compared to the bone marrow-derived MSCs [55]. Taken together, DNA methylation status could represent the cellular identities and differentiation potentials of MSCs. It has been reported that global DNA methylation was changed in long-term cultured MSCs that might correlate with their altered differentiation capacity [17]. Changes in global methylation caused by demethylation agents have been documented to accelerate the osteogenic [3] or neuronal cell-like [2] differentiation of MSCs. However, it is unclear whether DNA methylation changes are sufficient to set the stage for MSC cell fate changes. It has been reported that predeposited DNA methylation within different isolated MSCs defined the oncogenic SYT-SSX1 fusion protein expression and limited its function in MSCs [30]. On the other hand, methylation profiling of adipogenic promoters from freshly cultured adipose stem cells to the senescence state did not correlate with their reduced differentiation potential [90, 91]. The absence of a targeted methylation method has hindered our understanding of how DNA methylation

determines the cell fate of MSCs. A solution is to find a way to methylate a bivalent gene in MSC and observe if the cell fate changed after targeting.

10.5 TRIP10 as a Model

Trip10 (also known as *CIP4*) encodes Cdc42-interacting protein 4, which was identified to be associated with Cdc42 and to regulate the cytoskeleton and membrane trafficking. Trip10 interacts with the Rho family GTPase TC-10 in adipocytes to regulate the translocation of insulin-stimulated glucose transporter 4 (Glu4) to the plasma membrane and finally to increase the uptake of glucose [26, 81]. In the brain of human Huntington's disease (HD) [52], Trip10 is reported to be a modulator of cell survival in the adjustment of DNA damage [140]. To guard against DNA damage, *Trip10* expression is decreased in hepatocyte growth factor/scatter factor (HGF/SF)-mediated cell protection, but *Trip10* level is significantly increased during hyperbaric oxygen-induced neuroprotection [51]. Overexpression of Trip10 was also observed in human HD brain striatum and the neuronal Trip10 immunoreactivity increased with neuropathological severity in the neostriatum of HD patients [52]. In addition, increased cell death was found in rat striatal neurons transfected with Trip10 [52], suggesting that Trip10 is toxic to striatal neurons. These data suggest that the effect of Trip10 in cell survival and growth is tissue specific. These diverse and sometimes contrary roles of Trip10 could be attributed in part to its splicing variants; equally important is the fact that they are the outcomes between Trip10 interaction with distinct signaling components in different cell settings.

In human bone marrow-derived MSCs, *Trip10* is hypomethylated in the undifferentiated stage and becomes hypermethylated during MSC-to-liver differentiation, but remains hypomethylated during MSC-to-adipocyte differentiation. Therefore, the methylation state of *Trip10* varies in different tissues and becomes a candidate biomarker to track MSC differentiation [55, 57]. We reasoned that the stemness state of Trip10 is maintained by the Polycomb group protein in the MSCs and that changes of chromatin structure, especially by DNA methylation, could restrict the cell lineages of MSCs. The differentiation or death of MSCs was thus predicted to be affected by *Trip10* methylation, and this model could be tested using targeted *Trip10* methylation.

10.6 Targeted DNA Methylation and MSC Differentiation

It has been hypothesized that DNA methylation within certain loci is sufficient to transform or differentiate cells, but this hypothesis had not been proved since there was no method to directly methylate specific loci [54]. Normal or abnormal methylation changes have been identified during cellular differentiation or transformation, but it remains to be elucidated whether all or any of the detected methylation changes

can affect cell fate. Moreover, if we can determine whether DNA methylation within certain loci is sufficient to determine the cell fate, this will provide additional information to evaluate the target genes that control cellular differentiation and transformation.

DNA methylation is initiated and maintained by DNA methyltransferase (DNMT) in mammalian cells [27, 40, 130]. As illustrated in Fig. 10.1a, during the cellular replication, DNMTs are recruited by the semimethylated old template and methylate the newly synthesized strand of DNAs [103]. The newly synthesized strand will then possess the same DNA methylation as the old strand. We reasoned that, by providing a methylated strand of DNA that is complementary with target loci, we might be able to recruit DNMT to the target loci and initiate targeted DNA methylation in the cell (Fig. 10.1b, [55, 56, 78]). A stretch of cloned *Trip10* promoter was in vitro methylated using commercial bacterial methylase, *Sss*I. These methylated inserts were then purified, denatured, and used to transfect MSCs. Unmethylated inserts served as the negative control; they did not induce any methylation at the *Trip10* promoter. Liposome-based transfection agents that were conjugated with florescent compounds were used for transfection in order to calculate the transfection efficiency. Also, the methylated/unmethylated inserts were labeled with Cy-dyes to track if the inserts entered the cell nuclei, because the denatured inserts need to be present and docked in the nuclei for the recruitment of DNMTs. Repeated transfection was needed to ensure the targeted DNA methylation. The promoter insert from another gene like *Casp8AP2* was used as a specificity control, as the methylated *Casp8AP2* inserts did not induce methylation at the *Trip10* promoter [55].

Targeted *Trip10* methylation was detected by semiquantitative methylation-specific PCR and bisulfite sequencing and the reduced *Trip10* expression was determined by RT-PCR and visualized by immunostaining. A two-component reporter gene system was established to validate the methylation-induced silencing at the transcription level and visualize the onset of DNA methylation in live cells. The two-component reporter system [55, 56] consists of two parts: (1) a cloned *Trip10* promoter that is linked with and regulates the expression of the *Tet* repressor (*TetR*) gene; and (2) a CMV promoter that is linked with, and regulates the expression of, the reporter gene enhanced green florescent protein (*EGFP*), with an intervening TetR binding site, $TetO_2$. Both constructs were transfected into a cell line simultaneously and colonies of cells that possess both inserted constructs were selected. Colonies of selected cells were then transfected with in vitro methylated or unmethylated *Trip10* inserts. The unmethylated *Trip10* promoter within the first construct will continue to express *TetR* that in turn represses the expression of *EGFP*. In contrast, targeted DNA methylation at the exogenous *Trip10* promoter silences the *TetR* expression which leads to the expression of the EGFP reporter. This induced EGFP expression could be reversed by adding of 5-Aza, suggesting that the original expression was caused by DNA methylation. With this reporter system, the targeted DNA methylation can be visualized in live cells.

During neuronal induction of MSCs, *Trip10* expression was greatly reduced and its distribution was confined to the peri-nuclei region in these induced cells [57]. Similar to the neuronal induction, targeted *Trip10* DNA methylation caused reduced *Trip10* expression and re-distribution and prompted the MSC-to-neuron

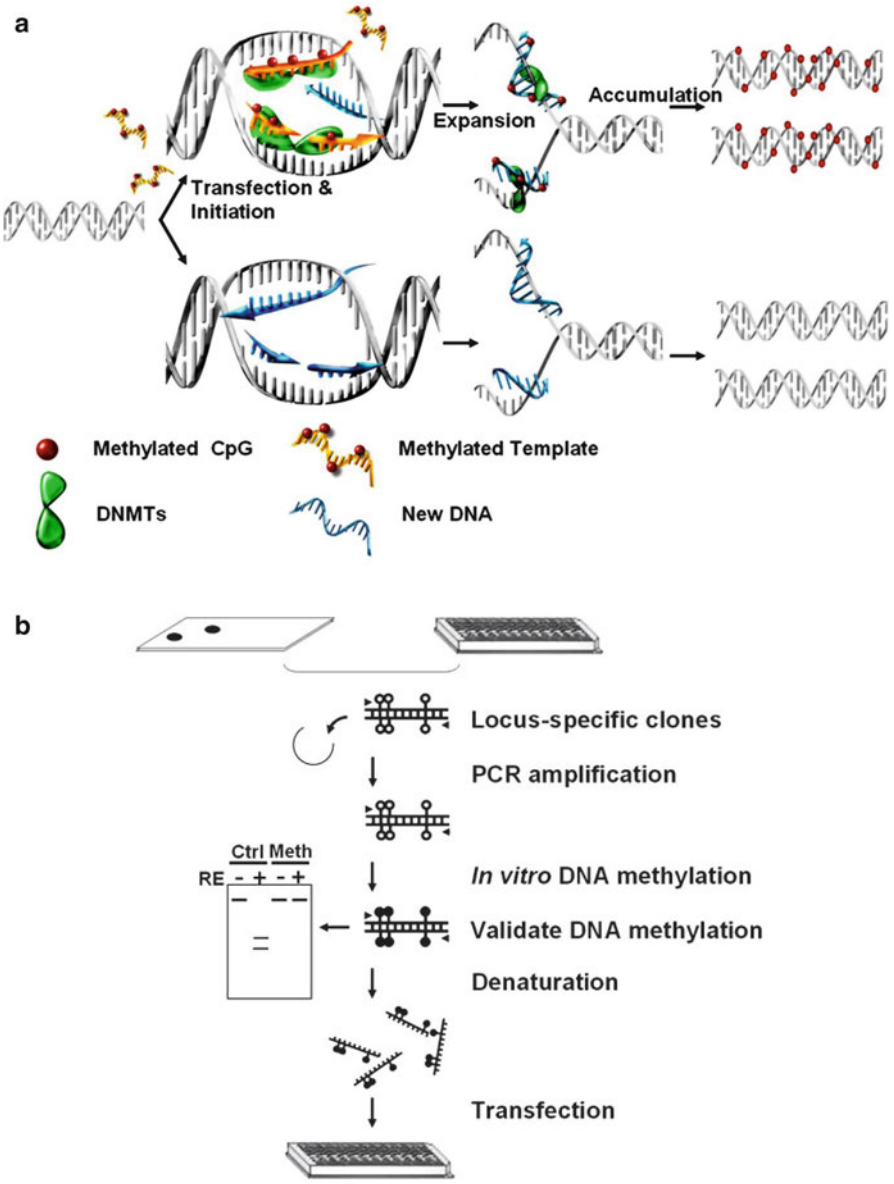

Fig. 10.1 Targeted DNA methylation. (**a**) Illustration of targeted DNA methylation. DNA methylation is maintained by DNMTs during cellular replication. *Upper*, the original unmethylated locus like *Trip10* will not recruit DNMTs to the newly synthesized strands of DNA; therefore, they remain hypomethylated. If the original strand was methylated, then the old template of DNA will recruit DNMTs to the newly synthesizing DNAs and add the methyl group to the new strand of DNAs. Targeted DNA methylation method transfects the cells with a denatured, in vitro methylated DNA with its sequence complemented to the target loci (*upper*). The provided methylated DNAs will pair with the old templates and recruit DNMTs to the newly synthesizing sites and methylate the new strands of DNAs. The seeded DNA methylation then will be spread and maintained during the following replications. (**b**) Flow of targeted DNA methylation (details in text). Templates of targeted DNA methylation could be from the CpG island library or cloned from the cultured cells. RE: methylation sensitive restriction enzymes like *Hpa*II and *BstU*I, etc

differentiation. This preferential cellular differentiation is specific since the same *Trip10* targeted DNA methylation prevented the MSC-to-adipocyte induction (Fig. 10.2a) [55]. These data indicate that DNA methylation within one of the bivalent loci is sufficient to control cellular differentiation.

10.7 DNA Methylation and Tumorigenesis of MSC

It is generally accepted that abnormal hypermethylation of tumor suppressor genes can transform normal cells [7, 12]. To support this theory, *HIC1* and *RassF1A*, two tumor suppressor genes that are methylated in several cancers but are not associated with Polycomb group protein in MSC, were in vitro methylated and then transfected into MSCs. Targeted methylation of *HIC1* or *RassF1A* alone is insufficient to transform the MSCs but concurrent *HIC1* and *RassF1A* methylation transforms the MSCs [125]. However, methylation of nine genes within the Salvador–Warts–Hippo pathway (including *RassF1A*) is insufficient to transform the MSCs [125], indicating that the *HIC1* and *RassF1A* methylation-caused transformation is not random. The transformed MSCs (named *me-H&R* MSCs) can still be differentiated into different cells including osteocytes, neurons, and adipocytes. Immunodeficient mice inoculated with a low number of *me-H&R* MSCs rapidly developed tumors. The developed tumors consisted of several clones of cells that express different cell surface markers, including mesenchymal and epithelial ones. 5-Aza treatment reversed the transformation and the tumoral properties of *me-H&R* MSCs, demonstrating that the transformation was caused by DNA methylation. Taken together, these findings suggest that the *me-H&R* MSCs become cancer stem cell (CSC)-like since they possess both tumoral and stem cell characters [125]. These results also imply that mal-maintained DNA methylation directly contributes to tumorigenesis.

10.8 Application of the Targeted DNA Methylation Technique

Epigenomic profiling in diverse cells including MSCs has revealed many cellular physiologies that are versatile and even personal [14, 17, 55, 59, 116]. Targeted DNA methylation is a direct validation of the profiling results and proves that epigenetic changes like DNA methylation are sufficient to direct MSC differentiation and tumorigenesis. As illustrated in Fig. 10.2a, MSCs could be differentiated into osteocyte, adipocyte, neuron, etc. Targeted *Trip10* methylation limits the differentiation potency of MSCs and accelerates their neural and osteogenic differentiation. On the other hand, targeted *HIC1* and *RassF1A* methylation transforms MSCs into CSC-like cells; targeted DNA methylation within nine loci in the Salvador–Warts–Hippo pathway cannot transform the MSCs but can keep the MSCs proliferating. These results indicate that CSC-like cells might arise from somatic stem cells-like MSCs (Fig. 10.2b), and the tumorigenesis and the immortalization could be dissected by the epigenetic modifications. In summary, using targeted DNA methylation, the differentiation

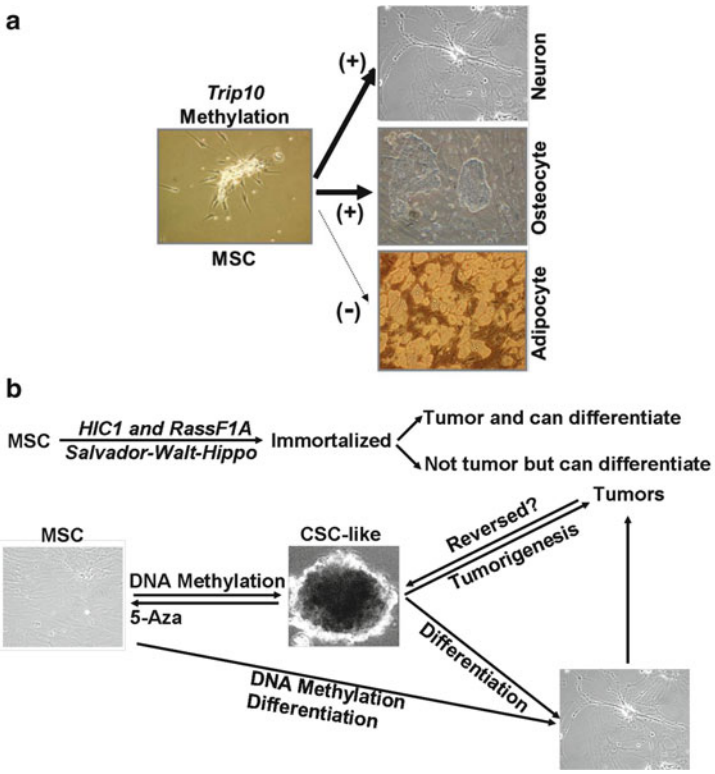

Fig. 10.2 Reprogramming of MSC. (**a**) *Trip10* methylation accelerates MSCs differentiation. Targeted DNA methylation within the *Trip10* promoter accelerates the MSCs to neuron or osteocyte differentiation but blocks their differentiation into adipocytes. (**b**) Summary of MSCs reprogramming. After methylation within the *HIC1* and *RassF1A*, the MSCs became tumors and still can differentiate. After the targeted methylation of nine genes in the Salvador–Warts–Hippo signaling pathway, the MSCs can be stably passaged and differentiated but they are not tumorigenic (*top*). Targeted methylation of *HIC1* and *RassF1A* caused the MSCs to become CSC-like (*bottom*) and this process can be reversed by 5-Aza. The CSC-like MSCs can be further developed into tumor in immunodeficient mice or differentiated into different cells like neurons. Thus, the differentiation, proliferation, and tumorigenic state of MSCs can be controlled by DNA methylation

(*Trip10*), proliferation (Salvador–Warts–Hippo), and tumorigenic (*HIC1* and *RassF1A*) characteristics of MSCs could be revealed.

References

1. Agger K, Cloos PA et al (2007) UTX and JMJD3 are histone H3K27 demethylases involved in HOX gene regulation and development. Nature 449:731–734
2. Alexanian AR (2007) Epigenetic modifiers promote efficient generation of neural-like cells from bone marrow-derived mesenchymal cells grown in neural environment. J Cell Biochem 100:362–371

3. Arnsdorf EJ, Tummala P et al (2010) The epigenetic mechanism of mechanically induced osteogenic differentiation. J Biomech 43:2881–2886
4. Asahara T, Masuda H et al (1999) Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. Circ Res 85:221–228
5. Balch C, Nephew KP et al (2007) Epigenetic "bivalently marked" process of cancer stem cell-driven tumorigenesis. Bioessays 29:842–845
6. Barrand S, Andersen IS et al (2010) Promoter-exon relationship of H3 lysine 9, 27, 36 and 79 methylation on pluripotency-associated genes. Biochem Biophys Res Commun 401:611–617
7. Baylin SB (2005) DNA methylation and gene silencing in cancer. Nat Clin Pract Oncol 2:S4–S11
8. Bender CM, Pao MM et al (1998) Inhibition of DNA methylation by 5-aza-2′-deoxycytidine suppresses the growth of human tumor cell lines. Cancer Res 58:95–101
9. Bianco P, Gehron Robey P (2000) Marrow stromal stem cells. J Clin Invest 105:1663–1668
10. Bird AP (1980) DNA methylation and the frequency of CpG in animal DNA. Nucleic Acids Res 8:1499–1504
11. Bird A (1999) DNA methylation de novo. Science 286:2287–2288
12. Bird A (2002) DNA methylation patterns and epigenetic memory. Genes Dev 16:6–21
13. Bird A, Macleod D (2004) Reading the DNA methylation signal. Cold Spring Harb Symp Quant Biol 69:113–118
14. Bloushtain-Qimron N, Yao J et al (2009) Epigenetic patterns of embryonic and adult stem cells. Cell Cycle 8:809–817
15. Boland GM, Perkins G et al (2004) Wnt 3a promotes proliferation and suppresses osteogenic differentiation of adult human mesenchymal stem cells. J Cell Biochem 93:1210–1230
16. Boquest AC, Noer A et al (2006) Epigenetic programming of mesenchymal stem cells from human adipose tissue. Stem Cell Rev 2:319–329
17. Bork S, Pfister S et al (2010) DNA methylation pattern changes upon long-term culture and aging of human mesenchymal stromal cells. Aging Cell 9:54–63
18. Brazelton TR, Rossi FM et al (2000) From marrow to brain: expression of neuronal phenotypes in adult mice. Science 290:1775–1779
19. Buhring HJ, Battula VL et al (2007) Novel markers for the prospective isolation of human MSC. Ann N Y Acad Sci 1106:262–271
20. Burdach S, Plehm S et al (2009) Epigenetic maintenance of stemness and malignancy in peripheral neuroectodermal tumors by EZH2. Cell Cycle 8(13):1991–1996
21. Burgold T, Spreafico F et al (2008) The histone H3 lysine 27-specific demethylase Jmjd3 is required for neural commitment. PLoS One 3(8):e3034
22. Cameron EE, Bachman KE et al (1999) Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. Nat Genet 21:103–107
23. Cao Q, Yu J et al (2008) Repression of E-cadherin by the polycomb group protein EZH2 in cancer. Oncogene 27:7274–7284
24. Carvin CD, Kladde MP (2004) Effectors of lysine 4 methylation of histone H3 in Saccharomyces cerevisiae are negative regulators of PHO5 and GAL1-10. J Biol Chem 279:33057–33062
25. Caudill MA, Wang JC et al (2001) Intracellular S-adenosylhomocysteine concentrations predict global DNA hypomethylation in tissues of methyl-deficient cystathionine beta-synthase heterozygous mice. J Nutr 131:2811–2818
26. Chang L, Adams RD et al (2002) The TC10-interacting protein CIP4/2 is required for insulin-stimulated Glut4 translocation in 3T3L1 adipocytes. Proc Natl Acad Sci USA 99:12835–12840
27. Cheng X, Blumenthal RM (2008) Mammalian DNA methyltransferases: a structural perspective. Structure 16:341–350
28. Christophersen NS, Helin K (2010) Epigenetic control of embryonic stem cell fate. J Exp Med 207:2287–2295

29. Chuang JC, Warner SL et al (2010) S110, a 5-Aza-2′-deoxycytidine-containing dinucleotide, is an effective DNA methylation inhibitor in vivo and can reduce tumor growth. Mol Cancer Ther 9:1443–1450
30. Cironi L, Provero P et al (2009) Epigenetic features of human mesenchymal stem cells determine their permissiveness for induction of relevant transcriptional changes by SYT-SSX1. PLoS One 4:e7904
31. Cohen NM, Dighe V et al (2009) DNA methylation programming and reprogramming in primate embryonic stem cells. Genome Res 19:2193–2201
32. Collas P (2009) Epigenetic states in stem cells. Biochim Biophys Acta 1790:900–905
33. Cross SH, Bird AP (1995) CpG islands and genes. Curr Opin Genet Dev 5:309–314
34. da Silva ML, Chagastelles PC et al (2006) Mesenchymal stem cells reside in virtually all post-natal organs and tissues. J Cell Sci 119:2204–2213
35. de Boer J, Siddappa R et al (2004) Wnt signaling inhibits osteogenic differentiation of human mesenchymal stem cells. Bone 34:818–826
36. De Boer J, Wang HJ et al (2004) Effects of Wnt signaling on proliferation and differentiation of human mesenchymal stem cells. Tissue Eng 10:393–401
37. De Miguel MP, Fuentes-Julian S et al (2010) Pluripotent stem cells: origin, maintenance and induction. Stem Cell Rev 6:633–649
38. Dolinoy DC, Weidman JR et al (2006) Maternal genistein alters coat color and protects Avy mouse offspring from obesity by modifying the fetal epigenome. Environ Health Perspect 114:567–572
39. Dwyer RM, Kerin MJ (2010) Mesenchymal stem cells and cancer: tumor-specific delivery vehicles or therapeutic targets? Hum Gene Ther 21:1506–1512
40. El-Osta A (2003) DNMT cooperativity—the developing links between methylation, chromatin structure and cancer. Bioessays 25:1071–1084
41. Ferrari G, Cusella-De Angelis G et al (1998) Muscle regeneration by bone marrow-derived myogenic progenitors. Science 279:1528–1530
42. Fingerman IM, Wu CL et al (2005) Global loss of Set1-mediated H3 Lys4 trimethylation is associated with silencing defects in Saccharomyces cerevisiae. J Biol Chem 280:28761–28765
43. Gan Q, Yoshida T et al (2007) Concise review: epigenetic mechanisms contribute to pluripotency and cell lineage determination of embryonic stem cells. Stem Cells 25:2–9
44. Gangaraju VK, Lin H (2009) MicroRNAs: key regulators of stem cells. Nat Rev Mol Cell Biol 10:116–125
45. Glinsky GV (2008) "Stemness" genomics law governs clinical behavior of human cancer: implications for decision making in disease management. J Clin Oncol 26:2846–2853
46. Gussoni E, Soneoka Y et al (1999) Dystrophin expression in the mdx mouse restored by stem cell transplantation. Nature 401:390–394
47. Hanna JH, Saha K et al (2010) Pluripotency and cellular reprogramming: facts, hypotheses, unresolved issues. Cell 143:508–525
48. Hansen KH, Bracken AP et al (2008) A model for transmission of the H3K27me3 epigenetic mark. Nat Cell Biol 10:1291–1300
49. Hematti P (2011) Human embryonic stem cell-derived mesenchymal progenitors: an overview. Methods Mol Biol 690:163–174
50. Hemberger M, Dean W et al (2009) Epigenetic dynamics of stem cells and cell lineage commitment: digging Waddington's canal. Nat Rev Mol Cell Biol 10:526–537
51. Hirata T, Cui YJ et al (2007) The temporal profile of genomic responses and protein synthesis in ischemic tolerance of the rat brain induced by repeated hyperbaric oxygen. Brain Res 1130:214–222
52. Holbert S, Dedeoglu A et al (2003) Cdc42-interacting protein 4 binds to huntingtin: neuropathologic and biological evidence for a role in Huntington's disease. Proc Natl Acad Sci USA 100:2712–2717
53. Hong S, Cho YW et al (2007) Identification of JmjC domain-containing UTX and JMJD3 as histone H3 lysine 27 demethylases. Proc Natl Acad Sci USA 104:18439–18444

54. Hsiao SH, Huang TH et al (2009) Excavating relics of DNA methylation changes during the development of neoplasia. Semin Cancer Biol 19:198–208
55. Hsiao SH, Lee KD et al (2010) DNA methylation of the Trip10 promoter accelerates mesenchymal stem cell lineage determination. Biochem Biophys Res Commun 400:305–312
56. Hsu CC, Li HP et al (2010) Targeted methylation of CMV and E1A viral promoters. Biochem Biophys Res Commun 402:228–234
57. Hsu CC, Leu YW et al (2011) Functional characterization of Trip10 in cancer cell growth and survival. J Biomed Sci 18:12
58. Ji H, Ehrlich LI et al (2010) Comprehensive methylome map of lineage commitment from haematopoietic progenitors. Nature 467:338–342
59. Jones PA, Martienssen R (2005) A blueprint for a Human Epigenome Project: the AACR Human Epigenome Workshop. Cancer Res 65:11241–11246
60. Jones PA, Taylor SM et al (1983) Inhibition of DNA methylation by 5-azacytidine. Recent Results Cancer Res 84:202–211
61. Jung JW, Lee S et al (2010) Histone deacetylase controls adult stem cell aging by balancing the expression of polycomb genes and jumonji domain containing 3. Cell Mol Life Sci 67:1165–1176
62. Kalari S, Pfeifer GP (2010) Identification of driver and passenger DNA methylation in cancer by epigenomic analysis. Adv Genet 70:277–308
63. Kang TH, Lee JH et al (2007) Epigallocatechin-3-gallate enhances CD8+ T cell-mediated antitumor immunity induced by DNA vaccination. Cancer Res 67:802–811
64. Kierszenbaum AL (2002) Genomic imprinting and epigenetic reprogramming: unearthing the garden of forking paths. Mol Reprod Dev 63:269–272
65. Kim D, Yang JY et al (2009) Overexpression of alpha-catenin increases osteoblastic differentiation in mouse mesenchymal C3H10T1/2 cells. Biochem Biophys Res Commun 382:745–750
66. Kirmizis A, Bartley SM et al (2004) Silencing of human polycomb target genes is associated with methylation of histone H3 Lys 27. Genes Dev 18:1592–1605
67. Klopp AH, Gupta A et al (2011) Concise review: dissecting a discrepancy in the literature: do mesenchymal stem cells support or suppress tumor growth? Stem Cells 29:11–19
68. Krause DS, Theise ND et al (2001) Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. Cell 105:369–377
69. Lakshmipathy U, Hart RP (2008) Concise review: microRNA expression in multipotent mesenchymal stromal cells. Stem Cells 26:356–363
70. Lan F, Bayliss PE et al (2007) A histone H3 lysine 27 demethylase regulates animal posterior development. Nature 449:689–694
71. Lau PN, Cheung P (2011) Histone code pathway involving H3 S28 phosphorylation and K27 acetylation activates transcription and antagonizes polycomb silencing. Proc Natl Acad Sci USA 108:2801–2806
72. Lee KD, Kuo TK et al (2004) In vitro hepatic differentiation of human mesenchymal stem cells. Hepatology 40:1275–1284
73. Lee OK, Kuo TK et al (2004) Isolation of multipotent mesenchymal stem cells from umbilical cord blood. Blood 103:1669–1675
74. Leu YW, Rahmatpanah F et al (2003) Double RNA interference of DNMT3b and DNMT1 enhances DNA demethylation and gene reactivation. Cancer Res 63:6110–6115
75. Leu YW, Yan PS et al (2004) Loss of estrogen receptor signaling triggers epigenetic silencing of downstream targets in breast cancer. Cancer Res 64:8184–8192
76. Li E (2002) Chromatin modification and epigenetic reprogramming in mammalian development. Nat Rev Genet 3:662–673
77. Lin Y, Weisdorf DJ et al (2000) Origins of circulating endothelial cells and endothelial outgrowth from blood. J Clin Invest 105:71–77
78. Lin YS, Shaw AY et al (2011) Identification of novel DNA methylation inhibitors via a two-component reporter gene system. J Biomed Sci 18:3

79. Liu YZ, Shao Z et al (2010) Prediction of Polycomb target genes in mouse embryonic stem cells. Genomics 96:17–26
80. Locke M, Feisst V et al (2011) Concise review: human adipose-derived stem cells (ASC): separating promise from clinical need. Stem Cells 29:404–411
81. Lodhi IJ, Chiang SH et al (2007) Gapex-5, a Rab31 guanine nucleotide exchange factor that regulates Glut4 trafficking in adipocytes. Cell Metab 5:59–72
82. Lopez MJ, Spencer ND (2011) In vitro adult rat adipose tissue-derived stromal cell isolation and differentiation. Methods Mol Biol 702:37–46
83. Martin DI, Cropley JE et al (2008) Environmental influence on epigenetic inheritance at the Avy allele. Nutr Rev 66:S12–S14
84. Mathieu O, Probst AV et al (2005) Distinct regulation of histone H3 methylation at lysines 27 and 9 by CpG methylation in Arabidopsis. EMBO J 24:2783–2791
85. Messmer S, Franke A et al (1992) Analysis of the functional role of the Polycomb chromo domain in Drosophila melanogaster. Genes Dev 6:1241–1254
86. Miller SA, Mohn SE et al (2010) Jmjd3 and UTX play a demethylase-independent role in chromatin remodeling to regulate T-box family member-dependent gene expression. Mol Cell 40:594–605
87. Mohn F, Weber M et al (2008) Lineage-specific polycomb targets and de novo DNA methylation define restriction and potential of neuronal progenitors. Mol Cell 30:755–766
88. Momin EN, Vela G et al (2010) The oncogenic potential of mesenchymal stem cells in the treatment of cancer: directions for future research. Curr Immunol Rev 6:137–148
89. Morgan HD, Sutherland HG et al (1999) Epigenetic inheritance at the agouti locus in the mouse. Nat Genet 23:314–318
90. Noer A, Boquest AC et al (2007) Dynamics of adipogenic promoter DNA methylation during clonal culture of human adipose stem cells to senescence. BMC Cell Biol 8:18
91. Noer A, Lindeman LC et al (2009) Histone H3 modifications associated with differentiation and long-term culture of mesenchymal adipose stem cells. Stem Cells Dev 18:725–736
92. Okitsu CY, Hsieh JC et al (2010) Transcriptional activity affects the H3K4me3 level and distribution in the coding region. Mol Cell Biol 30:2933–2946
93. Opavsky R, Wang SH et al (2007) CpG island methylation in a mouse model of lymphoma is driven by the genetic configuration of tumor cells. PLoS Genet 3:1757–1769
94. Pacini S, Carnicelli V et al (2010) Constitutive expression of pluripotency-associated genes in mesodermal progenitor cells (MPCs). PLoS One 5:e9861
95. Papp B, Muller J (2006) Histone trimethylation and the maintenance of transcriptional ON and OFF states by trxG and PcG proteins. Genes Dev 20:2041–2054
96. Pasini D, Malatesta M et al (2010) Characterization of an antagonistic switch between histone H3 lysine 27 methylation and acetylation in the transcriptional regulation of Polycomb group target genes. Nucleic Acids Res 38:4958–4969
97. Petersen BE, Bowen WC et al (1999) Bone marrow as a potential source of hepatic oval cells. Science 284:1168–1170
98. Pittenger MF, Mackay AM et al (1999) Multilineage potential of adult human mesenchymal stem cells. Science 284:143–147
99. Pontikoglou C, Deschaseaux F et al (2011) Bone marrow mesenchymal stem cells: biological properties and their role in hematopoiesis and hematopoietic stem cell transplantation. Stem Cell Rev 7:569–589
100. Prockop DJ (1997) Marrow stromal cells as stem cells for nonhematopoietic tissues. Science 276:71–74
101. Rajasekhar VK, Begemann M (2007) Concise review: roles of polycomb group proteins in development and disease: a stem cell perspective. Stem Cells 25:2498–2510
102. Reik W (2007) Stability and flexibility of epigenetic gene regulation in mammalian development. Nature 447:425–432
103. Robert MF, Morin S et al (2003) DNMT1 is required to maintain CpG methylation and aberrant gene silencing in human cancer cells. Nat Genet 33:61–65

104. Ross SA (2003) Diet and DNA methylation interactions in cancer prevention. Ann N Y Acad Sci 983:197–207
105. Salem HK, Thiemermann C (2010) Mesenchymal stromal cells: current understanding and clinical status. Stem Cells 28:585–596
106. Sanchez-Ramos J, Song S et al (2000) Adult bone marrow stromal cells differentiate into neural cells in vitro. Exp Neurol 164:247–256
107. Sasaki H, Matsui Y (2008) Epigenetic events in mammalian germ-cell development: reprogramming and beyond. Nat Rev Genet 9:129–140
108. Sawarkar R, Paro R (2010) Interpretation of developmental signaling at chromatin: the Polycomb perspective. Dev Cell 19:651–661
109. Schlesinger Y, Straussman R et al (2007) Polycomb-mediated methylation on Lys27 of histone H3 pre-marks genes for de novo methylation in cancer. Nat Genet 39:232–236
110. Schubeler D (2009) Epigenomics: methylation matters. Nature 462:296–297
111. Schwartz YB, Kahn TG et al (2010) Alternative epigenetic chromatin states of polycomb target genes. PLoS Genet 6:e1000805
112. Seki Y, Yamaji M et al (2007) Cellular dynamics associated with the genome-wide epigenetic reprogramming in migrating primordial germ cells in mice. Development 134:2627–2638
113. Sethe S, Scutt A et al (2006) Aging of mesenchymal stem cells. Ageing Res Rev 5:91–116
114. Shafa M, Krawetz R et al (2010) Returning to the stem state: epigenetics of recapitulating pre-differentiation chromatin structure. Bioessays 32:791–799
115. Sibani S, Melnyk S et al (2002) Studies of methionine cycle intermediates (SAM, SAH), DNA methylation and the impact of folate deficiency on tumor numbers in Min mice. Carcinogenesis 23:61–65
116. Siddiqi S, Mills J et al (2010) Epigenetic remodeling of chromatin architecture: exploring tumor differentiation therapies in mesenchymal stem cells and sarcomas. Curr Stem Cell Res Ther 5:63–73
117. Simile MM, Pascale R et al (1994) Correlation between S-adenosyl-L-methionine content and production of c-myc, c-Ha-ras, and c-Ki-ras mRNA transcripts in the early stages of rat liver carcinogenesis. Cancer Lett 79:9–16
118. Simon JA, Kingston RE (2009) Mechanisms of polycomb gene silencing: knowns and unknowns. Nat Rev Mol Cell Biol 10:697–708
119. Sparmann A, van Lohuizen M (2006) Polycomb silencers control cell fate, development and cancer. Nat Rev Cancer 6:846–856
120. Spencer ND, Lopez MJ (2011) In vitro adult canine adipose tissue-derived stromal cell growth characteristics. Methods Mol Biol 702:47–60
121. Spivakov M, Fisher AG (2007) Epigenetic signatures of stem-cell identity. Nat Rev Genet 8:263–271
122. Su Y, Deng B et al (2011) Polycomb group genes in stem cell self-renewal: a double-edged sword. Epigenetics 6:16–19
123. Surani MA, Hayashi K et al (2007) Genetic and epigenetic regulators of pluripotency. Cell 128:747–762
124. Tate PH, Bird AP (1993) Effects of DNA methylation on DNA-binding proteins and gene expression. Curr Opin Genet Dev 3:226–231
125. Teng IW, Hou PC et al (2011) Targeted methylation of two tumor suppressor genes is sufficient to transform mesenchymal stem cells into cancer stem/initiating cells. Cancer Res 71:4653–4663
126. Theise ND, Badve S et al (2000) Derivation of hepatocytes from bone marrow cells in mice after radiation-induced myeloablation. Hepatology 31:235–240
127. Theise ND, Nimmakayalu M et al (2000) Liver from bone marrow in humans. Hepatology 32:11–16
128. Tiwari VK, McGarvey KM et al (2008) PcG proteins, DNA methylation, and gene repression by chromatin looping. PLoS Biol 6:2911–2927
129. Trento C, Dazzi F (2010) Mesenchymal stem cells and innate tolerance: biology and clinical applications. Swiss Med Wkly 140:w13121

130. Turek-Plewa J, Jagodzinski PP (2005) The role of mammalian DNA methyltransferases in the regulation of gene expression. Cell Mol Biol Lett 10:631–647
131. Vincent A, Van Seuningen I (2009) Epigenetics, stem cells and epithelial cell fate. Differentiation 78:99–107
132. Wei Y, Xia W et al (2008) Loss of trimethylation at lysine 27 of histone H3 is a predictor of poor outcome in breast, ovarian, and pancreatic cancers. Mol Carcinog 47:701–706
133. Wei G, Wei L et al (2009) Global mapping of H3K4me3 and H3K27me3 reveals specificity and plasticity in lineage fate determination of differentiating CD4+ T cells. Immunity 30:155–167
134. Weinhofer I, Hehenberger E et al (2010) H3K27me3 profiling of the endosperm implies exclusion of polycomb group protein targeting by DNA methylation. PLoS Genet 6:e1001152
135. Wolff GL, Kodell RL et al (1998) Maternal epigenetics and methyl supplements affect agouti gene expression in Avy/a mice. FASEB J 12:949–957
136. Wu SC, Zhang Y (2010) Active DNA demethylation: many roads lead to Rome. Nat Rev Mol Cell Biol 11:607–620
137. Yamada Y, Watanabe A (2010) Epigenetic codes in stem cells and cancer stem cells. Adv Genet 70:177–199
138. Yamasaki-Ishizaki Y, Kayashima T et al (2007) Role of DNA methylation and histone H3 lysine 27 methylation in tissue-specific imprinting of mouse Grb10. Mol Cell Biol 27:732–742
139. Yang XF (2007) Immunology of stem cells and cancer stem cells. Cell Mol Immunol 4:161–171
140. Yuan R, Fan S et al (2001) Altered gene expression pattern in cultured human breast cancer cells treated with hepatocyte growth factor/scatter factor in the setting of DNA damage. Cancer Res 61:8022–8031
141. Zager RA, Johnson AC (2010) Progressive histone alterations and proinflammatory gene activation: consequences of heme protein/iron-mediated proximal tubule injury. Am J Physiol Renal Physiol 298:F827–F837
142. Zeng X (2007) Human embryonic stem cells: mechanisms to escape replicative senescence? Stem Cell Rev 3:270–279
143. Zheng C, Yang S et al (2009) Human multipotent mesenchymal stromal cells from fetal lung expressing pluripotent markers and differentiating into cell types of three germ layers. Cell Transplant 18:1093–1109
144. Jackson KA, Majka SM et al (2001) Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells. JCI 107:1395–1402

Part III
Clinical Implications and Analysis Methods

Chapter 11
Environmental Toxicants, Epigenetics, and Cancer

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Abstract Tumorigenesis, a complex and multifactorial progressive process of transformation of normal cells into malignant cells, is characterized by the accumulation of multiple cancer-specific heritable phenotypes triggered by the mutational and/or non-mutational (i.e., epigenetic) events. Accumulating evidence suggests that environmental and occupational exposures to natural substances, as well as man-made chemical and physical agents, play a causative role in human cancer. In a broad sense, carcinogenesis may be induced through either genotoxic or non-genotoxic mechanisms; however, both genotoxic and non-genotoxic carcinogens also cause prominent epigenetic changes. This review presents current evidence of the epigenetic alterations induced by various chemical carcinogens, including arsenic, 1,3-butadine, and pharmaceutical and biological agents, and highlights the potential for epigenetic changes to serve as markers for carcinogen exposure and cancer risk assessment.

11.1 Introduction

Tumorigenesis is a complex and multifactorial progressive process of transformation of normal cells into malignant ones. It is characterized by the accumulation of multiple cancer-specific heritable phenotypes, including persistent proliferative

Note: The views expressed in this chapter do not necessarily represent those of the U.S. Food and Drug Administration.

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signaling, resistance to cell death, evasion of growth suppression, replicative immortality, inflammatory response, deregulation of energy metabolism, genomic instability, induction of angiogenesis, and activation of invasion ultimately resulting in metastases [1]. The acquisition of these cancer-specific alterations may be triggered by the mutational and/or non-mutational (i.e., epigenetic) events in the genome which, in turn, affect gene expression and the downstream phenotypes listed above [1, 2]. Furthermore, it has been suggested that epigenetic alterations may play as important or even more prominent role in tumor development [3].

Epigenetic events, most prominently manifested by stable and heritable changes in gene expression that are not due to any alteration in the primary DNA sequence [4], signify the fundamental molecular principles in which genetic information is organized and read [5]. Epigenetic modifications include change in methylation patterns of cytosines in DNA [6, 7], modifications of the proteins that bind to DNA [8, 9], and the nucleosome positioning along DNA [4]. These epigenetic marks are tightly and interdependently connected and are essential for the normal development and the maintenance of cellular homeostasis and functions in adult organisms, particularly for X-chromosome inactivation in females, genomic imprinting, silencing of repetitive DNA elements, regulation of chromatin structure, and proper expression of genetic information [10]. The epigenetic status is well-balanced in normal cells, but may be altered in many ways in cancer cells. Additionally, growing evidence indicates that a number of lifestyle and environmental factors may disrupt this epigenetic balance and compromise the stability of the epigenome in normal cells leading to the development of a wide range of pathologies, including cancer.

11.2 Epigenetic Alterations in Cancer Cells

The unifying molecular feature of neoplastic cells is a profoundly reshaped genome characterized by global genomic *hypo*-methylation, gene-specific *hyper*- or *hypo*-methylation, and altered histone modification patterns [2, 11].

DNA demethylation signifies one of the two major DNA methylation states and refers to a state in which there is a decrease in the number of methylated cytosine bases from the "normal" methylation level. Demethylation of DNA can be achieved either passively or actively. Passive loss of methylated marks in the genome may be a consequence of limited availability of the universal methyl donor S-adenosyl-L-methionine (SAM), compromised integrity of DNA, and altered expression and/or activity of DNA methyltransferases [12]. Until recently, evidence for existence of an active replication-independent DNA demethylation process was controversial and inconclusive [7, 13]. However, recent studies provide compelling experimental evidence that active loss of DNA methylation is associated with the function of DNA repair machinery [14–17].

Global hypomethylation of DNA was the first epigenetic abnormality identified in cancer more than a quarter of century ago [18, 19]. It continues to be one of the

most common molecular alterations found in all human cancers [20, 21]; however, the molecular mechanisms behind cancer-linked global demethylation of the genome remain largely unknown. The loss of DNA methylation in cancer primarily affects stable, methylated areas of the genome composed predominantly of repetitive elements, genes, and intergenic regions [22].

There are several molecular consequences of global demethylation of DNA that may contribute to tumorigenesis. First, genomic hypomethylation causes significant elevation in mutation rates [23], activation of normally silenced tumor-promoting genes [24], and loss of imprinting [25]. Second, demethylation of the repetitive DNA sequences, such as long interspersed nucleotide elements (LINE)-1 and short interspersed nucleotide elements (SINE), retroviral intracisternal A particle (IAP), and Alu elements located at centromeric, pericentromeric, and subtelomeric chromosomal regions induces their activation and transposition leading to chromosomal instability [26–29]. For example, recent findings have demonstrated that DNA hypomethylation causes permissive transcriptional activity at the centromere [28]. Subsequently, the accumulation of small minor satellite transcripts that impair centromeric architecture and function is observed. Likewise, hypomethylation of the repetitive elements at the subtelomeric regions is associated with enhanced transcription of the telomeres [29].

Gene-specific loss of DNA methylation is also a finding for oncogenes and imprinted genes. In addition, many genes that are normally well-methylated, particularly cancer-germline genes, including B melanoma antigen family (*BAGE*), cancer testis antigen (*CAGE*), melanoma antigen family *A* (*MAGE-A*), X antigen family (*XAGE*), and other single-copy genes, including S100 calcium binding protein A4 (*S100A4*), flap endonuclease 1 (*FEN1*), and synuclein-gamma (*SNCG*), undergo progressive hypomethylation, which is accompanied by their increased expression, in human cancers [12, 21].

Despite the large body of evidence indicating that cancer-associated DNA demethylation is an important early event in tumor development, it is still less clear if the loss of DNA methylation is a cause, or a consequence of the malignant transformation [30]. The notion that DNA hypomethylation is playing a role in causation and/or promotion of cancer is based on the results of studies with nutritional "lipogenic methyl-deficient diet" [31–33], genetically engineered *Dnmt*- and *Lsh*-deficient mice [34, 35], and several models of chemical carcinogenesis [36]. In contrast, there is also evidence that cancer-linked DNA hypomethylation may be a passive inconsequential side effect of carcinogenesis [30, 37]. The latter is evidenced by facts that not all tumors exhibit DNA hypomethylation and not all carcinogenic processes are accompanied by the loss of DNA methylation [38]. Indeed, it is highly unlikely to expect that development and progression of diverse types of tumors are all associated with DNA hypomethylation. Furthermore, there is growing evidence that DNA hypomethylation suppresses development of certain tumor types, especially intestinal, gastric, and prostate carcinomas [39–41].

DNA hypermethylation is the state where the methylation of normally undermethylated DNA domains, those that predominantly consist of CpG islands [22], increases. CpG islands are defined as the genomic regions that contain the high

G + C content, have high frequency of CpG dinucleotides, are at least 400–500 bp long, and can be located either at intragenic and intergenic, or at the 5′ ends of genes [42–44]. However, only CpG islands that span 5′ promoters are mainly unmethylated. For instance, less than 3% of CpG islands in gene promoters are methylated [44].

It is well-established that hypermethylation of promoter-located CpG islands causes permanent and stable transcriptional silencing of a range of protein-coding genes [45], which, along with DNA hypomethylation, plays a critical role in cancer development [2, 11]. One of the most compelling examples of the link between DNA hypermethylation and carcinogenesis is epigenetic silencing of critical tumor-suppressor genes, including cyclin-dependent kinase inhibitor 2A (*CDKN2A*; $p16^{INK4A}$), secreted frizzled-related protein (*SFRPs*) genes, adenomatous polyposis coli (*APC*), and GATA binding protein 4 (*GATA4*). The aberrant silencing of these genes allows for survival and clonal expansion of the initiated cells. Additionally, hypermethylation of several DNA repair genes, including O^6-methylguanine-DNA methyltransferase (*MGMT*), xeroderma pigmentosum group C (*XPC*), MutL homolog 1 (*MLH1*), and breast cancer 1 and 2 (*BRCA1* and *BRCA2*) genes results in insufficient DNA repair leading to reduction in genomic stability and various genetic aberrations, particularly, the elevation of mutation rates in critical cancer-related genes [46, 47]. For example, the epigenetic silencing of *MGMT* leads to a greater mutation rate in *K-RAS* and *p53* genes during human colorectal carcinogenesis [48, 49]. Likewise, transcriptional inactivation of the *BRCA1* and *MLH1* genes caused by promoter hypermethylation results in elevated *p53* gene mutation frequency in human sporadic breast cancer [50] and microsatellite instability in sporadic colorectal cancer [51], respectively.

In addition to the vital role that DNA methylation state may play in the etiology and pathogenesis of cancer, it has been shown that disruption of normal patterns of covalent histone modifications is an epigenetic change frequently found in tumor cells. Histones are evolutionary conserved proteins that have globular carboxy-terminal domains critical to nucleosome formation, and flexible amino-terminal tails that protrude from the nucleosome core and contact adjacent nucleosomes to form higher order chromatin structures. At least eight different classes of post-translational modifications, including methylation, acetylation, phosphorylation, ubiquitination, sumoylation, biotinylation, and ADP-ribosylation have been identified on the core histones H2A, H2B, H3, H4, and the H1 family of linker histones [8, 9]. These histone marks are essential for organizing chromatin, maintaining genome stability, silencing repetitive DNA elements, regulating cell cycle progression, recognizing DNA damage sites and repair, and maintenance of proper expression of genetic information.

Accumulating evidence clearly indicates that cancer cells are characterized by a profoundly disturbed pattern of global and/or gene-specific histone modifications accompanied by alterations in the functioning of enzymes that are associated with those marks. There are various combinations of cancer-linked histone modifications that differ according to tumor type; however, one of the most characteristic examples of global changes in histone modifications is loss of histone H4 lysine 20

trimethylation and H4 lysine 16 acetylation, which is a common hallmark of human cancers [52].

Additionally, extensive studies in the past decade have indicated the existence and importance of another epigenetic mechanism of regulation of gene function by means of small non-coding microRNAs (miRNAs). Currently, miRNAs are recognized as one of the major regulatory gatekeepers of protein-coding genes in human genome [53, 54]. MiRNAs are small 16–29 nucleotide-long non-coding RNAs that primarily function as negative gene regulators at the post-transcriptional level [55]. MiRNAs are generated by RNA polymerase II or RNA polymerase III as long primary transcripts, primary miRNAs. Following transcription, primary miRNAs form a stem-loop structure, which is recognized and processed by the RNase III-type enzyme Drosha creating precursor miRNAs. These precursor miRNAs are transported from the nucleus to the cytoplasm by Exportin-5. In the cytoplasm, the pre-miRNAs are further processed by Dicer, an RNase III enzyme, generating miRNA:miRNA hybrids. After unwinding, one strand of the duplex is degraded, and another strand becomes a mature miRNA. MiRNAs can induce mRNA cleavage if complementary to 3′-untranslated region of targets is perfect or translational repression if complementarity is imperfect [53].

Currently there are more than 700 mammalian miRNAs that can potentially target up to one-third of protein-coding genes involved in the development, cell differentiation, metabolic regulation, signal-transduction, cell proliferation, and apoptosis. As the deregulation of these very same biological processes is a hallmark of cancer [1], it has been suggested that changes in miRNA expression might have significance in cancer [56–58]. In tumors, aberrant expression of miRNAs inhibits tumor suppressor genes or inappropriately activates oncogenes have been experimentally associated with most aspects of tumor biology, including tumor progression, invasiveness, metastasis, and acquisition of resistance of malignant cells to various chemotherapeutic agents [58]. This leads to the suggestion that altered expression of miRNAs is an important mechanism of carcinogenesis [57, 59].

11.3 Role of Epigenetic Alterations in Chemical Carcinogenesis

Many environmental and occupational exposures to natural substances, man-made chemical and physical agents are considered to be causative of human cancer [60–62]. In a broad sense, carcinogenesis may be induced through either genotoxic or non-genotoxic mechanisms. Genotoxic carcinogens are agents that interact directly or after metabolic activation with DNA, causing mutations and leading to tumor formation. Non-genotoxic carcinogens are a diverse group of chemical compounds that are known to cause tumors by mechanisms other than direct damage to DNA. The emphasis in carcinogenesis research, until recently, has focused mainly on the investigation of various molecular signaling events, DNA damage, DNA adduct repair, and genetic aberrations, despite the fact that the importance of epigenetic mechanisms in carcinogenic process was first suggested by Miller in 1970 [63].

Accumulating evidence suggests that regardless of the mechanism of action, both genotoxic and non-genotoxic carcinogens may also lead to prominent epigenetic abnormalities in tissues that are susceptible to carcinogenesis as a result of exposure [64–68]. The following sections present an overview of the epigenetic alterations induced by several carcinogens.

11.3.1 Arsenic

Arsenic is a naturally occurring element and a ubiquitous environmental contaminant which is a public health issue world-wide [69]. The major source of human exposure to arsenic is contaminated food and drinking water. Inorganic arsenic was one of the earliest identified human carcinogens [69, 70]. It is widely accepted that exposure to arsenic is associated with skin, lung, and bladder cancers [71]. Additionally, accumulating evidence indicates that long-term exposure to arsenic causes development of liver tumors [72].

Arsenic was classified as a known human carcinogen by the International Agency for Research on Cancer (IARC) in 2004, when sufficient evidence for human carcinogenicity became available [71]; even though limited evidence for animal carcinogenicity of arsenic existed. This may be explained mainly by the absence of adequate relevant animal models to study arsenic carcinogenesis. However, the experiments in transgenic mice, e.g., v-Ha-ras (Tg.AC), keratin VI/ornithine decarboxylase (K6/ODC), and $p53^{+/-}$, or inbred mouse strains that are prone to spontaneous cancer development provided evidence for the carcinogenicity of arsenic in animal studies. For instance, administration of arsenic to A/J mice, a strain that exhibits a susceptibility to different pulmonary pathological states including lung cancer, enhances lung tumor multiplicity and size [70, 73]. Similarly, in utero arsenic exposure of C3H/HeJ mice, which are prone to hepatocarcinogenesis, resulted in increased incidence and multiplicity of hepatocellular carcinomas in adults [74]. The most convincing evidence for the carcinogenicity of arsenic in animals has been presented in a recent report by Tokar et al. [75] that demonstrated that “whole-life” exposure of CD1 mice to arsenic causes induction of various tumors, including lung and liver.

The molecular mechanisms behind the cancer-inducing property of arsenic are not fully elucidated and remain a subject of debate. Several potential mechanisms have been proposed to explain arsenic-induced carcinogenesis, including induction of oxidative stress, DNA–protein crosslinking, chromosomal aberrations [70], disruption of signaling pathways, and epigenetic dysregulation, particularly DNA demethylation [76]. The first evidence demonstrating an association between arsenic tumorigenicity and global DNA hypomethylation was reported by Zhao et al. [77] who showed that exposure of rat liver epithelial TRL-1215 cells to arsenic in vitro led to their malignant transformation and was paralleled by global DNA demethylation. Importantly, the extent of DNA hypomethylation in the transformed cells was positively correlated with the tumorigenicity of the cells upon inoculation

into nude mice, suggesting that loss of DNA methylation may be a causative factor in arsenic-induced carcinogenesis [77]. Since then, a large amount of data has documented a substantial target organ-specific loss of global DNA methylation and repetitive element and gene-specific methylation in various in vitro and in vivo models of arsenic-induced tumorigenesis [78–80].

Several possible explanations exist for the mechanism of DNA demethylation after exposure to arsenic. First, arsenic-induced DNA hypomethylation can be explained by the absolute requirement of SAM for the biomethylation of inorganic arsenic and DNA methylation reactions [76, 81]. Therefore, the biomethylation of inorganic arsenic reduces availability of SAM for DNA and histone methylation. Second, arsenic exposure increases generation of reactive oxygen species that may cause direct damage to DNA [82, 83]. The presence of oxidative lesions in DNA (e.g., 8-oxodeoxyguanosine and 5-hydroxymethylcytosine) severely compromises the ability of DNA methyltransferases to methylate the target cytosine and leads to passive demethylation of DNA [84]. In addition, activation of DNA repair pathway promotes active demethylation of DNA [14–17]. Third, arsenic-induced oxidative stress causes depletion of the level of intracellular reduced glutathione. This consequently leads to the enhanced glutathione biosynthesis in a transsulfuration pathway, which impairs SAM biosynthesis and perturbs DNA and histone methylation reactions [85].

In addition to global and gene-specific DNA hypomethylation, arsenic exposure causes concurrent methylation-induced transcriptional silencing of a number of tumor suppressor genes, including *p53*, *CDKN2A* (*$p16^{INK4A}$*), Ras association domain family member 1 (*RASSF1A*), and death-associated protein kinase (*DAPK*) [73, 86, 87], various histone modification changes [88], and alterations in miRNA expression [89].

It is of note that growing evidence suggests that carcinogenesis induced by an environmental chronic exposure to other metals, such as nickel, chromium, cadmium, and mercury, may also involve molecular epigenetic alterations caused by the ability of these metals to induce damage to DNA and strongly influence intracellular molecular and metabolic alterations [90, 91].

11.3.2 1,3-Butadiene

The gaseous olefin 1,3-butadiene is a major industrial chemical monomer widely used in production of synthetic rubber, resins, and plastic. Additionally, this highly volatile agent is present in industrial and automobile exhaust, cigarette smoke, and ambient air in urban locations and industrial complexes [92]. Based on the results of numerous comprehensive epidemiological studies, the IARC has classified 1,3-butadiene as a known human carcinogen [92–94]. In rodents, it causes tumor formation at several target sites, including the hematopoietic system, lungs, heart, and liver [93]. Importantly, the hematopoietic system, lungs and liver are the most common sites of 1,3-butadiene-induced tumor formation in both humans and mice [93].

It is well-established that the mechanism of tumor induction caused by 1,3-butadiene-exposure is due to genotoxic reactivity of its metabolic epoxides: 1,2-epoxy-3-butene, 1,2:3,4-diepoxybutane, and 3,4-epoxy-1,2-butanediol that interact directly with DNA to form mutagenic DNA adducts [94]. However, recent evidence demonstrates that short-term inhalational exposure of C57BL/6J mice to 1,3-butadiene, in addition to DNA adduct formation, also causes extensive concurrent epigenetic changes. These include a marked reduction of global DNA and repetitive element methylation and a profound loss of histone H3K9, H3K27, and H4K20 trimethylation in the livers of C57BL/6J mice [95].

It is well-established that methylation of lysine residues 9 and 27 at histone H3 and lysine 20 at histone H4 plays a fundamental role in the formation of a condensed heterochromatin structure and transcriptional repression [96–98]. Hence, loss of H3K9 and H4K20 trimethylation induced by 1,3-butadiene-exposure may compromise genomic stability via chromatin relaxation and activation of mobile repetitive elements. Indeed, a recent report showing decondensation of chromatin and activation of main repetitive elements in the livers of 1,3-butadiene-exposed C57BL/6J mice support this suggestion [99]. Additionally, an open chromatin structure may increase further vulnerability of DNA to the genotoxicity of reactive 1,3-butadiene metabolites.

The elucidation of the mechanisms of carcinogenicity is usually carried out in genetically homogeneous in vivo models in order to fix as many variables as possible. This provides information in a single strain, yet the extrapolation of such data to the population effects is constrained by the inference from a single genome to model complex human phenotypes. To overcome this important limitation, panels of genetically defined animals may be used to determine genetic causes of inter-individual variability in cancer susceptibility [100]. In a recent study, Koturbash et al. [99] have demonstrated substantial differences in hepatic genetic and epigenetic response among mouse strains to short-term inhalational exposure to 1,3-butadiene. More importantly, the strain differences were associated with alterations in chromatin structure, mainly in the variability in histone H3K9, H3K27, and H4K20 methylation.

11.3.3 Pharmaceuticals

Diethylstilbestrol is a synthetic non-steroidal estrogen that was widely used to prevent potential miscarriages and as emergency contraceptive (morning-after pill) [101]. Currently, diethylstilbestrol is classified by the IARC as a known human carcinogen [101]. Breast is the main target organ for diethylstilbestrol-induced carcinogenesis in women who were exposed during pregnancy. Additionally, diethylstilbestrol also causes development of adenocarcinoma in the uterus and cervix of women who were exposed in utero.

In addition to the established mechanistic genotoxic and estrogen receptor-mediated carcinogenic events, epigenetic programming also plays a substantial role.

Perinatal exposure to diethylstilbestrol causes persistent demethylation and transcriptional activation of several critical cancer-related genes in the mouse uterus, including lactoferrin (*Lf*), nucleosomal binding protein 1 (*Nsbp1*), and c-*fos* [102–104]. The mechanism of these demethylation events is associated with the ability of diethylstilbestrol to inhibit expression of the maintenance (*Dnmt1*) and de novo (*Dnmt3a* and *Dnmt3b*) DNA methyltransferases in the mouse uterus [105]. Additionally, recent evidence indicates that diethylstilbestrol exposure causes epigenetically induced down-regulation of miRNA-9 in human breast epithelial cells [106], one of the frequently down-regulated miRNAs in human breast cancer [107].

Tamoxifen, a selective non-steroidal anti-estrogen, is a widely used drug for chemotherapy and for chemoprevention of breast cancer worldwide [108]. However, recently the IARC classified tamoxifen as a known human carcinogen based on evidence for endometrial cancer [101]. One of the possible mechanisms of carcinogenic effects of tamoxifen in the uterus is tamoxifen-induced gene expression changes [109], particularly, hypomethylation-linked activation of paired box 2 (*PAX2*) gene [110].

Additionally, a number of studies have demonstrated that tamoxifen is a potent hepatocarcinogen in rats with both tumor initiating and promoting properties [111]. The mechanism of tamoxifen-induced hepatocarcinogenesis is associated with its genotoxic [112, 113] and epigenetic effects [114]. These non-genotoxic epigenetic alterations include demethylation of the entire genome and the repetitive elements, loss of global histone H4 lysine 20 trimethylation [114, 115], and altered expression of miRNAs [116]. The results of these studies further emphasize the importance of non-genotoxic mechanisms in chemical carcinogenesis induced by genotoxic carcinogens.

Phenobarbital, the most widely used anticonvulsant worldwide, is a well-established mitogenic non-genotoxic rodent liver carcinogen. It is known to increase cell proliferation, alter cell cycle checkpoint control, including delaying and attenuating the G1 checkpoint, inhibit the induction of p53, thereby resulting in accumulation of DNA damage, and induce extensive epigenetic abnormalities. Treatment with phenobarbital leads to rapid and progressive accumulation of altered DNA methylation regions in the livers of C57BL/6 and B6C3F1 mice [117]. These changes were more pronounced in livers of tumor-prone B6C3F1 and CAR (constitutive androstane receptor) wild-type mice [118]. Interestingly, the number of hypermethylated regions was noticeably smaller than hypomethylated regions, among which cytochrome P450, family 2, subfamily b, polypeptide 10 (*Cyp2b10)* gene is concomitantly hypomethylated and transcriptionally activated early after phenobarbital treatment [119].

Oxazepam is widely used as a sedative-hypnotic and antianxiety drug. Chronic exposure of B6C3F1 mice to oxazepam induces development of hepatoblastoma and hepatocellular carcinoma in mice [120]. Interestingly, oxazepam, similar to phenobarbital, causes induction of *Cyp2b10* gene in the livers of B6C3F1 mice [121, 122]. Also, oxazepam-induced tumors display a decreased expression of *Apc* and phosphatase and tensin (*Pten*) homolog tumor suppressor genes and genes involved in regulation of DNA methylation and histone modification [122].

11.3.4 Biological Agents

Mycotoxins are a structurally diverse class of molecules of fungal origin that are common contaminants of the human and animal food products [123]. Three of the most ubiquitous mycotoxins, aflatoxin B_1, fumonisin B1, and ochratoxin, are classified by the IARC as known and possible human carcinogens [124, 125]. It is well-established that aflatoxin B_1, fumonisin B1, and ochratoxin A are genotoxic carcinogens [123, 126, 127]; however, accumulating evidence indicates that their carcinogenicity involves also a complex network of epigenetic alterations [128–134].

Aflatoxin B_1 induces several epigenetic abnormalities that may induce and promote tumor development. Specifically, exposure to aflatoxin B_1 causes methylation-induced transcriptional silencing of *MGMT, p16^{INK4A}*, and *RASSF1A* genes, a fundamental epigenetic event in liver carcinogenesis [128–130]. Conversely, aflatoxin B_1 is a strong inducer of epigenetically regulated *SNCG* gene [131]. Additionally, a study conducted by Hu et al. [134] has demonstrated that cytosine methylation at the CpG site at codon 14 of the *K-ras* gene is the major reason for preferential aflatoxin B_1-induced DNA-adduct formation at this codon in normal human bronchial epithelial cells.

Fumonisin B_1, in addition to various genotoxic and non-genotoxic alterations, increases the level of 5-methylcytosine in genomic DNA from 5 to 9% in human intestinal Caco-2 cells [132].

Helicobacter pylori infection is associated with development of gastric cancer, one of the most prevalent human cancers worldwide [135]. The results of several comprehensive studies indicate that *H. pylori* infection causes marked DNA methylation changes in infected normal or preneoplastic gastric mucosa. *H. pylori* infection causes significant aberrant DNA methylation in a number of the promoter CpG island-containing genes, including *p16^{INK4A}*, lipoxygenase (*LOX*), heart and neural crest derivatives expressed 1 (*HAND1*), thrombomodulin (*THBD*), and actin related protein 2/3 complex, subunit p41 (*p41ARC*) gastric cancer-associated genes in gastric mucosa [136–139]. Importantly, hypermethylation of some genes, e.g., *THBD* persisted in gastric mucosa after *H. pylori* eradication [140].

11.4 Epigenetic Alterations and the Evaluation of Cancer Risk

Recognition of the fundamental role of epigenetic alterations in cancer has resulted in the identification of numerous epigenetic abnormalities that may be used as potential biomarkers for the molecular diagnosis of cancer and prognosis of survival or treatment outcomes. Despite a lack of conclusive information to clarify whether or not epigenetic changes are involved directly in neoplastic cell transformation, evidence highlighted above suggests that epigenetic alterations may be used as early indicators of carcinogenesis for both genotoxic and non-genotoxic carcinogens. Importantly, several research groups have argued that epigenetic alterations may be

used as biomarkers in the evaluation of the carcinogenic potential of the environmental factors [5, 67, 68, 141].

Incorporation of the epigenetic biomarkers into the studies on cancer risk of exposures holds a number of advantages over traditionally used methods, such as evaluation of the carcinogen-induced DNA damage, DNA adduct formation, or bacterial mutagenicity. Specifically, we reason that the following features are in favor of greater integration of epigenetic biomarkers in studies of the carcinogenic potential of the environmental exposures: (1) early appearance; (2) stability; (3) target tissue-specificity; (4) relatively low cost of the assays needed to detect these changes; (5) applicability to both genotoxic and non-genotoxic agents; and, more importantly, (6) a greater number of detectable epigenetic changes as compared to the genetic alterations after exposure.

Also, the incorporation of epigenetic technologies into the studies of cancer risk promises to enhance substantially the efficiency of carcinogenicity testing. More importantly, the reversibility of epigenetic alterations opens novel mechanism-based approaches not only to cancer treatment but also to the timely prevention of cancer [142]. However, despite a very promising outlook on the benefits of epigenetic biomarkers, additional studies are still needed to better define the nature and mechanisms of epigenetic abnormalities with respect to carcinogenic processes [60, 143, 144]. Although extensive studies have identified a number of cancer-related epigenetic abnormalities that are associated with carcinogen exposure, there is no consensus on the role of changes in tumorigenesis.

Additionally, it is possible that not all these aberrant epigenetic events are equally important for the tumorigenic process. It is highly unlikely that all of these epigenetic changes play a causative role in tumorigenesis. For example, some epigenetic changes may drive other epigenetic events that contribute to the formation of a transformed phenotype, while others may be passenger epigenetic events that accompany the transformation process [145]. In this respect, the identification of those epigenetic events that drive cell transformation is crucially important for understanding mechanisms of tumorigenesis and for cancer prevention.

References

1. Hanahan D, Weinberg RA (2011) Hallmarks of cancer. Cell 144:646–674
2. Jones PA, Baylin SB (2007) The epigenomics of cancer. Cell 128:683–692
3. Ushijima T, Asada K (2010) Aberrant DNA methylation in contrast with mutations. Cancer Sci 101:300–305
4. Sharma S, Kelly TK, Jones PA (2010) Epigenetics in cancer. Carcinogenesis 31:27–36
5. Marlowe J, Teo SS, Chibout SD, Pognan F, Moggs JJ (2009) Mapping the epigenome—impact for toxicology. EXS 99:259–288
6. Kim JK, Samaranayake M, Pradhan S (2009) Epigenetic mechanisms in mammals. Cell Mol Life Sci 66:596–612
7. Ooi SK, O'Donnell AH, Bestor TH (2009) Mammalian cytosine methylation at a glance. J Cell Sci 122:2787–2791

8. Chen ZX, Riggs AD (2011) DNA methylation and demethylation in mammals. J Biol Chem 286:18347–18353
9. Jenuwein T, Allis CD (2001) Translating the histone code. Science 293:1074–1080
10. Kouzarides T (2007) Chromatin modifications and their function. Cell 128:693–705
11. Esteller M (2008) Epigenetics in cancer. N Engl J Med 358:1148–1159
12. Pogribny IP, Beland FA (2009) DNA hypomethylation in the origin and pathogenesis of human diseases. Cell Mol Life Sci 66:2249–2261
13. Ooi SK, Bestor TH (2008) The colorful history of active DNA demethylation. Cell 133:1145–1148
14. Ma DK, Guo JU, Ming GL, Song H (2009) DNA excision repair proteins and Gadd45 as molecular players for active DNA demethylation. Cell Cycle 8:1526–1531
15. He YF, Li BZ, Li Z, Wang Y, Tang Q, Ding J, Jiz Y, Chen Z, Li L, Sun Y, Li X, Dai Q, Song CX, Zhang K, He C, Xu GL (2011) Tet-mediated formation of 5-hydroxylcytosine and its excision by TDG in mammalian DNA. Science 333:1303–1307
16. Ito S, Shen L, Dai Q, Wu SC, Collins LB, Swenberg JA, He C, Zhang Y (2011) Tet proteins can convert 5-methylcytosine to 5-formylcytosine and 5-carboxylcytosine. Science 333: 1300–1333
17. Cortellino S, Xu J, Sannai M, Moore R, Caretti E, Cigliano A, Le Coz M, Devarajan K, Wessels A, Soprano D, Abramowitz LK, Bartolomei MS, Rambow F, Bassi MR, Bruno T, Fanciulli M, Renner C, Klein-Szanto AJ, Matsumoto Y, Kobi D, Davidson I, Alberti C, Larue L, Bellacosa A (2011) Thymine DNA glycosylase is essential for active DNA demethylation by linked deamination-base excision repair. Cell 146:67–79
18. Feinberg AP, Tycko B (2004) The history of cancer epigenetics. Nat Rev Cancer 4:143–153
19. Ehrlich M (2009) DNA hypomethylation in cancer cells. Epigenomics 1:239–259
20. Wilson AS, Power BE, Molloy PL (2007) DNA hypomethylation and human diseases. Biochim Biophys Acta 1775:138–162
21. De Smet C, Loriot A (2010) DNA hypomethylation in cancer: Epigenetic scars of a neoplastic journey. Epigenetics 5:206–213
22. Rollins RA, Haghighi F, Edwards JR, Das R, Zhang MQ, Ju J, Bestor TH (2006) Large-scale structure of genomic methylation patterns. Genome Res 16:157–163
23. Chen RZ, Pettersson U, Beard C, Jackson-Grusby L, Jaenisch R (1998) DNA hypomethylation leads to elevated mutation rates. Nature 395:89–93
24. Ehrlich M (2002) DNA methylation in cancer: too much, but also too little. Oncogene 21:5400–5413
25. Feinberg AP, Cui H, Ohlsson R (2002) DNA methylation and genomic imprinting: insights from cancer into epigenetic mechanisms. Semin Cancer Biol 12:389–398
26. Howard G, Eiges R, Gaudet F, Jaenisch R, Eden A (2008) Activation and transposition of endogenous retroviral elements in hypomethylation induced tumors in mice. Oncogene 27:404–408
27. Eden A, Gaudet F, Waghmare A, Jaenisch R (2003) Chromosomal instability and tumors promoted by DNA hypomethylation. Science 300:455
28. Wong NC, Wong LH, Quach JM, Canham P, Craig JM, Song IZ, Clark SJ, Choo KH (2006) Permissive transcriptional activity at the centromere through pockets of DNA hypomethylation. PLoS Genet 2:e17
29. Vera E, Canela A, Fraga MF, Esteller M, Blasco MA (2008) Epigenetic regulation of telomeres in human cancer. Oncogene 27:6817–6833
30. Wild L, Flanagan JM (2010) Genome-wide hypomethylation in cancer may be a passive consequence of transformation. Biochim Biophys Acta 1806:50–57
31. Wainfan E, Poirier LA (1992) Methyl groups in carcinogenesis: effects on DNA methylation and gene expression. Cancer Res 52:2071s–2077s
32. Christman JK (1995) Dietary effects on DNA methylation: do they account for hepatocarcinogenic properties of lipotrope diets? Adv Exp Med Biol 369:141–154
33. Pogribny IP, James SJ, Jernigan S, Pogribna M (2004) Genomic hypomethylation is specific for preneoplastic liver in folate/methyl deficient rats and does not occur in non-target tissues. Mutat Res 548:53–59

34. Gaudet F, Hodgson JG, Eden A, Jackson-Grusby L, Dausman J, Gray JW, Leonhardt H, Jaenisch R (2003) Induction of tumors in mice by genomic hypomethylation. Science 300:489–492
35. Fan T, Schmidtmann A, Xi S, Briones V, Zhu H, Suh HC, Gooya J, Keller JR, Xu H, Roayaei J, Anver M, Ruscetti S, Muegge K (2008) DNA hypomethylation caused by Lsh deletion promotes erythroleukemia development. Epigenetics 3:134–142
36. Counts JL, Goodman JI (1994) Hypomethylation of DNA: an epigenetic mechanism involved in tumor promotion. Mol Carcinog 11:185–188
37. Wild L, Funes JM, Boshoff C, Flanagan JM (2010) In vitro transformation of mesenchymal stem cells induces gradual genomic hypomethylation. Carcinogenesis 31:1854–1862
38. Bagnyukova TV, Tryndyak VP, Montgomery B, Churchwell MI, Karpf AR, James SR, Muskhelishvili L, Beland FA (2008) Genetic and epigenetic changes in rat preneoplastic liver tissue induced by 2-acetylaminofluorene. Carcinogenesis 29:638–646
39. Yamada Y, Jackson-Grusby L, Linhart H, Meissner A, Eden A, Lin H, Jaenish R (2005) Opposing effects of DNA hypomethylation on intestinal and liver carcinogenesis. Proc Natl Acad Sci USA 102:13580–13585
40. Tomita H, Hirata A, Yamada Y, Hata K, Oyama T, Mori H, Yamashita S, Ushijima T, Hara A (2010) Suppressive effect of global DNA hypomethylation on gastric carcinogenesis. Carcinogenesis 31:1627–1633
41. Kinney SR, Moser MT, Pascual M, Greally JM, Foster BA, Karpf AR (2010) Opposing roles of Dnmt1 in early- and late-stage murine prostate cancer. Mol Cell Biol 30:4159–4174
42. Maunakea AK, Nagarajan RP, Bilenky M, Ballinger TJ, D'Souza C, Fouse SD, Johnson BE, Hong C, Nielsen C, Zhao Y, Turecki G, Delaney A, Varhol R, Thiessen N, Shchors K, Heine VM, Rowitch DH, Xing X, Fiore C, Schillebeeckx M, Jones SJM, Haussler D, Marra MA, Hirst M, Wang T, Costello JF (2010) Conserved role of intragenic DNA methylation in regulating alternative promoters. Nature 466:253–257
43. Takai D, Jones PA (2002) Comprehensive analysis of CpG islands in human chromosomes 21 and 22. Proc Natl Acad Sci USA 99:3740–3745
44. Illingworth RS, Bird AP (2009) CpG islands—"a rough guide". FEBS Lett 583:1713–1720
45. Deaton AM, Bird A (2011) CpG islands and the regulation of transcription. Genes Dev 25:1010–1022
46. Jacinto FV, Esteller M (2007) Mutator pathways unleashed by epigenetic silencing in human cancer. Mutagenesis 22:247–253
47. Lahtz C, Pfeifer GP (2011) Epigenetic changes of DNA repair genes in cancer. J Mol Cell Biol 3:51–58
48. Esteller M, Toyota M, Sanchez-Cespedes M, Capella G, Peinado MA, Watkins DN, Issa JP, Sidransky D, Baylin SB, Herman JG (2000) Inactivation of the DNA repair gene O6-methylguanine-DNA methyltransferase by promoter hypermethylation is associated with G to A mutations in K-ras in colorectal tumorigenesis. Cancer Res 60:2368–2371
49. Esteller M, Riques RA, Toyota M, Capella G, Moreno V, Peinado MA, Baylin SB, Herman JG (2001) Promoter hypermethylation of the DNA repair gene O6-methylguanine-DNA methyltransferase is associated with the presence G:C to A:T transition mutations in p53 in human colorectal tumorigenesis. Cancer Res 61:4689–4692
50. Birgisdottir V, Stefansson OA, Bodvarsdottir SK, Hilmarsdottir H, Jonasson JG, Eyfjorf JE (2006) Epigenetic silencing and deletion of the BRCA1 gene in sporadic breast cancer. Breast Cancer Res 8:R38
51. Herman JG, Umar A, Polyak K, Graff JR, Ahuja N, Issa JP, Markowitz S, Willson JK, Hamilton SR, Kinzler KW, Kane MF, Kolodner RD, Vogelstein B, Kunkel TA, Baylin SB (1998) Incidence and functional consequences of hMLH1 promoter hypermethylation in colorectal carcinoma. Proc Natl Acad Sci USA 95:6870–6875
52. Fraga MF, Ballestar E, Villar-Garea A, Boix-Chornet M, Espada J, Schotta G, Bonaldi T, Haydon C, Ropero S, Petrie K, Lyer NG, Pérez-Rosado A, Calvo E, Lopez JA, Cano A, Calasanz MJ, Colomer D, Piris MA, Ahn N, Imhof A, Caldas C, Jenuwein T, Esteller M (2005) Loss of acetylation at Lys16 and trimethylation at Lys20 of histone H4 is a common hallmark of human cancer. Nat Genet 37:391–400

53. Bartel DP (2009) MicroRNAs: target recognition and regulatory functions. Cell 136:215–233
54. Carthew RW, Sontheimer EJ (2009) Origins and mechanisms of miRNAs and siRNAs. Cell 136:642–655
55. Guo H, Ingolia NT, Weissman JS, Bartel DP (2010) Mammalian microRNAs predominantly act to decrease target mRNA levels. Nature 466:835–840
56. Garzon R, Calin GA, Croce CM (2009) MicroRNAs in cancer. Annu Rev Med 60:167–179
57. Ventura A, Jacks ST (2009) MicroRNAs and cancer: short RNAs go a long way. Cell 136:586–591
58. Di Leva G, Croce CM (2010) Roles of small RNAs in tumor formation. Trends Mol Med 16:257–267
59. Pogribny IP (2009) MicroRNA dysregulation during chemical carcinogenesis. Epigenomics 1:281–290
60. Loeb LA, Harris CC (2008) Advances in chemical carcinogenesis: a historical review and prospective. Cancer Res 68:6863–6872
61. Wild CP (2009) Environmental exposure measurement in cancer epidemiology. Mutagenesis 24:117–125
62. Clapp RW, Jacobs MM, Loechler EL (2008) Environmental and occupational causes of cancer: new evidence 2005–2007. Rev Environ Health 23:1–37
63. Miller JA (1970) Carcinogenesis by chemicals: and overview—G.H.A. Clowes memorial lecture. Cancer Res 30:559–576
64. Herceg Z (2007) Epigenetics and cancer: towards an evaluation of the impact of environmental and dietary factors. Mutagenesis 22:91–103
65. Pogribny IP, Rusyn I, Beland FA (2008) Epigenetics aspects of genotoxic and non-genotoxic hepatocarcinogenesis: studies in rodents. Environ Mol Mutagen 49:9–15
66. Bollati V, Baccareli A (2010) Environmental epigenetics. Heredity 105:105–112
67. Nakajima T, Enomoto S, Ushijima T (2008) DNA methylation: a marker for carcinogen exposure and cancer risk. Environ Health Prev Med 13:8–15
68. Ziech D, Franco R, Pappa A, Malamou-Mitsi V, Georgakila S, Georgakitas AG, Panayiotidis MI (2010) The role of epigenetics in environmental and occupational carcinogenesis. Chem Biol Interact 188:340–349
69. Hughes MF, Beck BD, Chen Y, Lewis AS, Thomas DJ (2011) Arsenic exposure and toxicology: a historical perspective. Toxicol Sci 123:305–332
70. Rossman TG (2003) Mechanism of arsenic carcinogenesis: an integrated approach. Mutat Res 533:37–65
71. IARC Monographs on the Evaluations of Carcinogenic Risks to Humans (2004) Some drinking-water disinfectants and contaminants, including arsenic, vol 84. IARC, Lyon
72. Liu J, Waalkes MP (2008) Liver is a target of arsenic carcinogenesis. Toxicol Sci 105:24–32
73. Cui X, Wakai T, Shirai Y, Hatakeyama K, Hirano S (2006) Chronic oral exposure to inorganic arsenate interferes with methylation status of p16INK4A and RASSF1A and induces lung cancer in A/J mice. Toxicol Sci 91:372–381
74. Waalkes MP, Ward JM, Liu J, Diwan BA (2003) Transplacental carcinogenicity of inorganic arsenic in the drinking water: induction of hepatic, ovarian, pulmonary, and adrenal tumors in mice. Toxicol Appl Pharmacol 186:7–17
75. Tokar EJ, Diwan BA, Ward JM, Delker DA, Waalkes MP (2011) Carcinogenic effects of "whole-life" exposure to inorganic arsenic in CD1 mice. Toxicol Sci 119:73–83
76. Reichard JF, Puga A (2010) Effects of arsenic exposure on DNA methylation and epigenetic gene regulation. Epigenomics 2:87–104
77. Zhao CQ, Young MR, Diwan BA, Coogan TP, Waalkes MP (1997) Association of arsenic-induced malignant transformation with DNA hypomethylation and aberrant gene expression. Proc Natl Acad Sci USA 94:10907–10912
78. Chen H, Li S, Liu J, Diwan BA, Barrett JC, Waalkes MP (2004) Chronic inorganic arsenic exposure induces hepatic global and individual gene hypomethylation: implications for arsenic hepatocarcinogenesis. Carcinogenesis 25(9):1779–1786

79. Jensen TJ, Novak P, Elbin KE, Gandolfi AJ, Futscher BW (2008) Epigenetic remodeling during-arsenical-induced malignant transformation. Carcinogenesis 29:1500–1508
80. Ren X, McHale CM, Skibola CF, Smith AH, Smith MT, Zhang L (2011) An emerging role for epigenetic dysregulation in arsenic toxicity and carcinogenesis. Environ Health Perspect 119:11–19
81. Coppin JF, Qu W, Waalkes MP (2008) Interplay between cellular methyl metabolism and adaptive efflux during oncogenic transformation from chronic arsenic exposure in human cells. J Biol Chem 283:19342–19350
82. Kitchin KT, Ahmad S (2003) Oxidative stress as a possible mode of action for arsenic carcinogenesis. Toxicol Lett 137:3–13
83. Kojima C, Ramirez DC, Tokar EJ, Himeno S, Drobná Z, Stýblo M, Mason RP, Waalkes MP (2009) Requirement of arsenic biomethylation for oxidative DNA damage. J Natl Cancer Inst 101:1670–1681
84. Valinluck V, Sowers LC (2007) Endogenous cytosine damage products alter the site selectivity of human DNA maintenance methyltransferase DNMT1. Cancer Res 67:946–950
85. Lee DH, Jacobs DR Jr, Porta M (2009) Hypothesis: a unifying mechanism for nutrition and chemicals as lifelong modulators of DNA hypomethylation. Environ Health Perspect 117:1799–1802
86. Mass MJ, Wang L (1997) Arsenic alters cytosine methylation patterns of the promoter of the tumor suppressor gene p53 in human lung cells: a model for a mechanism of carcinogenesis. Mutat Res 386:263–277
87. Huang YC, Huang WC, Chen WT, Yu HS, Chai CY (2009) Sodium arsenite-induced DAPK hypermethylation and autophagy via ERK1/2 phosphorylation in human uroepithelial cells. Chem Biol Interact 181:254–262
88. Zhou X, Sun H, Ellen TP, Chen H, Costa M (2008) Arsenite alters global histone H3 methylation. Carcinogenesis 29:1831–1836
89. Beezhold K, Liu J, Kan H, Meighan T, Castranova V, Shi X, Chen F (2011) miR-190-mediated downregulation of PHLP contributes to arsenic-induced Akt activation and carcinogenesis. Toxicol Sci 123(2):411–420
90. Salnikow K, Zhitkovich A (2008) Genetic and epigenetic mechanisms in metals carcinogenesis and cocarcinogenesis: nickel, arsenic, and chromium. Chem Res Toxicol 21:28–44
91. Martinez-Zamudio R, Ha HC (2011) Environmental epigenetics in metal exposure. Epigenetics 6(7):820–827
92. IARC Monographs on the Evaluations of Carcinogenic Risks to Humans (2008) 1,3-Butadiene, ethylene oxide and vinyl halides (vinyl fluoride, vinyl chloride, and vinyl bromide), vol 97. IARC, Lyon
93. Melnick RL, Sills RC (2001) Comparative carcinogenicity of 1,3-butadiene, isoprene and chloroprene in rats and mice. Chem Biol Interact 135–136:27–42
94. Walker VE, Walker DM, Meng Q, McDonald JD, Scott BR, Selikop SK, Claffey DJ, Upton PB, Powley MW, Swenberg JA, Henderson RF, Committee HR (2009) Genotoxicity of 1,3-butadiene and its epoxy intermediate. Res Rep Health Eff Inst 144:3–79
95. Koturbash I, Scherhag A, Sorrentino J, Sexton K, Bodnar W, Tryndyak V, Latendresse JR, Swenberg JA, Beland FA, Pogribny IP, Rusyn I (2011) Epigenetic alterations in liver of C57BL/6J mice after short-term inhalational exposure to 1,3-butadiene. Environ Health Perspect 119:635–640
96. Schotta G, Lachner M, Sarma K, Ebert A, Sengupta R, Reuter G, Reinberg D, Jenuwein T (2004) A silencing pathway to induce H3-K9 and H4-K20 trimethylation at constitutive heterochromatin. Genes Dev 18:1251–1262
97. Martens JH, O'Sullivan RJ, Braunschweig U, Opravil S, Radolf M, Steinlein P, Jenuwein T (2005) The profile of repeat-associated histone lysine methylation states in the mouse epigenome. EMBO J 24:800–812
98. Jenuwein Y (2006) The epigenetic magic of histone lysine methylation. FEBS J 273:3121–3135
99. Koturbash I, Scherhag A, Sorrentino J, Sexton K, Bodnar W, Swenberg JA, Beland FA, Pardo-Manuel Devillena F, Rusyn I, Pogribny IP (2011) Epigenetic mechanisms of mouse

interstrain variability in genotoxicity of the environmental toxicant 1,3-butadiene. Toxicol Sci 122:448–456
100. Rusyn I, Gatti DM, Wilshire T, Kleeberger SR, Threadgill DW (2010) Toxicogenomics: population-based testing of drug and chemical safety in mouse models. Pharmacogenomics 11:1127–1136
101. IARC Monographs on the Evaluations of Carcinogenic Risks to Humans (2011) A review of human carcinogens, vol 100, Part A: Pharmaceuticals. IARC, Lyon
102. Li S, Washburn KA, Moore R, Uno T, Teng C, Newbold RR, McLachlan JA, Negishi M (1997) Developmental exposure to diethylstilbestrol elicits demethylation of estrogen-responsive lactoferrin gene in mouse uterus. Cancer Res 57:4356–4359
103. Li S, Hansman R, Newbold R, Davis B, McLachlan JA, Barrett JC (2003) Neonatal diethylstilbestrol exposure induces persistent elevation of c-fos expression and hypomethylation in its exon-4 in mouse uterus. Mol Carcinog 38:78–84
104. Tang WY, Newbold R, Mardilovich K, Jefferson W, Cheng RY, Medvedovic M, Ho SM (2008) Persistent hypomethylation in the promoter of nucleosomal binding protein 1 (Nsbp1) with overexpression of Nsbp1 in mouse uteri neonatally exposed to diethylstilbestrol or genistein. Endocrinology 149:5922–5931
105. Sato K, Fukata H, Kogo Y, Ohgane J, Shiota K, Mori C (2009) Neonatal exposure to diethylstilbestrol alters expression of DNA methyltransferases and methylation of genomic DNA in the mouse uterus. Endocr J 56:131–139
106. Hsu PY, Detherage DE, Rodriguez BA, Liyanarachchi S, Weng YI, Zuo T, Liu J, Cheng AS, Huang TH (2009) Xenoestrogen-induced epigenetic repression of microRNA-9-3 in breast epithelial cells. Cancer Res 69:5936–5945
107. Lehmann U, Hasemeier B, Christgen M, Müller M, Römermann D, Länger F, Kreipe H (2008) Epigenetic inactivation of microRNA gene has-mir-9-1 in human breast cancer. J Pathol 214:17–24
108. Jordan VC (2006) Tamoxifen (ICI146,474) as a target therapy to treat and prevent breast cancer. Br J Pharmacol 147:S269–S276
109. Shang Y (2006) Molecular mechanisms of oestrogen and SERMs in endometrial carcinogenesis. Nat Rev Cancer 6:360–368
110. Wu H, Chen Y, Liang J, Shi B, Wu G, Zhang Y, Wang D, Li R, Yi X, Zhang H, Sun L, Shang Y (2005) Hypomethylation-linked activation of PAX2 mediates tamoxifen-stimulated endometrial carcinogenesis. Nature 438:981–987
111. Wogan GN (1997) Review of the toxicology of tamoxifen. Semin Oncol 24:S87–S97
112. Phillips DH (2001) Understanding the genotoxicity of tamoxifen? Carcinogenesis 22:839–849
113. Gamboa da Costa G, McDaniel-Hamilton LP, Heflich RH, Margues MM, Beland FA (2001) DNA adduct formation and mutant induction in Sprague–Dawley rats treated with tamoxifen and its derivatives. Carcinogenesis 22:130701315
114. Tryndyak VP, Muskhelishvili L, Kovalchuk O, Rodriguez-Juarez R, Churchwell MI, Ross SA, Beland FA, Pogribny IP (2006) Effect of long-term tamoxifen exposure on genotoxic and epigenetic changes in rat liver: implications for tamoxifen-induced hepatocarcinogenesis. Carcinogenesis 27:1713–1720
115. Tryndyak VP, Kovalchuk O, Muskhelishvili L, Montgomery B, Rodriguez-Juarez R, Melnyk S, Ross SA, Beland FA, Pogribny IP (2007) Epigenetic reprogramming of liver cells in tamoxifen-induced rat hepatocarcinogenesis. Mol Carcinog 46:187–197
116. Pogribny IP, Tryndyak VP, Boyko A, Rodriguez-Juarez R, Beland FA, Kovalchuk O (2007) Induction of microRNAome deregulation in rat liver by long-term tamoxifen exposure. Mutat Res 619:30–37
117. Bachman AN, Phillips JM, Goodman JI (2006) Phenobarbital induces progressive patterns of GC-rich and gene-specific altered DNA methylation in the liver of tumor-prone B6C3F1 mice. Toxicol Sci 91:393–405
118. Phillips JM, Goodman JI (2009) Multiple genes exhibit Phenobarbital-induced constitutive active/androstane receptor-mediated DNA methylation changes during liver tumorigenesis and in liver tumors. Toxicol Sci 108:273–289

119. Lempiäinen H, Müller A, Brasa S, Teo SS, Roloff TC, Morawiec L, Zamurovic N, Vicart A, Funhoff E, Couttet P, Schübeler D, Grenet O, Marlowe J, Moggs J, Terranova R (2011) Phenobarbital mediates an epigenetic switch at the constitutive androstane receptor (CAR) target gene Cyp2b10 in the liver of B6C3F1 mice. PLoS One 6:e18216
120. Bucher JR, Shackelford CC, Haseman JK, Johnson JD, Kurtz PJ, Persing RL (1994) Carcinogenicity studies of oxazepam in mice. Fundam Appl Toxicol 23:280–297
121. Iida M, Anna CH, Hartis J, Bruno M, Wetmore B, Dubin JR, Sieber S, Bennett L, Cunningham ML, Paules RS, Tomer KB, Houle CD, Merrick AB, Sills RC, Devereux TR (2003) Changes in global gene and protein expression during early mouse liver carcinogenesis induced by non-genotoxic model carcinogens oxazepam and Wyeth-14,643. Carcinogenesis 24:757–770
122. Lahousse SA, Hoenerhoff M, Collins J, Ton TV, Massinde T, Olson D, Rebolosso Y, Koujitani T, Tomer KB, Hong HH, Bucher J, Sills RC (2011) Gene expression and mutation assessment provide clues of genetic and epigenetic mechanisms in liver tumors of oxazepam-exposed mice. Vet Pathol 48:678–699
123. Ferguson LR, Philpott M (2008) Nutrition and mutagenesis. Annu Rev Nutr 28:313–329
124. IARC Monographs on the Evaluations of Carcinogenic Risks to Humans (2002) Some traditional herbal medicines, some mycotoxins, naphthalene and styrene, vol 82. IARC, Lyon
125. Wild CP, Gong YY (2010) Mycotoxins and human disease: a largely ignored global health issue. Carcinogenesis 31:71–82
126. Knasmüller S, Bresgen N, Kassie F, Mersch-Sundermann V, Gelderblom W, Zöhrer E, Eckl PM (1997) Genotoxic effects of three Fusarium mycotoxins, fumonisin B1, moniliformin and vomitoxin in bacteria and primary cultures of rat hepatocytes. Mutat Res 391:39–48
127. Wang JS, Groopman JD (1999) DNA damage by mycotoxins. Mutat Res 424:167–181
128. Zhang YJ, Ahsan H, Chen Y, Lunn RM, Wang LY, Chen SY, Lee PH, Chen CJ, Santella RM (2002) High frequency of promoter hypermethylation of RASSF1A and p16 and its relationship to aflatoxin B1-DNA adduct levels in human hepatocellular carcinoma. Mol Carcinog 35:85–92
129. Zhang YJ, Chen Y, Ahsan H, Lunn RM, Chen SY, Santella RM (2003) Inactivation of the DNA repair gene O6-methylguanine-DNA methyltransferase by promoter hypermethylation and its relationship to aflatoxin B1-DNA adducts and the p53 mutations in hepatocellular carcinoma. Int J Cancer 103:440–444
130. Su H, Zhao J, Xiong Y, Xu T, Zhou F, Yuan Y, Zhang Y, Zhuang SM (2008) Large-scale analysis of the genetic and epigenetic alterations in hepatocellular carcinoma from Southeast China. Mutat Res 641:27–35
131. Zhao W, Liu H, Liu W, Wu Y, Chen W, Jiang B, Zhou Y, Xue R, Luo C, Wang L, Jiang JD, Liu J (2006) Abnormal activation of the synuclein-gamma gene in hepatocellular carcinomas by epigenetic alteration. Int J Oncol 28:1081–1088
132. Kouadio JH, Dano SD, Moukha S, Mobio TA, Creppy EE (2007) Effects of combinations of Fusarium mycotoxins on the inhibition of macromolecular synthesis, malondialdehyde levels, DNA methylation and fragmentation, and viability in Caco-2 cells. Toxicon 49:306–317
133. Marin-Kuan M, Cavin C, Delatour T, Schilter B (2008) Ochratoxin A carcinogenicity involves a complex network of epigenetic mechanisms. Toxicon 52:195–202
134. Hu W, Feng Z, Tang M (2003) Preferential carcinogen-DNA adduct formation at codons 12 and 14 in the human K-ras gene and their possible mechanisms. Biochemistry 42: 10012–10023
135. IARC Monographs on the Evaluations of Carcinogenic Risks to Humans (2011) A review of human carcinogens, vol 100, Part B: Biological agents. IARC, Lyon
136. Maekita T, Nakazawa K, Mihara M, Nakajima T, Yanaoka K, Iguchi M, Arii K, Kaneda A, Tsukamoto T, Tatematsu M, Tamura G, Saito D, Sugimura T, Ichinose M, Ushijima T (2006) High levels of aberrant DNA methylation in Helicobacter pylori-infected gastric mucosae and its possible association with gastric cancer risk. Clin Cancer Res 12:989–995
137. Nakajima T, Yamashita S, Maekita T, Niwa T, Nakazawa K, Ushijima T (2009) The presence of methylation fingerprint of Helicobacter pylori infection in human gastric mucosae. Int J Cancer 124:905–910

138. Niwa T, Tsukamoto T, Toyoda T, Mori A, Tanaka H, Maekita T, Ichinose M, Tatematsu M, Ushijima T (2010) Inflammatory processes triggered by Helicobacter pylori infection cause aberrant DNA methylation in gastric epithelial cells. Cancer Res 70:1430–1440
139. Shin CM, Kim N, Jung Y, Park JH, Kang GH, Kim JS, Jung HC, Song IS (2010) Role of Helicobacter pylori infection in aberrant DNA methylation along multistep gastric carcinogenesis. Cancer Sci 101:1337–1346
140. Nakajima T, Enomoto S, Yamashita S, Ando T, Nakanishi Y, Nakazawa K, Oda I, Gotoba T, Ushijima T (2010) Persistence of a component of DNA methylation in gastric mucosa after Helicobacter pylori eradication. J Gastroenterol 45:37–44
141. LeBaron MJ, Rasoulpour RJ, Klapacz J, Ellis-Hutchings RG, Hollnagel HM, Gollapudi BB (2010) Epigenetics and chemical safety assessment. Mutat Res 705:83–95
142. Huang YW, Kuo CT, Stoner K, Huang TH, Wang LS (2011) An overview of epigenetics and chemoprevention. FEBS Lett 585:2129–2136
143. Goodman JI, Augustine KA, Cunningham ML, Dixon D, Dragan YP, Falls JG, Rasoulpour RJ, Sills RC, Storer RD, Wolf DC, Pettit SD (2010) What do we need to know prior to thinking about incorporating an epigenetic evaluation into safety assessments? Toxicol Sci 116:375–381
144. Rasoulpour RJ, LeBaron MJ, Ellis-Hutchings RG, Klapacz J, Gpllapudi BB (2011) Epigenetic screening in product safety assessment: are we there yet? Toxicol Mech Methods 21:298–311
145. Kalari S, Pfeifer GP (2010) Identification of driver and passenger DNA methylation in cancer by epigenomic analysis. Adv Genet 70:277–308

Chapter 12
Blood-Derived DNA Methylation Markers of Cancer Risk

Carmen Marsit and Brock Christensen

Abstract The importance of somatic epigenetic alterations in tissues targeted for carcinogenesis is now well recognized and considered a key molecular step in the development of a tumor. Particularly, alteration of gene-specific and genomic DNA methylation has been extensively characterized in tumors, and has become an attractive biomarker of risk due to its specificity and stability in human samples. It also is clear that tumors do not develop as isolated phenomenon in their target tissue, but instead result from altered processes affecting not only the surrounding cells and tissues, but other organ systems, including the immune system. Thus, alterations to DNA methylation profiles detectable in peripheral blood may be useful not only in understanding the carcinogenic process and response to environmental insults, but can also provide critical insights in a systems biological view of tumorigenesis. Research to date has generally focused on how environmental exposures alter genomic DNA methylation content in peripheral blood. More recent work has begun to translate these findings to clinically useful endpoints, by defining the relationship between DNA methylation alterations and cancer risk. This chapter highlights the existing research linking the environment, blood-derived DNA methylation alterations, and cancer risk, and points out how these epigenetic alterations may be contributing fundamentally to carcinogenesis.

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12.1 Introduction

Epigenetic alterations within cells that give rise to tumors are believed to be causal contributors to the development of malignancy [27, 38]. The most widely studied epigenetic mechanism in cancer is DNA methylation and it is well recognized that cancer cells concomitantly exhibit both gene-specific increases in DNA methylation and genome-wide hypomethylation compared to their normal tissue counterparts. Because DNA methylation is tissue-specific, perhaps it is no surprise that a multitude of studies seeking to detect tumor-specific DNA methylation for early detection/diagnosis have used cell-free fractions (serum, plasma) of peripheral blood. Studies measuring DNA methylation in serum and plasma aim to reduce the potential noise contributed by leukocyte methylation patterns in whole blood and to specifically detect tumor-derived DNA methylation. However, interindividual variability in leukocyte methylation patterns may be—akin to genetic variation—related to an individual's cancer risk while acquired alterations to leukocyte methylation may represent both a cause and consequence of carcinogenesis in solid tissues. As new measurement technologies and analytic strategies are being developed, and there is an improved understanding of the contribution of the immune system to solid tumor development, there may be great utility in peripheral blood methylation analysis for predicting cancer risk.

This chapter will cover evidence from human studies that peripheral blood DNA methylation states can inform cancer risk. First, investigations of repetitive element and global DNA methylation will be presented. Then, epimutation and gene-specific methylation markers of cancer risk will be discussed, followed by more recent and larger-scale investigations of blood methylation and cancer risk. Notably, as mentioned above, cancer epigenetics includes a large body of research on cell-free (plasma, serum) DNA methylation for diagnostic and prognostic purposes that is not within this chapter's scope. To end, potential mechanisms underlying the basis for blood-based methylation markers of cancer risk, and future directions for this avenue of research will be covered.

12.2 Repetitive Elements, Global Methylation, and Cancer Risk

12.2.1 Introduction to Global Methylation and Repetitive Elements

While the classic example of altered DNA methylation in cancer would likely describe promoter hypermethylation-induced gene silencing of a tumor suppressor gene, before this phenomenon was understood it was recognized that tumors are heavily hypomethylated relative to their normal tissue counterparts. In contrast to tumor suppressor gene promoters, moderately to highly repetitive,

non-coding sequences of the genome are normally methylated [26, 63]. Indeed, generally non-specific methylation of repeat and non-coding elements is considered an important part of normal development, cellular differentiation, and X-chromosome inactivation. Hence, changes in this methylation can lead to specific human disease states including cancer. In fact, genomic or global hypomethylation is now thought to occur early in tumorigenesis, including in pre-cancerous lesions [60, 66, 67], and may promote cancer development by contributing to genomic instability.

A few studies have directly assessed the relationship between total genomic methyl-cytosine in blood and cancer risk. Pufulete and colleagues measured genome-wide reduction in 5-methylcytosine content with a (relatively insensitive) ^{3}H-thymidine incorporation assay in peripheral blood lymphocytes and found that hypomethylation significantly increased risk for colon adenoma and indicated a trend in risk of colon cancer [60]. In an investigation of colorectal adenoma among women, Lim et al. measured total genomic leukocyte methylation utilizing DNA digestion followed by liquid chromatography and mass spectrometry for quantitation in 230 cases and controls. When setting the referent group as the women in the *lowest tertile of methylation*, women in the second tertile had a reduced risk of colorectal adenoma (odds ratio (OR): 0.72, 95% CI: 0.34–1.52), and women in highest methylation tertile had a significantly decreased risk of colorectal adenoma (OR: 0.17, 95% CI: 0.06–0.49) [47]. Around the same time, a hospital-based case–control study in Spain found that reduced total percent methyl-cytosine content (using high-performance capillary electrophoresis, *HpaII* digestion, and densitometry) was significantly associated with bladder cancer risk [54]: compared to the quartile of subjects with the highest percent 5-methyl-cytosine, the adjusted OR for subjects in the lowest quartile of methylation was 2.67 (95% CI: 1.8–4.0). Further, when stratifying by smoking status, global hypomethylation was a strong risk factor for bladder cancer in never smokers (OR: 6.4, 95% CI: 2.4–17.2).

Early links between genomic hypomethylation and pathogenesis generated great interest in developing additional methods to determine global DNA methylation. Total genomic methyl-cytosine content can be directly measured, though large amounts of substrate and highly specialized equipment are required. In the mid 1990s, founded on the basis of chemical modification of DNA with sodium bisulfite, a PCR-based method for measuring DNA methylation was developed: methylation-specific PCR (MSP) [30]. Later, a quantitative version of MSP known as MethyLight was developed in Peter Laird's lab [21]. Using MethyLight to measure *LINE-1*, *Alu*, and satellite element repeats, Weisenberger et al. showed that methylation of repetitive elements were reasonably well correlated with total methyl-cytosine content [74]. Around the same time, the first report of bisulfite sequencing for *LINE-1* and *Alu* elements was published and was claimed as a simple method to estimate global DNA methylation [80]. Alone, *LINE-1* and *Alu* elements comprise about 30% of the human genome, making them an attractive target for a surrogate measure of global methylation [80]. With these more accessible methods to measure "global methylation", many groups began evaluating global methylation. As a result, the term "global methylation" lost its specific meaning

and started being used to describe any of these assays even though their measures are potentially non-comparable.

Because repetitive elements such as *LINE-1* and *Alu* are used to signify global methyl-cytosine content, it is important to clarify what these elements are and to point out potential drawbacks of using these as surrogate measures of global methylation. Long interspersed nuclear elements (*LINE-1*) and short interspersed nuclear elements (SINEs, which include *Alu* elements and mammalian interspersed repeats (MIR)), and long terminal repeats (LTRs) are retrotransposons. Collectively, with tandem repeats such as satellite elements (SAT), LINE, SINE, and LTR retrotransposons comprise approximately half of the human genome. The majority of these elements are evolutionary remnants that are truncated or mutated and even if transcribed would have no phenotype. For instance, there are approximately 500,000 *LINE-1* elements in the genome; very few of these are full-length (6 kb) complete with an internal RNA polymerase II promoter in the 5′ UTR, two open reading frames that encode an RNA-binding protein and elements for retrotransposon activity, and a 3′ UTR with a polyadenylation signal [17]. Unlike *LINE-1*, *Alu* elements use an internal RNA polymerase III promoter and lack any coding sequence. For retrotransposition, *Alu* elements require the retrotransposon machinery encoded by *LINE-1* elements [19]. LTRs are considered endogenous retroviruses, and with over 400,000 copies, these repeat elements account for 8% of the human genome [43]. Lastly, satellite repeated sequences (SAT) are small DNA transposons that are the oldest type of transposable element, having arisen as a result of simple repeat amplification [39, 43].

Because repeat elements can have transposition activity, largely outnumber coding genes and make up a large fraction of the genome, it is critical that they are appropriately regulated. Hence, in normal cells repeat elements are maintained as silenced with relatively high levels of DNA methylation in their promoter regions. However, if methylation is lost at repeat elements they may be re-expressed and insert into various regions of the genome, possibly leading to the inactivation of tumor suppressor genes, or activation of oncogenes, thereby contributing to cancer as well as other human diseases [18, 41].

12.2.2 Satellite Elements and Long Terminal Repeats

Although satellite elements and long terminal repeats are numerous and make up a considerable portion of the human genome, their potential role in carcinogenesis remains understudied. Nonetheless, initial investigations into LTR repeats in tumors have indicated that inappropriate activation of LTR repeats is linked to cancers. The methylation status of one type of LTR, the endogenous retrovirus type K (HERV-K) was hypomethylated in bladder tumor tissue compared to normal bladder [23]. Similarly, in a small number of ovarian tumors, HERV-W was hypomethylated compared to non-tumor tissue [53]. More recently, an examination of satellite repeat expression in pancreatic ductal adenocarcinomas revealed that HSATII

transcripts were highly cancer-specific, alpha satellite transcripts were abundantly expressed, and that increased satellite expression in these cancers was likely due to loss of methylation [71]. Unfortunately, to our knowledge there have not yet been any studies examining methylation of satellite or LTRs in blood to test for association with risk of cancer. However, as large-scale sequencing efforts continue, non-coding elements are becoming better annotated and may allow for better-informed approaches to investigate the potential role of satellite and LTR repeat methylation in blood as it relates to cancer risk [1].

12.2.3 Long Interspersed Nuclear Elements and Alu elements

Using bisulfite pyrosequencing assays, a number of studies on *LINE-1* methylation in human peripheral blood have now been conducted. First, it is interesting to note that there are several studies investigating the association of *LINE-1* methylation in blood DNA with exposures that are etiologically relevant to human cancers. Examples of exposures that are associated with *LINE-1* hypomethylation include benzene [10], particulate matter including traffic particles [4, 68], polycyclic aromatic hydrocarbons [58], and persistent organic pollutants [62].

One of the first case–control studies of cancer to measure *LINE-1* methylation in blood was conducted in head and neck squamous cell carcinoma (HNSCC) [36]. Hsiung et al. measured *LINE-1* methylation with a modified version of combined bisulfite restriction analysis in over 800 HNSCC cases and controls. The between-subject variability in *LINE-1* methylation ranged from 54 to 87%, with a significant ($P<0.002$) increase in the *LINE-1* methylation in males compared to females, and significant increases in *LINE-1* methylation associated with positive HPV16 antibody serology and for subjects of non-Caucasian race compared to Caucasians ($P<0.02$ and $P<0.03$, respectively). In cases, controlling for age, gender, race, lifetime average drinks per week, and HPV16 serology, dietary folate in the lowest tertile, compared to the upper two tertiles, had a borderline significant reduction in *LINE-1* methylation. Similarly, subjects with the *MTHFR* 677 variant had a significant ($P<0.04$) reduction in *LINE-1* methylation; whereas, among cases, smoking was significantly associated ($P<0.04$) with increased *LINE-1* methylation. With respect to risk of HNSCC, patients in the lowest tertile of *LINE-1* methylation had a significant relative risk of HNSCC (OR: 1.6, 95% CI: 1.1–2.4), while those in the mid tertile showed an elevated OR of 1.3 (95% CI: 0.9–2.0) when controlling for age, gender, race, smoking, drinking, and HPV16 serology. Across tertiles there was a significant trend ($P<0.03$) for increased HNSCC risk with lower *LINE-1* methylation, and suggested that epigenetic variation, in this case extent of repetitive region methylation, is associated with disease risk [36].

In a study of breast cancer risk, Choi et al. measured *both* total methyl-cytosine content and *LINE-1* methylation in blood DNA from cases and controls [15]. With 176 cases and 173 controls, the authors first measured methyl-cytosine content and *LINE-1* methylation in a pilot subset of 19 cases and 18 controls, and found that

cases had significantly reduced methyl-cytosine content ($P=0.001$) compared to controls, whereas *LINE-1* methylation was not associated with case status or correlated ($r=-0.2$, $P=0.23$). Based on the results from the pilot cases and controls, the remaining cases and controls were evaluated for total methyl-cytosine only. Among several demographic factors examined (including age, race, BMI, smoking, parity, and menopausal status), high alcohol intake (>median) was the only factor significantly associated with reduced methyl-cytosine, and this was true in each of the case ($P<0.04$) and control groups ($P<0.04$). Further, among all cases and controls total methyl-cytosine content in blood DNA was significantly lower in cases than controls: when compared to women in the highest tertile of methylation, women in the lowest tertile of methylation had a significantly increased risk of breast cancer (OR: 2.9, 95% CI: 1.7–4.9). Despite the association between methyl-cytosine levels and alcohol intake, alcohol consumption did not affect the association between methyl-cytosine content and breast cancer risk. However, when stratifying on demographic and lifestyle factors, the authors found that risk was further increased by lower methyl-cytosine content in women with a family history of disease, as well as among women who were never smokers.

Studying the risk of gastric cancer in relation to repeat element methylation, Hou et al. used pyrosequencing and measured both *LINE-1* and *Alu* methylation in blood DNA from 302 gastric cancer cases and 421 age- and gender-matched controls [35]. This population-based case–control study enrolled participants from Warsaw, Poland. Methylation data were stratified into tertiles and in an analysis adjusted for age, sex, education level, smoking, and alcohol there were borderline significant associations between reduced methylation and gastric cancer risk for *LINE-1* (OR: 1.4, 95% CI: 0.9–2.0) and *Alu* (OR: 1.3, 95% CI: 0.9–1.9). Yet, in stratified analyses the association between *LINE-1* hypomethylation and gastric cancer risk was stronger for individuals with a family history of disease (OR: 3.1, 95% CI: 1.4–7.0), current drinkers of alcohol (OR: 1.9, 95% CI: 1.0–3.6), current smokers (OR: 2.3, 95% CI: 1.1–4.6), subjects who rarely or never consumed fruit, as well as carriers of either of two polymorphisms in 5-methyltetrahydrofolate-homocysteine methyltransferase reductase (*MTRR*). However, associations between *LINE-1* methylation and cancer risk were not modified by sex, infection with *Helicobacter pylori*, or intake of protein, vitamin B6, or folate.

An investigation of *LINE-1* blood DNA methylation and bladder cancer risk in a population-based case–control study in New Hampshire also indicated that reduced *LINE-1* methylation is associated with cancer risk [76]. Among 285 cases and 465 controls, *LINE-1* methylation values from bisulfite pyrosequencing ranged from 58 to 92%. Comparing subjects in the lowest methylation decile to all other subjects, controlling for age, gender, and smoking status indicated a significantly increased risk of bladder cancer for the lowest decile subjects (OR: 1.8, 95% CI: 1.1–2.9). In addition, these authors stratified their analysis by invasive and non-invasive disease and found that the lowest decile of *LINE-1* methlyation was associated with a significantly increased risk of non-invasive disease, but not invasive disease. Similar to the results from Hsiung et al., which showed that males had significantly higher *LINE-1* methylation levels, Wilhelm et al. stratified their analysis by gender and

found the association between *LINE-1* hypomethylation and bladder cancer to be stronger in women than in men (OR_{women}: 2.5, 95% CI: 1.2–5.2; OR_{men}: 1.5, 95% CI: 0.8–2.7). Finally, recalling the studies of *LINE-1* methylation and environmental exposures with etiologic relevance to cancer, these authors showed a significant association between high exposure to arsenic and reduced *LINE-1* methylation in control subjects.

Along with the studies of global and repeat element hypomethylation and bladder cancer risk in Europeans from Moore et al., and in American Caucasians from Wilhelm et al., a third study in Chinese subjects has also been published [13]. Among 510 cases and 528 controls from a case–control study based in Shanghai China, *LINE-1* methylation values from bisulfite pyrosequencing ranged from 73 to 93%. Notably, the low-end of *LINE-1* methylation in these subjects was higher than studies of Caucasians, 73% compared to 58% from [36], and 53% from [76]. Nonetheless, similar to previous research, men had significantly higher *LINE-1* methylation than women ($P = 0.004$), and perhaps some of the disparity in low-range methylation among studies may be attributable to a higher prevalence of men in this study (77%) compared to the studies from Hsiung et al. (69%) and Wilhelm et al. (69%). Among all cases and controls in adjusted models comparing tertiles of *LINE-1* methylation, the lowest methylation tertile compared to the highest revealed an elevated risk of bladder cancer that did not reach statistical significance (OR: 1.3, 95% CI: 0.95–1.7). However, when stratifying by smoking status, never smokers in the lowest tertile of *LINE-1* methylation had a significantly increased risk of bladder cancer (OR: 1.9, 95% CI: 1.2–3.1). Further, lifelong non-smokers with *GSTM1* and/or *GSTT1* null genotypes had an even higher risk of bladder cancer (OR: 2.4, 95% CI: 1.3–4.1).

12.2.4 Challenges and Caveats

Despite recent advances in measuring repetitive element methylation with bisulfite pyrosequencing, a full understanding of the biology of these elements is still lacking, and there are technical limitations that should be carefully considered. Although reported in numerous studies, the relatively greater extent of methylation of *LINE-1* elements in men compared to women is not understood, and may represent fundamental differences that need to be further explored. Though the CpGs targeted for methylation measurement in pyrosequencing assays are generally 3–6 CpG sites in the 5′ UTR, because it is unclear how many of the 500,000 *LINE-1* elements are full length (6 kb) it is not known *how many* copies of *LINE-1* elements are actually being measured in any given individual. From an evolutionary standpoint the newer *LINE-1* sequences are more likely to be fully intact, though the 5′ end of the repeat can be deleted and it is not yet known what the prevalence of these deletions are. In addition, the total number of newer *LINE-1* sequence elements is polymorphic in the population. Together, these issues make it difficult if not impossible to know how many *LINE* elements are being measured and whether the number is similar across samples

or individuals. So, although a number of studies are now identifying and confirming associations between epigenetic alterations to these elements and cancer, the biological mechanism towards carcinogenesis that these observations represent is not understood. A more in-depth treatment of the challenges associated with repeat element and global methylation measures is available in Nelson et al. [56].

12.3 Gene-Specific Methylation and Epimutation

12.3.1 Epimutation

There is growing recognition that gene-specific soma-wide and/or germline DNA methylation, often called epimutation, can predispose individuals to cancer [20, 31]. Initial work in the area of epimutation identified changes to gene imprinting status that was phenotypically equivalent to disease attributable to genetic alterations. For example, Wilm's tumor can derive from inherited mutation in the *IGF2* gene leading to a change in the imprinting status and therefore the biallelic expression of this gene. A change in the DNA methylation status of the maternally imprinted allele without change to the underlying sequence can lead to loss of imprinting (LOI) at the *H19/IGF2* locus, which similarly results in biallelic expression and risk for Wilm's tumor [52, 65]. A number of other imprinting disorders have been identified and have been linked not only with genetic etiology but also epimutations, including Beckwith–Wiedeman, Silver–Russel, Prader–Willi, and Angelman syndromes [28, 57]. Epimutations resulting in LOI are relatively rare due to the scarcity of imprinted genes in the genome.

Epimutations also have been shown to occur in the context of biallelic expression and such epimutations have been linked to cancer. For example, 37% of individuals presenting with Cowden's syndrome or with Cowden-like features, but without genetic alteration to the *PTEN* gene, harbor germline allele-specific DNA methylation upstream of *PTEN*. This leads to reduced expression of the *KILLIN* gene and a greatly increased risk of breast and renal cancer [7]. Similarly, a subset of familial breast and ovarian cancer without *BRCA1* or *BRCA2* mutation is linked to mosaic epimutation of *BRCA1* [78], and hereditary non-polyposis colorectal cancer (HNPCC) without germline mutation is observed with allele-specific mosaic methylation of *MLH1* or *MSH2* [14, 32–34]. In some cases, multiple generations of individuals within these HNPCC families could be identified [33, 55]. In other cases, the aberrant methylation present in the affected individuals germline (i.e., spermatozoa), could not be identified in family members, suggesting a potential de novo germline or early embryonic event [32, 66, 67]. This lack of a consistent direct inheritance of the epimutation itself, but the potential for familial transmission suggests that it may, in fact, be a predisposition to epimutation in general that is truly being inherited.

These are examples of highly penetrant but rare epimutation in genes known to contribute to specific disease. Such findings are analogous to decades of work in

genetic susceptibility to cancer which originated with studies of highly penetrant, rare mutations leading to rare genetic disease and provided the profound understanding of the key genes involved in tumorigenesis. As genetic susceptibility studies later evolved into the investigation of more common, polymorphic variation associated with sporadic cancer, so has the study of epimutation begun to move beyond these rare variants to studies of common epigenetic variability association with common disease.

12.3.2 Gene-specific methylation

A number of investigations of peripheral blood DNA methylation have focused on the examination of candidate tumor suppressor gene methylation, taking cues from the alterations detected in targeted solid tissues to define candidates. Often these studies are based upon the assumption that the alterations driving carcinogenesis in a target tissue will be identified in blood and potentially other non-target tissues, although the somatic nature of methylation would argue against such assumption. Nevertheless, there is a large and growing literature utilizing candidate approaches to examine in populations single and multi-gene panels of candidate tumor suppressor genes in peripheral blood as markers of solid tumor risk.

Wong and colleagues [77] measured *CDKN2B* methylation in buffy coat DNA from 15 hepatocellular carcinoma (HCC) patients, 15 patients without cancer but with chronic hepatitis/cirrhosis, and 20 healthy controls with MSP. Among the 15 HCC patients, eight had *CDKN2B* methylation buffy coat DNA, whereas none of the healthy controls or individuals with hepatitis/cirrhosis had methylated *CDKN2B*. Further, the eight HCC patients with *CDKN2B* methylation in blood DNA also had *CDKN2B* methylation in their tumor tissue.

In colon cancer, Ally and colleagues measured methylation in blood DNA from 27 cases, 30 individuals with adenoma, 16 with hyperplastic polyps, and 57 disease-free controls [2]. Using bisulfite pyrosequencing the authors examined seven CpG sites in the promoter region of the estrogen receptor alpha gene (*ESR1*) and across all subjects the methylation of *ESR1* ranged from 0 to 13% (median, 4.3%). Across disease groups there was not a difference in *ESR1* methylation ($P > 0.05$). However, *ESR1* methylation was 60% lower in peripheral blood samples than in normal colonic tissues. Further, the authors observed a correlation between colonic tissue methylation and blood methylation of *ESR1* that was independent of age, gender, disease status, and body mass index (BMI).

Another interesting example comes from studies of *BRCA1* methylation in peripheral blood DNA of cases with breast cancer. Germline *BRCA1* mutations are related to the development of hereditary breast cancers, which account for ~5–10% of cases, and generally present at a younger age and with a more aggressive phenotype. Although mRNA levels of *BRCA1* have been shown to be reduced in a subset of sporadic breast cancer cases [70], mutations of *BRCA1* in sporadic breast cancer are rarely (if ever) present [25, 42]. As *BRCA1* is known to contain a CpG island in

its promoter region, it was hypothesized that DNA methylation-induced silencing may be present in a subset of sporadic breast tumors. It has been shown that up to 44% of sporadic breast tumors are methylated at *BRCA1*, and tumors with methylated *BRCA1* share pathologic features of tumors with mutated *BRCA1* [9, 12]. In 2008, Snell and colleagues measured *BRCA1* methylation in blood DNA from seven familial breast cancer cases that did not have detectable *BRCA1* or *BRCA2* mutations and seven age-matched controls. These authors used several techniques to measure *BRCA* methylation including MethyLight, methylation-sensitive high-resolution melting, and a digital version of the latter. Three of the seven patients studied had >0% methylation of *BRCA1* in peripheral blood DNA and the corresponding tumors were found to be heavily methylated. Among the control subjects, six of seven had no detectable *BRCA1* methylation, only one subject had low-level *BRCA1* (0.1%) methylation [64].

Al-Moghrabi et al. measured *BRCA1* methylation with MSP in 47 breast tumor tissues, and in peripheral blood from seven breast cancer cases and 73 disease-free controls [3]. Among tumor tissues, 13 (27%) had *BRCA1* methylation. Similarly, two (29%) of the seven blood samples from breast cancer cases were methylated at *BRCA1*. Further, there was a significant association between a younger age at diagnosis (≤40 years) and *BRCA1* methylation ($P < 0.004$). However, 8 of the 73 (11%) disease-free controls also had *BRCA1* methylation in blood, which was not a significantly lower prevalence of *BRCA1* methylation than in cases. Nonetheless, with only seven breast cancer cases providing blood DNA, this study may have been underpowered to detect a significant association between *BRCA1* methylation in blood and risk of breast cancer. In addition, it is possible that the disease-free women with *BRCA1* methylation in blood are still at an increased risk of developing breast cancer.

Iwamoto and colleagues presented a similar study of *BRCA1* methylation in peripheral blood DNA from 200 cases and 200 controls [37]. In peripheral blood samples from cases and controls, *BRCA1* methylation was measured with quantitative MSP and found to be associated with a significantly increased risk of breast cancer (OR: 1.7, 95% CI: 1.01–2.96), controlling for age, family history, age at menarche, parity, menopausal status, and BMI. In addition, these authors also measured *BRCA1* methylation in 162 breast tumors where 31 (19%) were *BRCA1* methylation-positive and these tumors were more likely to be estrogen receptor and progesterone receptor-negative. When stratifying by presence of *BRCA1* methylation in tumors (and controlling for covariates above), peripheral blood methylation of *BRCA1* was highly associated with risk of developing *BRCA1* methylation-positive breast cancer (OR: 17.8, 95% CI: 6.7–47.1).

Blood DNA methylation of *BRCA1* in relation to the risk of ovarian cancer has also been reported. Bosivel and colleagues [11] measured blood DNA methylation of both *BRCA1* and *BRCA2* promoter regions in 51 ovarian cancer cases (without *BRCA* mutation) and 349 controls using quantitative analysis of methylated alleles. Although they did not observe an association between *BRCA2* methylation level and case status, these authors reported significantly *reduced BRCA1* methylation in ovarian cancer cases compared to controls. However, the implications of a significantly

hypomethylated *BRCA1* promoter region in association with ovarian cancer are somewhat counterintuitive and warrant further investigation.

In a case–control study of lung cancer, Li et al. measured methylation of the putative tumor suppressor gene *FHIT* in peripheral blood DNA samples from Han Chinese subjects with MSP [46]. Among 123 lung cancer cases, 42 (34%) had *FHIT* promoter methylation, whereas none of the 105 control subjects' blood DNAs were methylated, indicating a significantly increased risk of lung cancer associated with peripheral blood methylation of *FHIT* (OR: 2.3, 95% CI: 2.0–2.7). Additionally, these authors reported that blood methylation of *FHIT* was significantly associated with cases who had early stage (I) disease ($P < 0.05$), and not cases with high-stage (IV) disease.

12.3.3 Panels of candidate genes

Some groups have reported blood methylation data for panels of candidate genes. The heterogeneity in molecular alterations of specific tumor types could be motivation for studies that examine multiple gene-loci, and the results from Iwamoto et al. are apropos: peripheral blood methylation of *BRCA1* was highly associated with risk of developing *BRCA1* methylation-positive breast cancer [37]. Of course, within a particular tumor type, not every tumor will have the same repertoire of molecular alterations. Hence, a more comprehensive approach to study blood-based methylation markers of cancer risk would measure methylation of several genes known (or suspected) to be methylated in a moderate to high proportion of tumors.

One such study from Liu et al. used an approach directed at six genes on chromosome 3p because a previous report from these authors had demonstrated a CpG island methylator phenotype (CIMP) associated with genes on 3p in lung tumors [48, 49]. Here, the authors used peripheral blood DNA from 80 cases of non-small cell lung cancer (NSCLC) and 80 matched controls and measured methylation of six genes (*OGG1*, *RARB*, *SEMA3B*, *RASSF1A*, *BLU*, *FHIT*) on chromosome 3p with MSP. If at least three of these genes were methylated the sample was considered 3pCIMP+. The prevalence of methylation in blood DNA from cases was higher than controls for all genes except *FHIT* where the same number of cases and controls were methylated. Further, almost all case blood samples (78/80, 98%) had at least one methylated gene, whereas 78% (62/80) of control blood samples had at least one methylated gene. When comparing 3pCIMP status in cases and controls, 44% of NSCLC cases were 3pCIMP + and only 6% of control blood DNA samples were 3pCIMP + ($P < 0.001$). In a model adjusting for age, sex, and smoking status, subjects with 3pCIMP + blood DNA were at a significantly increased risk of NSCLC (OR: 12.8, 95% CU: 4.4–37.4) [49].

Another gene-panel approach to investigate the role of blood-based DNA methylation markers of lung cancer risk was recently published by Vineis and colleagues using nested cases and controls from the European Prospective Investigation into Cancer and Nutrition (EPIC) [72]. This group measured methylation of multiple

CpGs in five genes: *CDKN2A*, *RASSF1A*, *GSTP1*, *MTHFR*, and *MGMT* with a bisulfite pyrosequencing approach in 93 lung cancer cases and 99 controls. Stratifying pyrosequencing methylation data for each gene on the median, adjusted models revealed that increased *RASSF1A* methylation was associated with a significantly increased risk of lung cancer (OR: 1.9, 95% CI: 1.0–3.5), though none of the other genes, or combination thereof were associated with disease. The authors also reported that serum levels of B vitamins and one-carbon metabolites were associated with methylation; increased folate was associated with increased *RASSF1A* and *MTHFR* methylation, whereas increased methionine was associated with decreased *RASSF1A* methylation [72].

Prior to these works, a group in France published a comparison of blood DNA methylation of ten genes in a study of prostate cancer [61]. Using prostate cancer cases with disease relapse ($n=20$), patients without relapse ($n=22$), as well as control subjects ($n=22$), the authors measured methylation of ten genes; *RASSF1A*, *CDH1*, *APC*, *DAPK*, *MGMT*, *CDKN2A* (*p16* and *p14*), *GSTP1*, *RARB*, and *TIMP3* using quantitative MSP. Compared to all cases, methylation levels of all ten genes were lower in control subject blood DNA, and five were significantly lower; *DAPK* ($P=0.04$) *RASSF1A*, *GSTP1*, *APC*, and *RARB* (all $P<0.0001$).

An interesting final example of small gene-panel studies comes from Flanagan and colleagues who developed a tiling microarray with a methylation-sensitive enzyme-based approach to study 17 breast cancer susceptibility genes [22]. With the tiling array the authors took an unbiased approach to examining the promoter and gene-coding regions for the 17 candidate genes. In the pilot phase, 14 cases with bilateral breast cancer and 14 control subjects had their blood DNA methylation measured. Notably, the authors described 181 regions in the 17 genes analyzed that had significantly variable methylation ($P<0.001$) across all 28 individuals, and the majority of these regions were significantly closer (within 200 bp) to repetitive elements than would be expected ($P=7.4e-07$). As a follow up, the authors validate two regions of variable methylation 4 kb downstream of the *ATM* gene in 190 cases and 190 controls and observed significantly increased methylation of *ATM* variable region 2 in cases compared to controls ($P=0.002$). In an inter-quartile analysis of the methylation data from this same region, subjects in the highest quartile of methylation were at a significantly increased risk of breast cancer (OR: 3.2, 95% CI: 1.8–5.9) compared to subjects in the lowest quartile [22]. One of the key facets of this particular study is that unlike most other investigations, these authors did not restrict their methylation measurements to promoter regions and argues that future studies should consider the distribution of regions measured for methylation.

12.4 Larger Gene-Panels and Commercial Methylation Arrays

A separate class of studies that has undertaken larger-scale approaches (25 genes to genome-wide) to investigate blood-based markers of DNA methylation and cancer risk will be covered here. One such study from Widschwendter et al. used a

three-step approach to investigate blood DNA methylation and the risk of breast cancer [75]. First, these authors chose 49 estrogen receptor target (ERT) and polycomb group target (PCGT) genes and second, used MethyLight to measure methylation in 83 healthy post-menopausal women. Thirdly, based on the distribution of methylation in these individuals 25 of the 49 genes were selected for measurement in 353 cases and 730 controls. After controlling for age and family history of breast cancer, methylation of 5 of the 25 genes (*ZNF217*, *NEUROD1*, *SFRP1*, *TITF1* (officially *NKX2-1* as of 8/14/11), *NUP155*) was associated with a significantly increased risk of breast cancer (ORs range: 1.40–1.49, median OR: 1.48) [75]. This study provides further proof of principle for the utility of blood-based methylation markers of cancer risk. However, because methylation of five separate genes were independently associated with breast cancer, it would have been interesting to know whether an analytic approach that combined the methylation markers would have increased the effect estimate.

A similar study of small cell lung cancer (SCLC) risk from Wang et al. also used a multi-step approach to curate a group of genes measured for methylation in a small pilot group of cases and controls before expanding into additional cases and controls [73]. This study took advantage of recent technologic advances that allow for the simultaneous resolution of hundreds to hundreds of thousands of methylation events, providing an epigenotyping platform for rapid epigenetic profiling [8]. First, bisulfite-modified blood DNA from 44 cases and 44 controls was applied to the Illumina GoldenGate methylation array which measures 1,505 CpG sites associated with >800 cancer-related genes. Testing 1,332 CpGs (those with methylation states not associated with cancer treatment) the authors observed 62 CpG sites associated with 52 genes to be significantly associated with cases status (FDR $P<0.05$). To follow up, the authors chose nine of these 62 CpGs for validation by bisulfite pyrosequencing in 138 cases and 138 controls. Controlling for age, sex, and smoking history, the methylation status of the nine CpG sites collectively were able to correctly classify 86% of cases as being at a higher risk of SCLC. Further, when considering specific CpGs, for the risk of SCLC increased ~4-fold for each 5% decrease in *ERCC1* methylation (OR: 3.9, 95% CI: 2.0–6.1) and ~1.5-fold for each 5% decrease in *CSF3R* methylation (OR: 1.5, 95% CI: 1.1–2.0) [73].

A group from the Mayo Clinic in Minnesota also used a two-phase study and the GoldenGate array to study blood methylation and risk of cancer, though they focused on pancreatic cancer [59]. First, these authors measured blood DNA methylation with the array in 132 cases and 60 controls and reported 110 CpGs with significantly differential methylation between cases and controls (FDR $P<0.05$). Then, using analogous technology in a custom platform from Illumina (VeraCode), the top 96 CpGs associated with case control status were subjected to validation in a further 240 cases and 240 matched controls. Leveraging the potential of combining methylation measures a prediction model was built and included five CpG sites associated with five genes: *IL10*, *LCN2*, *ZAP70*, *AIM2*, and *TAL1*. Collectively, these five CpGs demonstrated good discrimination between pancreatic cancer cases and controls (c-statistic phase I = 0.85, phase II = 0.72) [59].

Teschendorff et al. published an investigation of blood methylation profiles to predict ovarian cancer using a more comprehensive array platform, the Illumina Infinium 27K array [69]. Following exclusions for batch effects and quality control, methylation array data from 148 controls, 113 pre-treatment, and 122 post-treatment cases from the UK Ovarian Cancer Population Study were included in the analysis. Comparing methylation among controls to pre-treatment cases, the authors identified 2,714 CpG sites that were significantly (FDR $P<0.05$) associated with ovarian cancer. Notably, among the top 50 CpGs, 87% were hypomethylated in cases compared to controls ($P=9e-09$). To construct a DNA methylation signature associated with ovarian cancer, these authors used a supervised approach to the data with 100 iterations of training and testing sets (each with 90 controls and 70 pre-*t* cases) and multivariate logistic regression. With these iterations and a cross-validation step, the top 100 CpG sites were determined to be an optimal number of CpG sites for their classifier. The performance of these 100 CpGs as a classifier for ovarian caner in a blinded test set was very good (AUC: 0.8, 95% CI: 0.74–0.87), and was validated in the post-treatment cases (AUC: 0.76, 95% CI: 0.72–0.81) [69].

In a New Hampshire population-based bladder cancer case–control study, Marsit et al. examined peripheral blood DNA methylation profiles using the Infinium 27K array. Using a novel, semi-supervised recursively partitioned mixture modeling (SS-RPMM) strategy [40] involving classifier training in a series of subjects consisting of 118 controls and 112 cases, and validation in an independent series of 119 controls and 111 cases, Marsit et al. identified a panel of 9 CpG loci whose profile of DNA methylation was significantly associated ($P<0.0001$) with bladder cancer [50]. Membership in any of the three classes of DNA methylation associated with risk demonstrated a 5.2-fold increased risk of bladder cancer (95% CI 2.8, 9.7), when controlled for subject age, gender, smoking status, and family history of bladder cancer. Notably, the methylation classes whose membership was predominantly bladder cancer cases had higher levels of mean methylation across the 9 CpG loci. Gene-set enrichment analysis of the loci most associated with bladder cancer demonstrated that transcription-factor binding sites related to immune modulation and forkhead family transcription were over-represented among regions whose methylation differed in bladder cases compared to controls. The key role of immune modulation in both aging and carcinogenesis, and particularly bladder carcinogenesis, lends mechanistic significance to these findings.

Using the same array platform and SS-RPMM analytical approach, the association between peripheral blood methylation profiles and HNSCC was assessed by Langevin et al. [44] in 96 HNSCC cases and 96 cancer-free control subjects. In this study, cases and controls were best differentiated by a methylation profile of six CpG loci (associated with *FGD4*, *SERPINF1*, *WDR39*, *IL27*, *HYAL2,* and *PLEKHA6*), and after adjustment for subject age, gender, smoking, alcohol consumption, and HPV16 serostatus, the AUC was 0.85 (95% CI: 0.76–0.92). Notably, the methylation classes whose membership was predominantly head and neck cancer cases had lower mean methylation across the 6 CpG loci. Although this is not yet adequate for use in clinical settings, these results further demonstrate the poten-

tial of DNA methylation measured in blood for development of non-invasive applications for detection of head and neck cancer and the utility of the proposed methods for the analysis of the array-based methylation data.

12.5 Mechanisms

Just as normal genetic variation is now understood to be associated with a predisposition to a vast array of human diseases [51], it is important to consider interindividual variation in tissue-specific DNA methylation to better understand the ability of this variation to inform disease risk. Epigenetic variation has been hypothesized to cause underlying differences in disease susceptibility among monozygotic twins, and young twin-pairs have been shown to be more epigenetically similar than older monozygotic twins [24]. The aging process and differences in environment have been hypothesized to influence clinically significant changes in methylation profiles as individuals accumulate varying exposures with age.

Marks of DNA methylation are entirely reprogrammed during in-utero development. This reprogramming, during the pre-implantation period, necessitates a rapid de-methylation of the genome, thought to be accomplished through an active process [29, 45], followed by appropriate, cell and tissue-specific methylation of the genome. The mechanisms through which these processes of de-methylation and reprogramming of the DNA methylation marks and particularly, the appropriate targeting of enzymes responsible for establishing those marks remains unclear. Importantly, epigenetic reprogramming during in-utero development constitutes a critical period during which environmental stimuli and insults can alter the establishment of cell-type-specific DNA methylation profiles and may constitute one point at which variation in methylation profiles is established. Therefore, alteration to epigenetic profiles has been posited as the molecular basis of the developmental origins of health and disease phenomenon, which links the environment (taken broadly) in-utero, with outcomes throughout the life course of the individual [5, 6].

Beyond the variation in DNA methylation profile which is established in-utero, additional variation may arise resulting from exposures and the environment encountered throughout life, or from the process of aging itself. Work from Christensen et al. [16] demonstrated that features of the patterns of age-associated methylation were conserved irrespective of tissue-type, suggesting a common mechanism or dysregulation to explain these alterations. Potential mechanisms include reduced fidelity of maintenance methyltransferases with aging leading to hypomethyation events. Although age-related methylation alterations may not functionally result in a pathologic process, drifts of normal epigenomes may nonetheless confer significantly increased risk of conversion to a pathologic phenotype by enhancing both the likelihood and frequency of subsequent methylation events that ultimately result in aberrant expression or altered genomic stability.

Particularly when considering profiles of methylation in a heterogeneous tissue sample such as blood, it should be recognized that the quantitative measure of

methylation truly represents the fraction of cells within the sampled population exhibiting a methyl-group at any CpG site. Therefore, differences in DNA methylation profiles could and likely do indicate aging or exposure-related changes to the underlying populations of cells comprising that mixture. In the case of blood these shifts may indicate changes to the profile of immune cells and thus alterations to the immune system permissive to or resulting from carcinogenesis. In fact, comparing *LINE1*, Sat2, and Alu methylation levels in whole blood, granulocytes, mononuclear cells, and lymphoblastoid lines with multiple methylation assays (MethyLight, luminometric methylation assay, and a methyl acceptance assay) Wu et al. have demonstrated differences in methylation dependent upon substrate and assay used [79]. As additional studies are conducted to identify differentially methylated regions among various leukocyte subtypes, it may soon be possible to identify proportional shifts in specific leukocyte subtypes that may contribute to cancer, or indicate immune response to an existing tumor.

12.6 Conclusions

The extent of variability of the cellular epigenome in non-pathologic tissues, particularly at gene promoter regions, remains a critical question; the amount of variation in genomic methylation across the population is not currently known. It is clear that epigenetic variability detectable in human blood is influenced, in part, by aging and exposures, and in turn, specific profiles of methylation in blood are associated with cancer risk (Fig. 12.1). The ease of collection of blood samples and the rapidly advancing technologies to assess DNA methylation in genomic DNA from this tissue make this an ideal focus of study for novel biomarkers of disease risk and of disease prognosis. Additionally, as we better understand functional consequences of altered methylation profiles, there will be an improved understanding at the systems level of the contribution of non-target tissues and systems on carcinogenesis, likely yielding novel approaches not only of diagnosis but treatment as well.

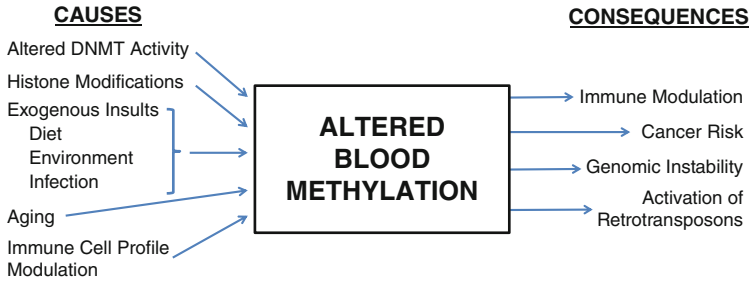

Fig. 12.1 Causes and consequences of altered blood DNA methylation

References

1. Alexander RP, Fang G et al (2010) Annotating non-coding regions of the genome. Nat Rev Genet 11(8):559–571
2. Ally MS, Al-Ghnaniem R et al (2009) The relationship between gene-specific DNA methylation in leukocytes and normal colorectal mucosa in subjects with and without colorectal tumors. Cancer Epidemiol Biomarkers Prev 18(3):922–928
3. Al-Moghrabi N, Al-Qasem AJ et al (2011) Methylation-related mutations in the BRCA1 promoter in peripheral blood cells from cancer-free women. Int J Oncol 39(1):129–135
4. Baccarelli A, Wright RO et al (2009) Rapid DNA methylation changes after exposure to traffic particles. Am J Respir Crit Care Med 179(7):572–578
5. Barker DJ (2004) Developmental origins of adult health and disease. J Epidemiol Community Health 58(2):114–115
6. Barker DJ (2004) The developmental origins of well-being. Philos Trans R Soc Lond B Biol Sci 359(1449):1359–1366
7. Bennett KL, Mester J et al (2010) Germline epigenetic regulation of KILLIN in Cowden and Cowden-like syndrome. JAMA 304(24):2724–2731
8. Bibikova M, Lin Z et al (2006) High-throughput DNA methylation profiling using universal bead arrays. Genome Res 16(3):383–393
9. Birgisdottir V, Stefansson OA et al (2006) Epigenetic silencing and deletion of the BRCA1 gene in sporadic breast cancer. Breast cancer research: BCR 8(4):R38
10. Bollati V, Baccarelli A et al (2007) Changes in DNA methylation patterns in subjects exposed to low-dose benzene. Cancer Res 67(3):876–880
11. Bosviel R, Michard E et al (2011) Peripheral blood DNA methylation detected in the BRCA1 or BRCA2 promoter for sporadic ovarian cancer patients and controls. Clin Chim Acta 412(15–16):1472–1475
12. Butcher DT, Rodenhiser DI (2007) Epigenetic inactivation of BRCA1 is associated with aberrant expression of CTCF and DNA methyltransferase (DNMT3B) in some sporadic breast tumours. Eur J Cancer 43(1):210–219
13. Cash HL, Tao L et al (2011) LINE-1 hypomethylation is associated with bladder cancer risk among nonsmoking Chinese. Int J Cancer 130(5):1151–1159
14. Chan TL, Yuen ST et al (2006) Heritable germline epimutation of MSH2 in a family with hereditary nonpolyposis colorectal cancer. Nat Genet 38(10):1178–1183
15. Choi JY, James SR et al (2009) Association between global DNA hypomethylation in leukocytes and risk of breast cancer. Carcinogenesis 30(11):1889–1897
16. Christensen BC, Houseman EA et al (2009) Aging and environmental exposures alter tissue-specific DNA methylation dependent upon CpG island context. PLoS Genet 5(8):e1000602
17. Cordaux R, Batzer MA (2009) The impact of retrotransposons on human genome evolution. Nat Rev Genet 10(10):691–703
18. Deininger PL, Batzer MA (1999) Alu repeats and human disease. Mol Genet Metab 67(3): 183–193
19. Dewannieux M, Esnault C et al (2003) LINE-mediated retrotransposition of marked Alu sequences. Nat Genet 35(1):41–48
20. Dobrovic A, Kristensen LS (2009) DNA methylation, epimutations and cancer predisposition. Int J Biochem Cell Biol 41(1):34–39
21. Eads CA, Danenberg KD et al (2000) MethyLight: a high-throughput assay to measure DNA methylation. Nucleic Acids Res 28(8):E32
22. Flanagan JM, Munoz-Alegre M et al (2009) Gene-body hypermethylation of ATM in peripheral blood DNA of bilateral breast cancer patients. Hum Mol Genet 18(7):1332–1342
23. Florl AR, Lower R et al (1999) DNA methylation and expression of LINE-1 and HERV-K provirus sequences in urothelial and renal cell carcinomas. Br J Cancer 80(9):1312–1321
24. Fraga MF, Ballestar E et al (2005) Epigenetic differences arise during the lifetime of monozygotic twins. Proc Natl Acad Sci USA 102(30):10604–10609

25. Futreal PA, Liu Q et al (1994) BRCA1 mutations in primary breast and ovarian carcinomas. Science 266(5182):120–122
26. Gama-Sosa MA, Wang RY et al (1983) The 5-methylcytosine content of highly repeated sequences in human DNA. Nucleic Acids Res 11(10):3087–3095
27. Gaudet F, Hodgson JG et al (2003) Induction of tumors in mice by genomic hypomethylation. Science 300(5618):489–492
28. Gicquel C, Rossignol S et al (2005) Epimutation of the telomeric imprinting center region on chromosome 11p15 in Silver-Russell syndrome. Nat Genet 37(9):1003–1007
29. Hajkova P, Erhardt S et al (2002) Epigenetic reprogramming in mouse primordial germ cells. Mech Dev 117(1–2):15–23
30. Herman JG, Graff JR et al (1996) Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci USA 93(18):9821–9826
31. Hitchins MP (2010) Inheritance of epigenetic aberrations (constitutional epimutations) in cancer susceptibility. Adv Genet 70:201–243
32. Hitchins M, Williams R et al (2005) MLH1 germline epimutations as a factor in hereditary nonpolyposis colorectal cancer. Gastroenterology 129(5):1392–1399
33. Hitchins MP, Wong JJ et al (2007) Inheritance of a cancer-associated MLH1 germ-line epimutation. N Engl J Med 356(7):697–705
34. Hitchins M, Owens S et al (2011) Identification of new cases of early-onset colorectal cancer with an MLH1 epimutation in an ethnically diverse South African cohort(dagger). Clin Genet 80(5):428–434
35. Hou L, Wang H et al (2010) Blood leukocyte DNA hypomethylation and gastric cancer risk in a high-risk Polish population. Int J Cancer 127(8):1866–1874
36. Hsiung DT, Marsit CJ et al (2007) Global DNA methylation level in whole blood as a biomarker in head and neck squamous cell carcinoma. Cancer Epidemiol Biomarkers Prev 16(1):108–114
37. Iwamoto T, Yamamoto N et al (2011) BRCA1 promoter methylation in peripheral blood cells is associated with increased risk of breast cancer with BRCA1 promoter methylation. Breast Cancer Res Treat 129(1):69–77
38. Jones PA, Laird PW (1999) Cancer epigenetics comes of age. Nat Genet 21(2):163–167
39. Jordan IK, Rogozin IB et al (2003) Origin of a substantial fraction of human regulatory sequences from transposable elements. Trends Genet 19(2):68–72
40. Koestler DC, Marsit CJ et al (2010) Semi-supervised recursively partitioned mixture models for identifying cancer subtypes. Bioinformatics 26(20):2578–2585
41. Kolomietz E, Meyn MS et al (2002) The role of Alu repeat clusters as mediators of recurrent chromosomal aberrations in tumors. Genes Chromosomes Cancer 35(2):97–112
42. Lancaster JM, Wooster R et al (1996) BRCA2 mutations in primary breast and ovarian cancers. Nat Genet 13(2):238–240
43. Lander ES, Linton LM et al (2001) Initial sequencing and analysis of the human genome. Nature 409(6822):860–921
44. Langevin SM, Koestler DC et al (2012) Peripheral blood DNA methylation profiles are predictive of head and neck squamous cell carcinoma: an epigenome-wide association study. Epigenetics 7(3):291–299
45. Lee J, Inoue K et al (2002) Erasing genomic imprinting memory in mouse clone embryos produced from day 11.5 primordial germ cells. Development 129(8):1807–1817
46. Li W, Deng J et al (2010) Association of 5′-CpG island hypermethylation of the FHIT gene with lung cancer in southern-central Chinese population. Cancer Biol Ther 10(10):997–1000
47. Lim U, Flood A et al (2008) Genomic methylation of leukocyte DNA in relation to colorectal adenoma among asymptomatic women. Gastroenterology 134(1):47–55
48. Liu Z, Zhao J et al (2008) CpG island methylator phenotype involving tumor suppressor genes located on chromosome 3p in non-small cell lung cancer. Lung Cancer 62(1):15–22
49. Liu Z, Li W et al (2010) CpG island methylator phenotype involving chromosome 3p confers an increased risk of non-small cell lung cancer. J Thorac Oncol 5(6):790–797

50. Marsit CJ, Koestler DC et al (2011) DNA methylation array analysis identifies profiles of blood-derived DNA methylation associated with bladder cancer. J Clin Oncol 29(9): 1133–1139
51. McCarthy MI, Hirschhorn JN (2008) Genome-wide association studies: potential next steps on a genetic journey. Hum Mol Genet 17(R2):R156–R165
52. McKay JD, Truong T et al (2011) A genome-wide association study of upper aerodigestive tract cancers conducted within the INHANCE consortium. PLoS Genet 7(3):e1001333
53. Menendez L, Benigno BB et al (2004) L1 and HERV-W retrotransposons are hypomethylated in human ovarian carcinomas. Mol Cancer 3:12
54. Moore LE, Pfeiffer RM et al (2008) Genomic DNA hypomethylation as a biomarker for bladder cancer susceptibility in the Spanish Bladder Cancer Study: a case–control study. Lancet Oncol 9(4):359–366
55. Morak M, Schackert HK et al (2008) Further evidence for heritability of an epimutation in one of 12 cases with MLH1 promoter methylation in blood cells clinically displaying HNPCC. Eur J Hum Genet 16(7):804–811
56. Nelson HH, Marsit CJ et al (2011) "Global methylation" in exposure biology and translational medical science. Environ Health Perspect 119(11):1528–1533
57. Netchine I, Rossignol S et al (2007) 11p15 imprinting center region 1 loss of methylation is a common and specific cause of typical Russell-Silver syndrome: clinical scoring system and epigenetic-phenotypic correlations. J Clin Endocrinol Metab 92(8):3148–3154
58. Pavanello S, Bollati V et al (2009) Global and gene-specific promoter methylation changes are related to anti-B[a]PDE-DNA adduct levels and influence micronuclei levels in polycyclic aromatic hydrocarbon-exposed individuals. Int J Cancer 125(7):1692–1697
59. Pedersen KS, Bamlet WR et al (2011) Leukocyte DNA methylation signature differentiates pancreatic cancer patients from healthy controls. PLoS One 6(3):e18223
60. Pufulete M, Al-Ghnaniem R et al (2003) Folate status, genomic DNA hypomethylation, and risk of colorectal adenoma and cancer: a case control study. Gastroenterology 124(5): 1240–1248
61. Roupret M, Hupertan V et al (2008) Promoter hypermethylation in circulating blood cells identifies prostate cancer progression. Int J Cancer 122(4):952–956
62. Rusiecki JA, Baccarelli A et al (2008) Global DNA hypomethylation is associated with high serum-persistent organic pollutants in Greenlandic Inuit. Environ Health Perspect 116(11): 1547–1552
63. Sano H, Imokawa M et al (1988) Detection of heavy methylation in human repetitive DNA subsets by a monoclonal antibody against 5-methylcytosine. Biochim Biophys Acta 951(1): 157–165
64. Snell C, Krypuy M et al (2008) BRCA1 promoter methylation in peripheral blood DNA of mutation negative familial breast cancer patients with a BRCA1 tumour phenotype. Breast Cancer Res 10(1):R12
65. Steenman MJ, Rainier S et al (1994) Loss of imprinting of IGF2 is linked to reduced expression and abnormal methylation of H19 in Wilms' tumour. Nat Genet 7(3):433–439
66. Suter CM, Martin DI et al (2004) Germline epimutation of MLH1 in individuals with multiple cancers. Nat Genet 36(5):497–501
67. Suter CM, Martin DI et al (2004) Hypomethylation of L1 retrotransposons in colorectal cancer and adjacent normal tissue. Int J Colorectal Dis 19(2):95–101
68. Tarantini L, Bonzini M et al (2009) Effects of particulate matter on genomic DNA methylation content and iNOS promoter methylation. Environ Health Perspect 117(2):217–222
69. Teschendorff AE, Menon U et al (2009) An epigenetic signature in peripheral blood predicts active ovarian cancer. PLoS One 4(12):e8274
70. Thompson ME, Jensen RA et al (1995) Decreased expression of BRCA1 accelerates growth and is often present during sporadic breast cancer progression. Nat Genet 9(4):444–450
71. Ting DT, Lipson D et al (2011) Aberrant overexpression of satellite repeats in pancreatic and other epithelial cancers. Science 331(6017):593–596

72. Vineis P, Chuang SC et al (2011) DNA methylation changes associated with cancer risk factors and blood levels of vitamin metabolites in a prospective study. Epigenetics 6(2):195–201
73. Wang L, Aakre JA et al (2010) Methylation markers for small cell lung cancer in peripheral blood leukocyte DNA. J Thorac Oncol 5(6):778–785
74. Weisenberger DJ, Campan M et al (2005) Analysis of repetitive element DNA methylation by MethyLight. Nucleic Acids Res 33(21):6823–6836
75. Widschwendter M, Apostolidou S et al (2008) Epigenotyping in peripheral blood cell DNA and breast cancer risk: a proof of principle study. PLoS One 3(7):e2656
76. Wilhelm CS, Kelsey KT et al (2010) Implications of LINE1 methylation for bladder cancer risk in women. Clin Cancer Res 16(5):1682–1689
77. Wong IH, Lo YM et al (2000) Frequent p15 promoter methylation in tumor and peripheral blood from hepatocellular carcinoma patients. Clin Cancer Res 6(9):3516–3521
78. Wong EM, Southey MC et al (2011) Constitutional methylation of the BRCA1 promoter is specifically associated with BRCA1 mutation-associated pathology in early-onset breast cancer. Cancer Prev Res (Phila) 4(1):23–33
79. Wu HC, Delgado-Cruzata L et al (2011) Global methylation profiles in DNA from different blood cell types. Epigenetics 6(1):76–85
80. Yang AS, Estecio MR et al (2004) A simple method for estimating global DNA methylation using bisulfite PCR of repetitive DNA elements. Nucleic Acids Res 32(3):e38

Chapter 13
Epigenetic Therapies in MDS and AML

Elizabeth A. Griffiths and Steven D. Gore

Abstract The use of low dose hypomethylating agents for patients with myelodysplastic syndrome (MDS) and secondary acute myeloid leukemia (AML) has had made a significant impact. In the past, therapies for these diseases were limited and patients who elected to receive treatment were subject to highly toxic, inpatient chemotherapeutics, which were often ineffective. In the era of hypomethylating agents (azacitidine and decitabine), a patient with high grade MDS or AML with multilineage dysplasia can be offered the alternative of outpatient, relatively low-toxicity therapy. Despite the fact that CR (CR) rates to such agents remain relatively low at 15–20%, a much larger percentage of patients will have clinically significant improvements in hemoglobin, platelet, and neutrophil counts while maintaining good outpatient quality of life. As our clinical experience with azanucleotides expands, questions regarding patient selection, optimal dosing strategy, latency to best response and optimal duration of therapy following disease progression remain, but there is no question that for some patients these agents offer, for a time, an almost miraculous clinical benefit. Ongoing clinical trials in combination and in sequence with conventional therapeutics, with other epigenetically active agents, or in conjunction with bone marrow transplantation continue to provide promise for optimization of these agents for patients with myeloid disease. Although the mechanism(s) responsible for the proven efficacy of these agents remain a matter of some controversy, activity is thought to stem from induction of DNA hypomethylation, direct DNA damage, or possibly even immune modulation; there is no question that they have become a permanent part of the armamentarium against myeloid neoplasms.

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13.1 Introduction

Myelodysplastic syndromes (MDS) are a heterogenous group of malignant myeloid disorders characterized by peripheral blood cytopenias in association with bone marrow hypercellularity and dysplasia [1]. Patients with high grade MDS (int-2 or high by IPSS criteria, Fig. 13.1) have a high rate of transformation to acute myeloid leukemia (AML) and poor long-term survival with a life expectancy in the absence of treatment between 0.4 and 1.8 years [2]. The International Prognostic Scoring System (IPSS) was developed as a tool for stratifying patient outcomes based upon readily available clinical characteristics. Figure 13.1 details the components necessary for the generation of an IPSS score and the expected survival for each designation [2]. "Secondary" AMLs such as those arising in patients with an antecedent MDS diagnosis are generally resistant to traditional chemotherapeutics and the overall survival (OS) in this group of patients is universally poor [3–5]. Both MDS and AML are diseases of the elderly with a majority of patients diagnosed when they are older than 60 years [5]. Although a small minority of patients with MDS will present with mild cytopenias and low grade disease, a majority do not [2]. Patients with MDS associated with multilineage cytopenias (anemia, thrombocytopenia, and neutropenia), high bone marrow blast percentages, or characteristic adverse chromosomal features often progress rapidly to AML and in the absence of bone marrow transplantation, ultimately die of their disease [2].

For these patients, and for a large number of older people who present with putatively de novo myeloid leukemias, but with unrecognized low grade cytopenias and

Prognostic Variable	Score value				
	0	0.5	1.0	1.5	2.0
Bone Marrow Blast %	<5	5-10		11-20	21-30
Karyotype	Good= normal,-y, del(5q), del(20q)	Intermediate= all others	Poor= ≥3 or any 7 abnormality		
Cytopenias	0/1	2/3			

IPSS risk group	Score	Median Survival
Low	0	5.7 years
Int-1	0.5-1	3.5 years
Int-2	1.5-2.0	1.2 years;
High	≥2.5	0.4 year

Fig. 13.1 Clinical criteria for and IPSS risk group classification of patients with myelodysplasia, from ref. [2]

bone marrow dysplasia, conventional induction chemotherapeutics (IC, with daunorubicin and cytarabine) have been in large measure disappointing [6]. Furthermore many such patients are unfit for intensive treatment and are offered instead low dose cytarabine, clinical trials or supportive care [7]. In this group the OS rates at 2 and 5 years remain only 10% and 2% respectively [3, 4]. Patients who are fit to receive traditional IC require long periods of time (often 4–6 weeks) in the hospital, and this treatment offers a complete remission rate of only 20–30%, with median survivals ranging between 5 and 13 months [6, 8, 9]. In addition to induction failure and early relapse, even in those who achieve remission, prolonged hospitalization can have the side effect of physical deconditioning and the 3 or more weeks of neutropenia resulting from this treatment can result in resistant bacterial and fungal infections [6]. These burdens create patients who are unable to return to good quality of life and who become ineligible for salvage therapy or clinical trials upon relapse due to poor performance status, organ dysfunction or infection. Even in those who retain an excellent performance status following induction, primary refractory AML remains a significant quality of life problem, requiring frequent blood transfusions, extensive prophylactic antibiotic regimens, and regular hospital visits [9].

Until recently, toxic traditional IC was the only real option for fit patients with high grade MDS or AML with MDS related changes [1]. Recently however, the epigenetically active drugs azacitidine (Aza, Vidaza, Celgene, Concord OH) and decitabine (Dac, Dacogen, Esai Inc., Mars, PA) have been approved both in the United States and Europe for the treatment of MDS and low blast count (<30%) AML [7, 10]. These drugs, both of which are incorporated into DNA resulting in the depletion of the intracellular methyltransferases (DNMTs) when given at low dose, were the first epigenetically active therapy to be approved for cancer. They have resulted in a significant change in the approach to patients with MDS and required the development of the International Working Group (IWG) response criteria in MDS in order to measure meaningful improvements in cytopenias that did not fit into the traditional response assessment which designated only complete (CR) or partial (PR) responses as meaningful [11, 12]. A summary of the IWG response criteria in MDS are provided in Table 13.1. In particular, Aza has been shown to improve OS, delay the transformation to AML in high-grade MDS patients, and produce significant responses in patients with low blast count AML [7]. Although a statistically significant survival benefit has not been demonstrated following treatment with Dac, this drug has been shown to produce both CRs and hematological improvements in both MDS and AML patients who receive it [10, 13]. Taken together these drugs offer an effective alternative to induction chemotherapy and have become the standard of care for patients with MDS as well as selected patients with AML.

As with conventional chemotherapeutic strategies for these patients, responses are usually limited to a year or two, but therapy is largely outpatient, with minimal end organ toxicity and few side effects [14]. Despite notable limitations, these drugs have made a significant impact upon quality of life for a large number of patients with high grade MDS and AML. Ongoing work to understand the mechanism responsible for the efficacy of these drugs and the ultimate loss of response observed

Table 13.1 Selected clinical trials with azacitidine (aza) or decitabine (dac) in MDS

Trial	CALGB 9221	D-0007	ICD03-180	AZA-001	US Oncology	ADOPT	EORTC 06011
Author (publication year)	Silverman (2002) [27]	Kantarjian (2006) [10]	Kantarjian (2007) [55]	Fenaux (2009) [7]	Lyons (2009) [36]	Steensma (2009) [56]	Lubbert (2011) [13]
Number enrolled	191	170	95	358	151	99	233
Number treated with study drug	150 (99 upfront, 51) crossovers	89	95	179	151	99	119
Phase	III	III	II	III	II	II	III
Study regimen	Aza SQ 75 mg/m^2×7 days	Dac IV 15 mg/m^2 q8h×3 days	Dac IV 10 mg/m^2×10 days IV 20 mg/m^2×5 days SQ 20 mg/m^2×5 days	Aza SQ 75 mg/m^2×7 days	Aza SQ 75 mg/m^2 ×5days–2 days off-2days ×5 days–2days off-5 days ×5 days	Dac IV 20 mg/m^2×5 days	Dac IV 15 mg/m^2 q8h×3days
Int-2 or high IPSS (%)	46	70	66	87	Not reported	46	93
Median cycles administered	4	3	7	9	6	5	4
CR % (by IWG 2000)	9	9	37	17	Not reported	15	13
CR+PR+HI% (by IWG 2000)	48	30	73	49	48	43	34

clinically is ongoing. Furthermore, the development of novel dosing strategies, combinations, and the appropriate use of allogenic transplantation provide hope for improving response duration and possibly even providing an opportunity for long-term remission to these unfortunate patients.

13.2 Single Agent "Hypomethylating" Therapy for MDS and AML

13.2.1 Azacitidine

Aza is a nucleoside analog of cytidine in which the carbon 5 position of the pyrimidine ring has been substituted with nitrogen (Fig. 13.2a) [15]. It is imported into cells by the action of nucleotide transporters, where it is activated by uridine–cytidine kinase and incorporated into RNA (Fig. 13.3) [15]. Sixty to 80% of the Aza dose given is incorporated into RNA and this has impacts upon protein synthesis and RNA metabolism [15]. Twenty to 40% of the dose is converted into the deoxyribonucleoside Dac by the action of ribonucleotide reductase [15]. This deoxyribonucleoside base is then phosphorylated and incorporated into DNA where it acts as a suicide substrate for DNMTs and induces DNA hypomethylation during cellular replication as well as DNA damage due to adduct formation [15]. Aza was first synthesized and tested in 1960s and 1970s [16, 17]. In early clinical trials as a traditional chemotherapeutic, it was demonstrated to be effective in myeloid malignancy, however its efficacy was limited by significant gastrointestinal toxicity and prolonged cytopenias [16–18]. Cytarabine or AraC was developed at about the same time. This drug, another nucleoside analog of cytidine whose activity is thought to result in chain termination, is among the most active drugs used for myeloid malignancy. Ultimately the toxicity of 5-Aza limited its further

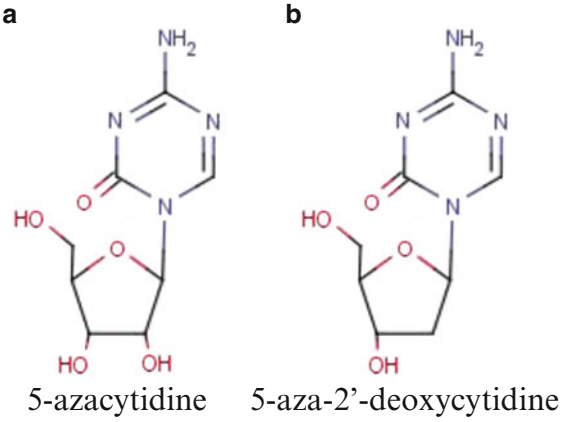

Fig. 13.2 Molecular structure of Aza (**a**) and Dac (**b**)

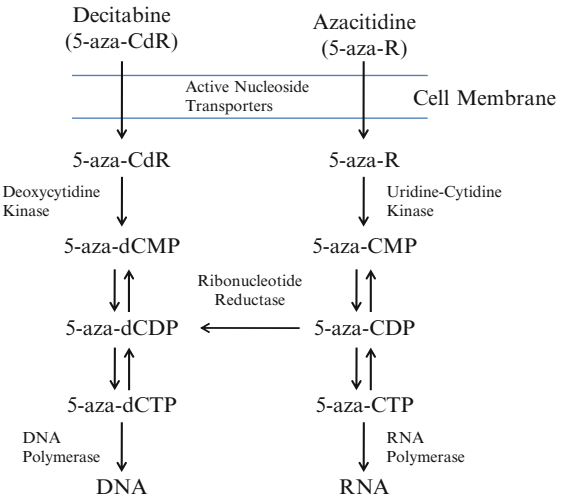

Fig. 13.3 Uptake and serial steps for the incorporation of Aza and Dac into RNA and DNA

clinical development, and cytarabine became the nucleoside analog of choice in myeloid malignancy [17, 18].

In 1978, Peter Jones and colleagues demonstrated that treatment of mouse embryo cells in vitro with Aza and its deoxy analog 5-aza-2′-deoxycytitine (Dac) could induce differentiation into functional myotubes [19]. Jones and Taylor went on to show that this differentiation resulted from changes in DNA methylation elicited by treatment with azanucleosides [20, 21]. Further work, by Dr. Jones and others, identified methylation as a common event in many malignancies, including the pre-leukemic condition known as MDS, a disease for which no treatment was available [22]. Although initially used as a laboratory tool to test gene and chromosome specific methylation changes, the identification of methylation as a potentially reversible cancer specific event spurred interest in the possibility that cancers treated with these drugs might be induced to differentiate and potentially to apoptose and die.

Ultimately in the 1990s, insights into methylation events common to MDS, specifically identification of recurrent methylation of tumor suppressor genes such as p15INK4B, resulted in the development of a number of phase I and II clinical trials of azanucleotides in this disease [23, 24]. Table 13.2 reviews the key published trials with single agent azanucleotides in MDS.

Among the first published trials with Aza for the treatment of MDS delivered the drug at 75 mg/m^2 as a continuous intravenous infusion for 7 days every 4 weeks [25]. This trial enrolled high grade MDS patients with symptomatic disease characterized by red cell and platelet transfusion dependence and poor life expectancy (refractory anemia with excess blasts (10–20%), or refractory anemia with excess blasts in transformation (20–30%). Forty three patients were evaluable and responses were seen in 21 (49%) of these patients [25]. Five patients (12%) achieved a CR, 11 (25%) achieved a partial response (PR), and 5 “improved” (a response characterized in the study as a ≥50% reduction in transfusion requirements, or improvement in platelets,

Table 13.2 Selected clinical trials with azacitidine (aza) or decitabine (dac) in MDS

Trial	CALGB 9221	D-0007	ICD03-180	AZA-001	US Oncology	ADOPT	EORTC 06011
Author (publication year)	Silverman (2002) [27]	Kantarjian (2006) [10]	Kantarjian (2007) [55]	Fenaux (2009) [7]	Lyons (2009) [36]	Steensma (2009) [56]	Lubbert (2011) [13]
Number enrolled	191	170	95	358	151	99	233
Number treated with study drug	150 (99 upfront, 51) crossovers	89	95	179	151	99	119
Phase	III	III	II	III	II	II	III
Study regimen	Aza SQ 75 mg/m^2×7 days	Dac IV 15 mg/m^2 q8h×3 days	Dac IV 10 mg/m^2×10 days IV 20 mg/m^2×5 days SQ 20 mg/m^2×5 days	Aza SQ 75 mg/m^2×7 days	Aza SQ 75 mg/m^2 ×5d–2 days off-2 days ×5 days–2 days off-5 days ×5 days	Dac IV 20 mg/m^2/days×5 days	Dac IV 15 mg/m^2 q8h×3 days
Int-2 or high IPSS (%)	46	70	66	87	Not reported	46	93
Median cycles administered	4	3	7	9	6	5	4
CR % (by IWG 2000)	9	9	37	17	Not reported	15	13
CR+PR+HI% (by IWG 2000)	48	30	73	49	48	43	34

hemoglobin or neutrophils) [25]. OS in these high risk patients was 13.3 months and for those achieving CR or PR was 14.7 months, and the chief toxicities were mild to moderate nausea [25]. A number of other clinical trials using this drug were published suggesting that Aza had significant activity in MDS and these results were sufficient to prompt two larger, randomized trials of Aza in MDS [26].

In 2004, the FDA approved Aza for the treatment of MDS based upon results from a single phase III clinical trial (described in detail below) [27]. A second trial demonstrating survival was required by European regulators, and this was published formally in 2009 [7]. These trials established Aza as the standard of care approach to patients with int-2 and high risk MDS by demonstrating a prolongation in the time to progression to AML, decreased transfusion requirements, improvements in neutropenia, and ultimately, improvements in OS.

13.2.1.1 CALGB 9221

The first phase III trial of Aza in patients with MDS was published by investigators from the CALGB [27]. CALGB 9221 enrolled 191 patients of median age 68 with French American British-defined MDS (reference for FAB classification), to receive either supportive care or Aza at a dose of 75 mg/m^2/day subcutaneously for 7 of 28 days. Patients were maintained on their randomized arm for 4 months, after which patients who were deemed to have progressed on the supportive care arm could crossover to the Aza arm. Patient characteristics were distributed evenly across both arms with 59% of the patients overall having RAEB or RAEB-T by FAB criteria (46% Int-2 or high by IPSS) [27]. Sixty five percent of the enrolled patients were red blood cell transfusion dependent (69% Aza arm, 61% supportive care arm) [27].

Responses were evaluated in both arms. Among patients randomized to receive supportive care, 5% met criteria for improvement; no patients on this arm achieved a CR or PR. Of the 99 patients randomized to receive Aza, 60% ($n=60$) achieved a response ($p<0.0001$) [28]. Responses were classified as CR in 7% ($n=7$), PR in 16% ($n=16$), and improvement in 37% ($n=37$). Of those patients demonstrating "improvement," 35% had increases in three cell lines inadequate to qualify as a PR, 30% had improvement in two cell lines, and 35% had improvement in only one cell line. Responses did not depend upon MDS sub-classification. Forty nine patients crossed over to receive Aza, of these 47% ($n=23$) responded and 10% ($n=5$) achieved a CR [27]. Patients treated with Aza had a median time to progression to AML or death of 21 months vs. 12 months in those patients treated with supportive care alone, and this was statistically significant ($p=0.007$), median OS in an intention to treat analysis was 20 months in the Aza treated patients vs. 14 months for those randomized to supportive care, although this difference was not statistically significant ($p=0.10$) [27].

Due to the design of this study, the survival analysis was confounded by the 49 patients who crossed over to receive Aza. In order to eliminate this bias, a landmark analysis at the 6 month date was performed. Three subgroups were identified, the

first included patients randomized to supportive care who did not crossover, or who crossed over after the six 6 month time point, the second were patients who were randomized to Aza, and the third were patients who crossed over after 4 months, but before 6 months [27]. This analysis excluded 36 patients who died before the landmark date. The median survival in these three groups was 11 (supportive care only), 14 (early crossover), and 18 (randomized to Aza) months respectively. A statistically significant difference in survival was observed between the Aza treated and supportive care groups ($p=0.03$), but not between supportive care and early crossovers [27].

Transfusion requirements were tracked in both groups. In the Aza treated group transfusion needs increased during the first cycle, and thereafter declined, whereas in the supportive care arm transfusion requirements remained stable or increased. Of the 99 patients initially randomized to receive Aza, 51% had an improvement in hemoglobin, 45% (29) became RBC transfusion independent, and 6 (9%) had a reduction in transfusion dependence by at least 50%. Improved platelet counts were observed in 47%, and increased white cell counts were seen in 40% of the Aza treated patients [27].

In addition to objective improvements in transfusion requirements, white cell counts, survival and prolonged time to AML transformation, patients treated with Aza on this trial experienced significant improvements in quality of life. These were reported as improvements in fatigue, physical functioning, dyspnea, psychological distress, and positive effect, all of which demonstrated statistical significance when compared to patients treated with supportive care alone with a p value ≤ 0.01 [27]. Similar results were observed in the patients who crossed over to Aza. Toxicities among the Aza treated patients were most frequently related to myelosuppression and were difficult to distinguish from the underlying disease. It was notable that treatment with Aza did not appear to increase the infection or bleeding rates above background, and furthermore only one treatment related death was reported on the study, emphasizing the safety of this therapy, even for older patients [27].

13.2.1.2 AZA-001

Although the data from CALGB 9221 was compelling, this study did not, in the final analysis, demonstrate a difference in OS between the patients randomized to receive Aza and those randomized to supportive care, likely as a result of the crossover trial design. The AZA-001 study was designed to address the question of whether Aza provided an OS benefit for high grade MDS patients [7]. This cleverly conceived, international, randomized trial definitively demonstrated that Aza 75 mg/m^2 given subcutaneously for 7 days of a 28 day schedule prolonged OS when compared with conventional care regimens (CCRs) as selected by the patients physician. The investigators aimed to provide at least six cycles of Aza to those patients randomized to the experimental arm. Conventional care was assigned by the patient's physician prior to randomization depending upon the patient's age, performance status co-morbidities and patient preference. CCR consisted of the three most

common treatments for patients with int-2 or high risk MDS: IC including cytarabine 100–200 mg/m^2/day × 7 days plus, daunorubicin 45–60 mg/m^2 × 3 days or idarubicin 9–12 mg/m^2/day × 3 days or mitoxantrone 8–12 mg/m^2/day, low dose cytarabine (LDAC) at a dose of 20 mg/m^2 for 14 days every 28 days, or best supportive care (BSC). All patients randomized received CCR as selected by their physician or Aza on trial. A total 358 patients were randomized. In this way a pre-specified subgroup analysis based upon physician assignment was possible and helped to eliminate differences in outcome based upon issues of performance status and patient fitness.

The primary OS endpoint of this study was met after a median follow-up of 21.1 months [7]. At this analysis the OS in the Aza treated patients was 24.5 months vs. 15 months for patients assigned to CCR and this result was found to be statistically significant ($p \leq 0.0001$). Two year OS also favored Aza, at 51% vs. 25% for CCRs ($p \leq 0.0001$) [7]. Predefined subgroup analysis was also done in order to compare Aza responses with each of the CCRs selected and within specific cytogenetic and IPSS risk groups. There were significant differences between Aza and BSC with an OS benefit for azacytidine treatment of 9.6 months (HR 0.58, $p = 0.0045$), as well as between Aza and LDAC with an OS benefit of 9.2 months (HR 0.36, $p = 0.0006$) [7]. No statistically significant differences in OS were seen when Aza was compared with IC; OS was prolonged by 9.4 months with a hazard ratio of 0.76, but the p value was not significant at 0.51 [7]. This apparent discrepancy was likely due to the low numbers in this subgroup ($n = 42$); 17 patients in this group were randomized to Aza and 25 to intensive chemotherapy.

No differences in response to Aza were seen across the IPSS risk groups enrolled (although most patients were int-2 or high risk $n = 313$ (87%)), nor within the cytogenetic risk groups identified by the IPSS (good, intermediate, poor). Patients with del-7 or del(7q), a group recognized to have particularly poor prognosis, had an OS of 13.1 months vs. 4.6 months in the CCR group [7, 29].

Responses on this trial were similar to those observed in CALGB 9221. Overall, 29% of those assigned to Aza achieved either CR (17%) or PR (12%) compared with 12% (8% CR, 4% PR) assigned to CCR ($p = 0.0001$) [7]. Any hematological improvement (HI) was observed in 49% of those treated with Aza vs. 29% of those treated with CCR ($p = 0.0001$) [7]. In addition, for those treated with Aza, major erythroid responses were seen in 40% of patients, major platelet responses in 33% and major neutrophil responses in 19%. By contrast, for those receiving CCR major erythroid responses were seen in 11% ($p < 0.0001$), major platelet responses were seen in 14% ($p < 0.0003$) and major neutrophil responses were seen in 18% ($p = 0.58$, not statistically significant) [7]. Patients treated with Aza experienced a statistically significant reduction in the need for intravenous antibiotics (33% relative risk reduction vs. CCR; RR 0.66 95% CI:0.49–0.87 $p = 0.0032$). Furthermore of the 111 patients with red cell transfusion dependence at the time of study enrollment, 50 (45%) became transfusion independent vs. 13 (11.4%) of the 114 patients randomized to receive CCR (p value significant at 0.0032) [7].

Secondary endpoints in this trial included time to AML transformation and hematological response according to the IWG 2000 criteria for MDS [11]. Treatment with Aza in the entire group was associated with delayed leukemic transformation;

the median time to transformation was 17.8 months in the Aza treated group vs. 11.5 months in the CCR group ($p < 0.0001$) [7].

Among the most notable findings on this trial was that achievement of CR or PR was not necessary in order to achieve an improvement in OS; any patient who achieved a hematological response showed a survival benefit.

13.2.1.3 AZA in AML

Changes in the diagnostic criteria for AML based upon the WHO guidelines published in 2008 resulted in the reclassification of patients enrolled on both the CALGB and AZA-001 from the previous FAB classification of Refractory Anemia with Excess Blasts in Transformation (RAEB-T; 20–30% bone marrow blasts) to a new diagnosis of AML [1, 30, 31]. The WHO now defines any patients with ≥20% blasts as having AML [30].

A pooled analysis of previously published CALGB studies including 9221, 8921, and 8421, in which enrolled patients treated with Aza would now be re-assigned as AML was published in 2006 [28]. This reported the response to Aza given either intravenously or subcutaneously at a dose of 75 mg/m^2/day for 7 days of a 28 day cycle in 103 patients who would now be classified as having AML, 91 of whom received Aza [28]. Of these patients 33 (36%) developed a response (8 CRs, 2 PRs, 23 HIs), with a median duration of response of 7.3 months (range 2.2–25.9 months) [28]. Formal comparison with supportive care alone across the three studies was not possible, but 27 patients enrolled in 9221 were randomized to upfront Aza and a further 13 crossed over to receive Aza before the 6 month analysis. Of these, 7% in the Aza group achieved CR or PR compared with 0% in the observation-only group [28]. Median survival time for the 27 patients assigned upfront to Aza was 19.3 months compared with 12.9 months for the 25 AML patients randomly assigned to observation. Of 13 patients with WHO AML at the time of study entry who crossed over to receive Aza, one achieved a PR, and one HI.

Of the 358 patients originally enrolled on AZA-001, a third would now be identified as having AML. A second analysis of these patients was undertaken in order to assess outcome in this group of older adults treated with either Aza or CCR [7, 32]. Of the 113 patients now designated as AML, 63 were assigned to BSC, 34 to LDAC and 16 to IC [32]. The median age in all groups was 70 years with a range of 58–80. Patients were evenly distributed with respect to age, cytogenetic risk group, and ECOG scores. Bone marrow blast percentages were similar in both groups at 23% with a range of 20–34%. In all, 55 patients were randomized to the Aza arm and 53 to CCR. After a median follow-up of 20.1 months, OS was significantly ($p = 0.005$) longer in those patients treated with Aza (24.5 months) than in those receiving CCR (16 months). The 2 year survival was also superior in the Aza group at 50% compared with16% in the CCR group ($p = 0.001$) [32]. Adverse events in this group of patients were primarily grade 3 and 4 cytopenias, which remain difficult to distinguish from the underlying disease. Four patients in the Aza group and three patients in the CCR group discontinued treatment as a result of adverse events.

Several prospective studies of Aza given on the conventional schedule of 75 mg/m^2/day for 7 days in patients identified as AML at diagnosis have been reported. One such study enrolled 82 patients with AML (27 (33%) with secondary disease) and a median age of 72 years (range, 29–87 years) [33]. Thirty-five patients (43%) received Aza as their first treatment, and 47 patients (57%) had previously received 1 or more lines of chemotherapy. The overall response rate in this group was 32% (26/82 patients) with 16 patients (20%) achieving a CR or a CR with incomplete count recovery, and 10 patients (12%) achieving a PR [33]. Untreated patients responded more often than those previously treated with 31% of untreated patients achieving either a CR or a CR with incomplete count recovery compared with only 9 (19%) such responses in the previously treated group ($p=0.006$). The response duration in untreated patients who achieved a response was 13 months with 1 and 2 year survivals of 58 and 24% respectively [33]. Another study from Germany evaluated medically unfit ($n=20$) or relapsed/refractory ($n=20$) patients with AML and a median bone marrow blasts count of 42% [34]. This study showed similar statistically significant differences in response between untreated patients, who demonstrated overall responses (CR + PR + HI) of 50%, and patients with relapsed or refractory disease, who had an overall response rate of only 10% ($p=0.008$) [34]. These response rates are striking and compare favorably with responses seen with induction therapy although additional data are necessary in order to determine whether Aza or Dac will end up the therapeutic agent of choice in this context [6, 35].

Results from the CALGB trials were sufficient in the United States and the AZA-001 trial satisfied the European regulators for the approval of Aza as standard therapy for patients with MDS and low blast count AML. In the United States, approval was granted for all IPSS defined MDS subtypes, while in Europe approval is confined to patients with Int-2 and high risk IPSS scores not eligible for bone marrow transplantation, those with CMML-2 and those with WHO defined AML with 20–30% blasts or multilineage dysplasia.

Both large phase III trials demonstrated this drugs activity in MDS and AML, and further showed that unlike previous therapies, DNMTi require prolonged exposure to elicit a clinical benefit. In the CALGB trials most responses were seen by cycle 4 (75%), with a median number of cycles to any response (CR, PR, HI) of three cycles [27]. The range for this response was 1–17 cycles, however and although 90% of patients achieved a response by cycle 6, some patients got their response as late as cycle 17 [27]. In the AZA-001 trial where the goal was to provide at least six cycles of therapy and there was no predefined stopping point, the investigators demonstrated that continuing the Aza dosing as long as possible can result in improvements in the observed responses, and these results were re-iterated by additional analysis of the studies conducted by the CALGB [28, 32]. The secondary analysis of CALGB studies demonstrated a response in 91 of 179 patients, and responders received a median of 14 cycles of therapy (range 2–30) [28]. The median time to first response in this study was slightly shorter than that seen in 9221, at 2 cycles (but with a range of 1–16) and although most responses (91%) were achieved by the sixth cycle, continuation of Aza was able to improve the quality of the first response in 48% of those treated, and this best response was seen in most patients (92%) by

the 12th cycle [28]. Overall 30 patients achieved a best response of CR 3.5 cycles beyond the first response (with a 95% CI of 3.0–6.0 cycles), and in 21 patients whose best response was PR, this was seen as a median of 3.0 cycles after the first response (95% CI was 1.0–3.0) [28].

13.2.1.4 Other Considerations of Dose and Schedule

Additional questions which remain about the use of single agent Aza therapy are related to administration schedule (to weekend or not to weekend, are 7 days enough) and optimal drug delivery (subcutaneous vs. intravenous vs. oral).

In community practice there is often difficulty in giving this drug on the FDA approved schedule due to inadequate availability of personnel to administer the drug on weekends. This practical consideration resulted in a trial of several schedules of Aza administered in a community setting during weekdays only [36]. In this trial, 151 patients, for the most part with lower risk MDS (low, int-1 in 63% of patients), were randomized to receive Aza on one of the three schedules: 75 mg/m^2 daily for 5 days, off 2 days and then on 2 days (5-2-2), 50 mg/m^2 daily for 5 days, off 2 days and then on for 5 further days (5-2-5), and lastly 75 mg/m^2 daily for 5 days alone (5-0-0) [36]. These schedules seemed to result in similar hematological improvement rates (44%, 45%, 56%, respectively), but this study was not designed to produce statistically significant results, nor have these schedules been directly compared with the approved 7 day schedule. Thus it is difficult to condone alteration of the schedule at this time, based upon the lack of survival data in these schedules and the demonstrated survival benefit with administration of these drugs on the approved schedule. One additional schedule question has been raised by the preliminary data from the Eastern Cooperative Oncology Group trial 1905, which was a randomized phase II trial comparing Aza 50 mg/m^2/day subcutaneously for 10 days to the same Aza schedule given in combination with the Histone deacetylase (HDAC) inhibitor entinostat (4 mg/m^2/day PO days 3 and day 10) [37]. This abstract reported only on patients with baseline cytogenetic abnormalities ($n = 40$ evaluable) but demonstrated complete cytogenetic responses of 13% and a partial cytogenetic responses of 23% for an overall response in this subgroup of 51% (21/40) [37]. No differences in response were seen between the two treatment groups. Notably the responses observed were significantly higher than those reported with conventional Aza dosing raising the question of whether a lower dose, longer administration schedule may be of some benefit. At present these data are insufficient to change practice, however as additional groups publish the results of ongoing clinical trials of different dosing schedules, practice changes may be in order.

With respect to optimal drug delivery there is only a single study which directly compares the pharmacokinetics of intravenous to subcutaneous dosing within individual patients. In this study the pharmacokinetic profile of intravenous administration was almost identical to that seen with subcutaneous dosing, although the peak drug concentration was higher in patients receiving intravenous drug [38]. Despite these

data, published clinical trials using 20 min IV infusion schedules are limited to two studies, one which gave Aza for 5 days and the other for 7 [39, 40]. Both of these studies demonstrated response rates which were similar to those seen with subcutaneous dosing (27% in the 5 day and 56% for the 7 day schedule), but neither of them was powered to detect a survival benefit [39, 40]. Despite the dearth of published response data, it seems reasonable to switch to intravenous administration in patients who suffer significant injection site reactions with subcutaneous dosing, and the FDA approved a New Drug Application for intravenous Aza in January 2007, supporting this practice [41].

Initial studies with oral Aza were limited by rapid catabolism of the compound in aqueous environments but the development of a film-coated formulation improved stability [42, 43]. Since that time the first phase I study of oral Aza has been published, demonstrating activity for the oral drug in patients with both MDS and CMML, with promising response rates [44]. Six of 17 (35%) previously treated patients had a response (CR + PR + HI) and 11 of 15 (73%) untreated patients responded (CR + PR + HI). This study demonstrated no overall response in the 8 patients with AML, however two patients had stable disease for 14 and 15 cycles [44]. Overall these results suggest that oral Aza may be a real possibility for the future and clinical trials of this drug are ongoing.

13.2.2 Dac

5-Aza-2′-deoxycytidine (Dac) is a deoxynucleoside analog of cytidine in which the carbon 5 position of the pyrimidine ring has been substituted with nitrogen (Fig. 13.2b) [15]. It is imported into cells by the action of nucleotide transporters, where it is activated by deoxycytidine kinase and then phosphorylated (Fig. 13.3) [15]. After its phosphorylation to the triphosphate form, 100% of the drug is incorporated into DNA, where it interrupts the action of DNA methyltransferases as described above for Aza. Similar to Aza, Dac has been demonstrated to cause both DNA hypomethylation and DNA damage, albeit at lower concentrations [45]. The identification of DNA hypomethylation as a functional consequence of exposure to both Aza and Dac, in conjunction with the recognition of DNA methylation changes as a frequent abnormality in cancer, spurred significant clinical interest in the development of these drugs for clinical use [20, 45].

Although effects upon DNA methylation were recognized and noted early in its development, initial clinical trials focused on conventional dosing strategies aimed at developing a maximum tolerated dose schedules [46–48]. These studies demonstrated considerable activity but with toxicity not significantly superior to cytarabine, with several studies performed investigating combinations with other chemotherapeutics in the salvage setting [49, 50].

Several early studies showed promising results with “low dose” Dac regimens, however these studies provided the drug at doses of 40–50 mg/m^2/day, and toxicity remained a serious problem [51–53]. The first study to investigate the “optimal”

lower dose Dac schedule for maximal demethylation was published in Blood in 2002 by Jean-Pierre Issa and colleagues [54]. This trial enrolled 48 patients at doses ranging from 5 to 20 mg/m^2/day for 10–20 days of a 6 week schedule depending upon count recovery. Most interestingly in this study, responses appeared to be superior for the lower dose schedules studied, prompting the authors to suggest further investigations of the drug be undertaken at truly lower dose schedules [54].

Based upon extensive phase I/II data at moderate to higher doses, the first large scale trial of Dac enrolled 170 patients with MDS between 2001 and 2004 and randomized them to either Dac (89 patients), given at 15 mg/m^2 iv every 8 h (45 mg/m^2/day) for 5 days, or BSC (81 patients) [10]. Patients were removed from the study for disease progression, transformation to AML, failure to achieve a PR after six cycles of therapy, or failure to achieve a CR after eight cycles of therapy. Additionally, patients who did achieve a CR were removed from therapy after two cycles of sustained CR. The groups were well matched for all important variables with a median age of 70 years (range, 30–85 years). A majority of the patients (71%) had int-2 or high risk disease by IPSS criteria. The primary study endpoints were overall response rate and time to AML transformation or death. Overall 30% ($n=27$) of patients experienced improvement on the study (CR + PR + HI) compared with 7% ($n=6$) patients randomized to BSC, and this difference was statistically significant $p=0.001$ [10]. In a retrospective central review of pathology nine patients enrolled on Dac and three patients on the supportive care arm were designated as having AML (by FAB criteria, >30% bone marrow blasts). Response rates in these nine patients were 56% (5/9), while none of the patients enrolled on the supportive care arm developed a response [10]. It is important to note that in this randomized controlled non-crossover trial there was no survival benefit for the use of Dac, although one might argue that the dose used (45 mg/m^2/day × 5 days) was not low enough to maximize hypomethylation over cytotoxicity and the median number of cycles administered was low (3).

Following the results of this trial (which were disappointing from a survival perspective, but represented the first active agent for patients with high grade myelodysplasia), in 2006 the FDA approved Dac for all MDS subtypes. Based upon the results of earlier studies suggesting that lower dose Dac dosing might be superior, two pivotal phase II studies were performed aimed at identifying the "optimal" hypomethylating dose for Dac [55, 56]. The first of these was published in 2007 and enrolled 95 patients, again with a majority (66%) of patients having int-2 or high risk disease [55]. All patients were randomized to receive one of the three different Dac schedules, 10 mg/m^2 intravenously over 1 h daily for 10 days, 20 mg/m^2 intravenously over 1 h daily for 5 days, or 20 mg/m^2 subcutaneously daily for 5 days. Patients received a median of seven cycles of treatment and the CR rate overall was significantly better than anticipated at 37%, and an overall improvement (including CR + PR + HI) was observed in a staggering 73% of patients [55]. The 5 day schedule was deemed superior with 25/64 patients on this arm achieving CR and this schedule was selected for further investigation in subsequent trials [55]. The second analogous trial published in 2009 by Steensma and colleagues enrolled 99 patients in a single arm trial of Dac 20 mg/m^2 over 1 h daily for 5 days [56]. A lower percentage of patients on this trial were high grade (46%), and the median number of administered courses were slightly

lower (5) than in the prior investigation. These authors observed a 15% CR rate and an overall response rate of 43% (CR + PR + HI) [56]. Both trials demonstrated that the lower dose schedule of Dac 20 mg/m^2/day for 5 days had at least equivalent efficacy when compared with the FDA approved schedule, and furthermore that maintaining 4 week dosing intervals and repeated cycles of therapy were important in order to maximize response.

One additional phase III study of Dac has been published [13]. It is important to note that this study did not employ the 5 day, 20 mg/m^2/day schedule described above. This trial was designed to demonstrate a survival benefit for the use of Dac in patients with MDS, comparable to that observed with Aza. Two-hundred and thirty-three patients with a median age of 70 years (range 60–90) were enrolled; 53% had poor-risk cytogenetics and 33% fulfilled WHO AML diagnostic criteria (≥20% blasts) [13]. The primary end point for this trial was OS. Patients were stratified by IPSS risk group, cytogenetics and enrollment site, and were randomly assigned to receive either Dac or BSC. This study design specifically prohibited patient crossover to the experimental arm in an effort to eliminate crossover bias. The Dac was given intravenously at a dose of 15 mg/m^2 every 8 h for 3 days. Cycles were scheduled to repeat every 6 weeks, but the interval could be extended up to 10 weeks for failure of count recovery, eight cycles of treatment were planned. In total 119 patients were randomized to receive decitabine and 114 patients were randomized to the control arm; only 21% of patients received the planned eight cycles of treatment. At the planned analysis point of 2 years, OS in the Dac treatment cohort was 10.1 months vs. 8.5 months in the supportive care arm, this difference was not statistically significant ($p = 0.38$, HR, 0.88; 95% CI, 0.66–1.17) [13]. Sixteen patients on the Dac arm (13%) achieved a CR and 25 patients (21%) improved (PR + HI), for an overall response rate of 34%. The median time to best response was 3.8 months (range, 1.4–11.8 months) for all responders, with a median of 5.8, 2.9, and 3.8 months to reach CR, PR, and HI, respectively. Two patients (2%) in the supportive care arm had a HI, there were no CRs or PRs in this group. Dac did not have a statistically significant impact upon time to AML transformation; patients on Dac transformed to AML after 8.8 months vs. 6.1 months in the supportive care arm (HR 0.85; 95% CI 0.64–1.12; $p = 0.24$) [13].

Disappointing results, in terms of survival benefit, from two large phase III trials of Dac in MDS have resulted in a significant shift in terms of practice away from Dac in this population [10, 13]. Despite these results, some clinicians continue to use Dac in the first line treatment of MDS patients, and it is certainly notable that none of the three phase III studies of Dac used the most common low dose schedule of Dac at 20 mg/m^2/day for 5 days, a dose schedule which is pharmacologically more consistent with the 75 mg/m^2 Aza dose demonstrated to prolong survival. Additionally, the European phase III trial delayed subsequent Dac cycles based upon cytopenias, a strategy which is increasingly recognized as inferior. As a result of these caveats it is likely that Dac has similar efficacy to Aza, although at present the data have not definitively demonstrated this equivalence.

13.2.2.1 DAC in AML

Despite disappointing results in patients with MDS, many clinicians favor Dac in patients presenting with AML, particularly in those with very proliferative disease, as a result of its relative cytotoxicity when compared with Aza. A dosing strategy employing 20 mg/m^2 for 10 days has been studied by investigators at the Ohio State James Cancer Center [35, 57]. This dose schedule was initially developed in a phase I trial designed to assess combination therapy with valproic acid, however a single agent response of 73% in a group of very elderly (median age 70) patients with high risk AML prompted phase II investigation (see below) [57]. The Phase II trial enrolled 53 patients of median age 74 years (range 60–85) with AML (16 complex karyotype, 19 with an antecedent hematological diagnosis) and produced a response rate of 64% (34/53) composed of 25 CRs and 9 CRs without count recovery [35]. Patients enrolled on study had a median survival of more than a year, suggesting that this strategy is similarly effective to conventional chemotherapeutics in this patient population [6, 8, 9]. These very promising results have produced an ongoing cooperative trial using this dose schedule in older patients with AML and may yet demonstrate statistically significant improvements in survival for this particular subgroup of elderly AML patients.

13.3 Azanucleotides and CMML

Dac remains the most studied drug in patients with CMML, a distinct entity within the WHO diagnostic criteria form MDS. Several studies have examined the activity of Dac both prospectively and retrospectively in this group. One recently published phase II study enrolled 39 patients of median age of 71 years with advanced CMML to receive Dac on the 20 mg/m^2/day intravenous schedule for 5 days of a 28 day cycle [58]. Enrolled patients received a median of ten cycles of drug (range, 1–24) and the overall response rate was 38%, composed of 4 (10%) CRs, 8 (21%) marrow responses, and 3(8%) His [58]. With a median on trial follow-up of 23 months the OS was 48%. Another study examined the response to Dac in 31 patients diagnosed with CMML who were treated on two phase II and one phase III clinical trials [59]. Patients included in the analysis had similar demographics and disease characteristics across the three studies. The median age was 70 and patients were predominantly male (71%). The overall response rate in this group was 36% (14% CR + 11% PR + 11% HI) [59]. Although Aza has also been shown to have activity in this disease, the number of published reports in this group are limited, and thus most experts would likely favor the use of Dac for patients with CMML outside the context of a clinical trial [60]. An ongoing clinical trial designed to prospectively enroll patients with CMML is ongoing in order to address the efficacy of Aza in this disease.

13.4 Outcomes Following Azanucleotide Failure

As we develop our experience with azanucleotides it has become clear that patients who lose their response to azanucleotides have a dismal prognosis [14]. As a result of these poor outcomes, current standard practice is to maintain patients on therapy with hypomethylating drugs on a monthly schedule indefinitely and to stop only in the context of overt progression. Unfortunately, analysis of patients enrolled on early studies of Aza who develop disease progression have now been published, showing that in patients who fail azanucleotides, survival is remarkably short with a median life expectancy of 5.6 months and a 2-year survival probability of 15% [61]. Similar results have been reported in patients who fail Dac [14, 62]. Outcomes in these reports suggest that enrollment on clinical trials and bone marrow transplantation may result in superior outcome in these patients, however in the absence of successful bone marrow transplantation the OS reported at 1 year remains a mere 28% [14, 61, 62].

13.5 Histone Deacetylase Inhibitors

Histone deacetylase inhibitors (HDACis) are a novel class of drugs whose putative mechanism of action depends upon the ability to alter gene expression. Intracellularly, DNA is stored in the form of "beads on a string" in which the DNA duplex winds around a nucleosome composed of eight histones (two each of H2A, H2B, H3 and H4) [63]. The DNA/histone unit (the nucleosome) is condensed to form higher order chromatin structures such as heterochromatin, which has densely packed nucleosomes and euchromatin, which has loosely packed nucleosomes [63]. Modifications, including ubiquitination, methylation, phosphorylation, poly(ADP) ribosylation, and acetylation, of specific amino acid residues within each histone make up the "histone code" which determines the state of the regional chromatin at specific genes and thus their transcriptional activity [63]. DNA methylation events are thought to induce changes within the local "histone code" which promote gene silencing, although whether methylation events or histone marks are primary remains a matter of some controversy. Perhaps the most studied histone modification is acetylation of lysine N-terminal tails which are common to most histones. Acetylation of lysine results in an open chromatin conformation and promotes gene transcription while deacetylation of lysine residues promotes gene silencing [63].

HDACs are enzymes that remove acetyl groups from a variety of different protein targets including histones. Increased HDAC activity has been described in cancer cells, and aberrant HDAC activity is characteristic of a number of well recognized recurrent genetic anomalies characteristic of leukemia including the core binding factor gene fusions (t(8;21)(q22;22) and inv(16)), and the sine qua non of acute promyelocytic leukemia t(15;17)(q24;21) [64–66]. The gene products of such fusions result in aberrant recruitment of HDACs to genes important for myeloid

differentiation. Recognition of HDACi as a potential novel therapy in myeloid malignancy resulted from the observation that drugs known to induce differentiation in vitro induced histone hyperacetylation, potentially leading to re-expression of epigenetically silenced genes [67]. Many different diverse chemical compounds can inhibit HDACs, including short chain fatty acids (e.g., phenylbutyrate), hydroxamic acid derivatives (e.g., vorinostat), non-hydroxamate small molecules (e.g., entinostat), and cyclic peptides (e.g., romidepsin) [68].

Most of the published clinical trials of HDACi in MDS and AML are phase I. As single agents the response rates observed have been relatively low, usually between 10 and 20% [68]. Toxicities with these agents demonstrate a common pattern and include fatigue, nausea, vomiting, and diarrhea. Although most of these studies evaluated the correlative endpoint of histone acetylation, no associations between hyperacetylation of histones and response to therapy have been demonstrated. For a more complete review of HDACi in cancer please see Chap. 3, Sect. 3.5 of this book.

13.6 Azanucleotides and HDACis

There has been significant enthusiasm for a combination strategy which includes azanucleotides in conjunction with HDACis. This stems from the observation in vitro that sequential exposure to Dac or Aza followed by HDACi result in synergistic re-expression of DNA methylation silenced genes [69]. Several studies evaluating such combinations have been published to date and the results remain mixed. Although some studies suggest a higher response rate than for single agent azanucleotides, most data are in the phase I or II setting, at a single center, and employ alternative dosing strategies for the azanucleotide making it difficult to distinguish whether these responses are truly superior. In those studies where a single agent arm was also enrolled response rates do not appear to be consistently superior [37, 57]. Although early correlative endpoints did demonstrate evidence to support a connection between reversal of methylation events and response to therapy, subsequent studies (even at the same institution by the same investigators) have failed to substantiate a correlation between gene specific reversal of methylation and response [70, 71].

The first two studies published reports on a combination of Aza at doses between 25 and 75 mg/m^2/day subcutaneously for 5–10 days [70, 72]. These studies enrolled a total of 42 patients with MDS (16) and AML (26), of median age 66. These studies reported that the combination was well tolerated and resulted in response rates of 34 (11/32, 5 CRs) and 50% (5/10, no CRs) respectively (CR + PR + stable disease) [70, 72]. The second study reported correlative epigenetic data in three responders and three non-responders, with those patients who developed a response showing robust demethylation of the tumor suppressor gene *p15^{INK4B}* while those who did not retained methylation at this locus, suggesting that changes in methylation were indeed a marker for responsiveness [70].

Two phase I/II studies have evaluated the combination of Dac with valproic acid. The first employed Dac 15 mg/m^2/day for 10 days with a dose escalation of valproic acid from 20 to 50 mg/kg/day for 10 days in patients with high grade MDS or AML [73]. Fifty four patients of median age 60 (range 5–80 years) were enrolled, 48 patients had AML and 6 had MDS, 11 patients were previously untreated. Twelve patients responded to therapy; 10 developed a CR and 2 a CR with incomplete platelet recovery. Median responses were seen after 2 months (range 29–130 days) and responders survived a median of 15.3 (range 4.6–20.2+) months vs. 4.9 (0.6–17.8+) months in non-responders [73]. Responders were more likely to have been randomized to a higher dose of valproic acid. Although changes in methylation (both gene specific events, including $p15^{INK4B}$, and genome wide methylation, by LINE-1 pyrosequencing) and gene expression were analyzed in the patients on this study no correlations with response were observed [73]. All patients experienced a decrease in genome wide methylation which correlated with Dac exposure. In a second study, this one employing Dac 20 mg/m^2/day for 10 days intravenously, responses were also encouraging with an overall response rate of 44% in 11 of 25 enrolled patients [57]. This trial enrolled 25 AML patients, in whom the median age was 70 years; 12 patients were untreated and 13 had relapsed disease. In this group of slightly older patients, encephalopathy was the principal toxicity and this was dose limiting at 20–25 mg/kg/day. In an intent-to-treat analysis, the response rate was 52% (13). CR was observed in 8 patients and PR in 4. Responses appeared similar for patients who received Dac alone and for those who received valproic acid in addition. In this study, re-expression of estrogen receptor was statistically significantly associated with clinical response ($p=0.05$), however although the investigators also demonstrated ER promoter demethylation, global DNA hypomethylation, depletion of DNA methyltransferase enzyme, and histone hyperacetylation, these markers did not correlate with response [57].

The combination of Aza with vorinostat (SAHA) has also been explored. In one phase I trial in patients with MDS and AML this combination produced an impressive overall response rate of 64%[74]. A second phase II trial of this combination in patients with MDS and AML has also been reported [75]. This trial enrolled 17 untreated patients and demonstrated an overall response rate of 41% ($n=7$) [75]. Similar outcomes (overall response of 37%) were observed in patients receiving a combination of Aza with the compound MGCD0103, an oral isotype-selective HDACi [76]. Although these responses appear to be encouraging, a majority of these combination studies have been published to date only in abstract form and larger studies are necessary in order to verify their superiority.

Data from one of the first randomized phase II studies to enroll patients either on single or double agent therapy was presented at the 2010 ASH meeting and reviewed in detail earlier in this manuscript (see Aza section under Sect. 5.2.1.4), this study, at least, suggests that combination therapy may not be superior [37]. In this trial patients with either MDS or AML with MDS related changes were randomized to receive either Aza at 50 mg/m^2 for 10 days subcutaneously alone or Aza in combination

with entinostat 4 mg/m^2 orally on days 3 and 10. Although the final results of this trial have not yet been published, it is important to note that the response rates for patients enrolled to receive Aza alone were indistinguishable from those who got the combination.

These results and others with a variety of HDACis may underestimate the value of combined therapy. It is important to note that among the many mechanisms postulated to be responsible for the efficacy of HDACis are induction of apoptosis and cell cycle arrest [77]. Since azanucleotides require DNA replication in order to produce DNA demethylation, it may be that administration of HDACi simultaneously or even in advance of the azanucleotide may result in diminished incorporation and limit responsiveness. Presently, a multi-institution phase II sequence study designed to address this question is open for enrollment [78].

13.7 Azanucleotides and Conventional Chemotherapy

One study has been published which explores the possible role of azanucleotide in "priming" leukemia cells for death [79]. This open label, phase I study was designed to address the safety and feasibility of Dac at a dose of 20 mg/m^2 either as a continuous infusion or a short infusion for 3, 5, or 7 days followed by standard dose 7 + 3 IC (cytarabine 100 mg/m^2/day continuous intravenous infusion for 7 days + daunorubicin 60 mg/m^2/day for 3 days). The study enrolled 30 patients of median age 55 (range 23–60) with newly diagnosed AML and a less than favorable karyotype (inv(16), t(8;21) and APL patients were excluded). Thirteen patients had complex, 11q23 or chromosome 7 abnormality associated leukemias and 8 had an antecedent hematological diagnosis. Toxicity was not dissimilar to that seen with 7 + 3 alone, although there appeared to be slightly more gastrointestinal toxicity in the group treated with 7 days of Dac priming, and there were no deaths. All subjects received consolidation, 20 patients went on to receive allogeneic bone marrow transplantation. Overall 27 (90%) of patients responded to one course of induction therapy, 17 patients achieved a CR and 10 a PR, patients scored as a PR all achieved hematological remission, but went on to receive a second course of induction resulting in a CR in 8/10 patients [79]. The overall CR rate following 1 or 2 cycles of induction therapy was therefore 83%. With a median follow-up of 32 months, 53% of patients (16/30) remained alive and in CR, 14 subjects died, 3 of complications related to allogeneic bone marrow transplant and the remainder died of relapsed or refractory AML [79]. The correlative DNA methylation analysis of this study revealed universal demethylation at both gene specific and genome wide loci with all schedules of Dac. The most potent hypomethylation was observed in patients treated with bolus, rather than continuous infusion schedules of Dac.

Although preliminary, this phase I trial demonstrated a remarkably good CR rate and a randomized phase II study designed to assess the two most potent demethylation schedules of Dac priming identified by this study should begin accrual in 2012.

13.8 Azanucleotides and Bone Marrow Transplantation

Allogeneic bone marrow transplant (allo-transplant) is the only curative strategy currently available for patients with MDS and high risk AML. Presently the role of hypomethylating agents both prior to and following transplant is under investigation.

Several small retrospective studies of azanucleotide induction prior to allo-transplant have been reported, two using Dac and two using Aza. The first of these reported outcomes in 17 patients with MDS of median age 55.5 (range 36–66) years undergoing allo-transplant (12 sibling donor, 5 unrelated donor) after prior therapy with Dac (various dosing regimens) [80]. These patients received predominantly reduced intensity conditioning and peripheral blood stem cells (13/17). With median follow-up of 12 (range 3–35) months, 8 patients remained in CR [80]. A second prospective study performed in Europe reported similar results in 15 patients of median age 69 (range 60–75) years with either MDS ($n=10$) or AML ($n=5$) [81]. All patients were treated with upfront Dac followed by reduced intensity allo-transplant (4 sibling donor, 11 unrelated donor). Fourteen patients achieved a CR (93%), with a median duration of 5 (range 1–51) months [81]. The relapse rate in this group was similar (4/15) to that reported retrospectively. The third study examined outcomes in 54 patients with MDS or CMML who either received (30) or did not receive (24) prior therapy with Aza [82]. Patients treated with Aza received a median of 4 (range 1–7) courses prior to transplant. The overall, relapse free and cumulative relapse 1 year following transplant were 47, 41, and 20%, for those patients treated with Aza and 60, 51, and 32% for untreated patients and these results were not statistically significantly different [82]. The final trial using Aza was a retrospective review of 68 patients undergoing allo-transplant for MDS or AML arising from MDS [83]. Thirty five patients received Aza followed by either myeloablative (40%) or reduced intensity (60%) conditioning. Thirty three patients received IC followed by allo-transplant. In these two, albeit somewhat different groups, the OS at 1 year was 57% in those treated with Aza and 36% in the IC group [83]. Overall these data suggest that Dac and Aza are a reasonable pre-transplantation strategy that does not adversely affect outcome when compared with high dose induction or supportive care. A phase II clinical trial of Dac prior to allo-transplant is ongoing in Singapore using the currently favored schedule of 20 mg/m^2/day for 5 days intravenously.

Post-transplant relapse remains a significant problem in MDS and high risk AML patients. Traditionally relapses in this population have been managed with donor lymphocyte infusions (DLI) (in those who do not demonstrate graft vs. host disease) or re-induction with traditional chemotherapeutic agents. Although limited prospective data exist on the use of azanucleotides for salvage of patients relapsing following allogeneic transplant, or as a preventive strategy following transplant, several small studies have been published, suggesting that these agents may have a significant role to play.

The first of these examined the efficacy of Aza at a flat dose of 100 mg subcutaneously days 1–3 followed by planned DLI on day 10 [84]. Cycles were repeated

every 22 days for a median of 2 (range 1–10) courses to 26 patients with relapsed AML ($n=24$) or CMML ($n=2$) following allo-transplant. Toxicity with this combination was as expected and consisted of infections and GVHD. Four patients (15%) were salvaged with a complete and lasting CR following this combination [84].

A second study, this one retrospective, described the results of salvage with Aza 100 mg/m^2 for 5 days in 22 patients of median age of 50 (range 28–69) years, with either AML (17) or MDS (5) relapsed following allo-transplant [85]. A majority (20/23) of these patients had received a myeloablative conditioning regimen and half (10/23) had a sibling donor. On average two cycles of Aza were administered (range 1–8). Most patients also received DLI (18/23). In this group, 5 patients (23%) achieved a CR lasting a median of 433 days (range 114–769) with a 2-year survival rate of 23%[85].

A third single institution study, retrospectively reviewed Aza 75 mg/m^2 for 5 or 7 days as salvage in 10 patients with MDS (9) or AML (1) of median age 55 (range 25–67) years [86]. Seven patients achieved CR or stable disease with this regimen, 3 of whom progressed after a median of 6 cycles. The median OS (OS) for the group was 422.5 days (range 127–1,411).

Taken together these results are encouraging and a variety of studies are ongoing to determine prospectively the role of azanucleotides both before and after allo-transplant [87].

13.9 Molecular Determinants of DNMTi Response in MDS and AML

Early on in the development of azanucleosides for the treatment of myeloid disease there was considerable enthusiasm for the identification of molecular markers of disease response. Initially several authors examined gene specific methylation reversal, including *p15^{INK4B}* and ER as discussed earlier in this manuscript [10, 55, 57, 70, 71]. Disappointingly, although reversal of methylation at many loci has been documented following azanucleotide exposure, it has not been demonstrate to correlate with or predict response to treatment, but rather seems to reflect duration of exposure to hypomethylating agents [88]. Another marker of response which has been studied is p53-inducible-ribonucleotide-reductase (p53R2), a gene identified in cell line screens to be induced following decitabine exposure [89, 90]. Link and colleagues demonstrated a statistically significant concordance between response to therapy and induction of p53R2 both at the mRNA and protein levels [90]. Although these results are thought provoking, they require sampling after many cycles of therapy and it is difficult to determine how useful a biomarker of response this would be clinically.

The identification of mutations in the genes encoding *TET2* (ten–eleven translocation2) and *DNMT3A* in patients with MDS and AML have raised questions about whether response to therapy may depend upon genetic characteristics of the underlying myeloid neoplasm. Recently a number of authors have demonstrated that up

to 26% of patients with MDS demonstrate mutations in TET2, and further that MDS patients with *TET2* mutations appear to have a superior prognosis (although this is not as clear in patients with AML) [91, 92]. Since *TET2* encodes a dioxygenase which functions to convert 5-methylcytosine to 5-hydroxymethylcytosine resulting in DNA demethylation at selective loci, defects in TET2 function would be expected to result in hypermethylation. One recent study suggests that patients bearing *TET2* mutations have a superior response (CR + PR + HI) to Aza treatment 82% vs. 45% ($p = 0.007$), although OS was not different in the two groups and these results have yet to be validated [93]. By contrast with mutations involving *TET2,* mutations in *DMNT3A* have been demonstrated to predict adverse outcome in both MDS and AML, although as yet no evaluation has been made of the impact of such mutations on response to epigenetic therapies [94–96].

13.10 Conclusions

Azanucleotides have changed the landscape of treatment for patients with MDS and AML with MDS related changes. Ongoing work with these agents in patients with a variety of myeloid diseases is likely to result in advances over the next few years. Despite the considerable efficacy of these drugs, patients with underlying myelodysplasia continue to have a remarkably poor outcome and novel strategies in these diseases remain essential. As we continue to develop insight into the mechanism(s) which underlie the activity of these drugs, perhaps we will be able to understand why they work so well for some patients and what strategies will maximize the longevity of these responses. Certainly it has become clear that single agent azanucleotides given on a conventional schedule are not a panacea. Whether responses can be optimized with continuous dosing strategies, combination with other drugs, or allogeneic bone marrow transplantation remains a question yet to be answered by well designed clinical trials.

References

1. Vardiman JW, Harris NL, Brunning RD (2002) The World Health Organization (WHO) classification of the myeloid neoplasms. Blood 100:2292–2302
2. Greenberg PL (1998) Risk factors and their relationship to prognosis in myelodysplastic syndromes. Leuk Res 22(Suppl 1):S3–S6
3. Menzin J, Lang K, Earle CC, Kerney D, Mallick R (2002) The outcomes and costs of acute myeloid leukemia among the elderly. Arch Intern Med 162:1597–1603
4. Lowenberg B, Downing JR, Burnett A (1999) Acute myeloid leukemia. N Engl J Med 341:1051–1062
5. Howlader N, Noone AM, Krapcho M, Neyman N, Aminou R, Waldron W, Altekruse SF, Kosary CL, Ruhl J, Tatalovich Z, Cho H, Mariotto A, Eisner MP, Lewis DR, Chen HS, Feuer EJ, Cronin KA, Edwards BK (2011) SEER Cancer Statistics Review, 1975-2008, National Cancer Institute. Bethesda, MD, based on November 2010 SEER data submission, posted to the SEER web site

6. Kantarjian H, O'brien S, Cortes J, Giles F, Faderl S, Jabbour E, Garcia-Manero G, Wierda W, Pierce S, Shan J, Estey E (2006) Results of intensive chemotherapy in 998 patients age 65 years or older with acute myeloid leukemia or high-risk myelodysplastic syndrome: predictive prognostic models for outcome. Cancer 106:1090–1098
7. Fenaux P, Mufti GJ, Hellstrom-Lindberg E, Santini V, Finelli C, Giagounidis A, Schoch R, Gattermann N, Sanz G, List A, Gore SD, Seymour JF, Bennett JM, Byrd J, Backstrom J, Zimmerman L, McKenzie D, Beach C, Silverman LR (2009) International Vidaza High-Risk MDS Survival Study Group: Efficacy of azacitidine compared with that of conventional care regimens in the treatment of higher-risk myelodysplastic syndromes: a randomised, open-label, phase III study. Lancet Oncol 10:223–232
8. Tilly H, Castaigne S, Bordessoule D, Casassus P, Le Prise PY, Tertian G, Desablens B, Henry-Amar M, Degos L (1990) Low-dose cytarabine versus intensive chemotherapy in the treatment of acute nonlymphocytic leukemia in the elderly. J Clin Oncol 8:272–279
9. Gardin C, Turlure P, Fagot T, Thomas X, Terre C, Contentin N, Raffoux E, de Botton S, Pautas C, Reman O, Bourhis JH, Fenaux P, Castaigne S, Michallet M, Preudhomme C, de Revel T, Bordessoule D, Dombret H (2007) Postremission treatment of elderly patients with acute myeloid leukemia in first complete remission after intensive induction chemotherapy: results of the multicenter randomized Acute Leukemia French Association (ALFA) 9803 trial. Blood 109:5129–5135
10. Kantarjian H, Issa JP, Rosenfeld CS, Bennett JM, Albitar M, DiPersio J, Klimek V, Slack J, de Castro C, Ravandi F, Helmer R III, Shen L, Nimer SD, Leavitt R, Raza A, Saba H (2006) Decitabine improves patient outcomes in myelodysplastic syndromes: results of a phase III randomized study. Cancer 106:1794–1803
11. Cheson BD, Bennett JM, Kantarjian H, Pinto A, Schiffer CA, Nimer SD, Lowenberg B, Beran M, de Witte TM, Stone RM, Mittelman M, Sanz GF, Wijermans PW, Gore S, Greenberg PL (2000) World Health Organization(WHO) international working group: Report of an international working group to standardize response criteria for myelodysplastic syndromes. Blood 96:3671–3674
12. Cheson BD, Cassileth PA, Head DR, Schiffer CA, Bennett JM, Bloomfield CD, Brunning R, Gale RP, Grever MR, Keating MJ (1990) Report of the National Cancer Institute-sponsored workshop on definitions of diagnosis and response in acute myeloid leukemia. J Clin Oncol 8:813–819
13. Lubbert M, Suciu S, Baila L, Ruter BH, Platzbecker U, Giagounidis A, Selleslag D, Labar B, Germing U, Salih HR, Beeldens F, Muus P, Pfluger KH, Coens C, Hagemeijer A, Eckart Schaefer H, Ganser A, Aul C, de Witte T, Wijermans PW (2011) Low-dose decitabine versus best supportive care in elderly patients with intermediate- or high-risk myelodysplastic syndrome (MDS) ineligible for intensive chemotherapy: final results of the randomized phase III study of the European Organisation for Research and Treatment of Cancer Leukemia Group and the German MDS Study Group. J Clin Oncol 29:1987–1996
14. Kadia TM, Jabbour E, Kantarjian H (2011) Failure of hypomethylating agent-based therapy in myelodysplastic syndromes. Semin Oncol 38:682–692
15. Yang X, Lay F, Han H, Jones PA (2010) Targeting DNA methylation for epigenetic therapy. Trends Pharmacol Sci 31:536–546
16. Weiss AJ, Stambaugh JE, Mastrangelo MJ, Laucius JF, Bellet RE (1972) Phase I study of 5-azacytidine (NSC-102816). Cancer Chemother Rep 56:413–419
17. Karon M, Sieger L, Leimbrock S, Finklestein JZ, Nesbit ME, Swaney JJ (1973) 5-Azacytidine: a new active agent for the treatment of acute leukemia. Blood 42:359–365
18. McCredie KB, Bodey GP, Burgess MA, Gutterman JU, Rodriguez V, Sullivan MP, Freireich EJ (1973) Treatment of acute leukemia with 5-azacytidine (NSC-102816). Cancer Chemother Rep 57:319–323
19. Constantinides PG, Taylor SM, Jones PA (1978) Phenotypic conversion of cultured mouse embryo cells by aza pyrimidine nucleosides. Dev Biol 66:57–71
20. Jones PA, Taylor SM (1980) Cellular differentiation, cytidine analogs and DNA methylation. Cell 20:85–93

21. Jones PA, Taylor SM (1981) Hemimethylated duplex DNAs prepared from 5-azacytidine-treated cells. Nucleic Acids Res 9:2933–2947
22. Quesnel B, Guillerm G, Vereecque R, Wattel E, Preudhomme C, Bauters F, Vanrumbeke M, Fenaux P (1998) Methylation of the p15(INK4b) gene in myelodysplastic syndromes is frequent and acquired during disease progression. Blood 91:2985–2990
23. Esteller M (2003) Profiling aberrant DNA methylation in hematologic neoplasms: a view from the tip of the iceberg. Clin Immunol 109:80–88
24. Voso MT, Scardocci A, Guidi F, Zini G, Di Mario A, Pagano L, Hohaus S, Leone G (2004) Aberrant methylation of DAP-kinase in therapy-related acute myeloid leukemia and myelodysplastic syndromes. Blood 103:698–700
25. Silverman LR, Holland JF, Weinberg RS, Alter BP, Davis RB, Ellison RR, Demakos EP, Cornell CJ Jr (1993) Carey RW, Schiffer C: Effects of treatment with 5-azacytidine on the in vivo and in vitro hematopoiesis in patients with myelodysplastic syndromes. Leukemia 7(Suppl 1):21–29
26. Chitambar CR, Libnoch JA, Matthaeus WG, Ash RC, Ritch PS, Anderson T (1991) Evaluation of continuous infusion low-dose 5-azacytidine in the treatment of myelodysplastic syndromes. Am J Hematol 37:100–104
27. Silverman LR, Demakos EP, Peterson BL, Kornblith AB, Holland JC, Odchimar-Reissig R, Stone RM, Nelson D, Powell BL, DeCastro CM, Ellerton J, Larson RA, Schiffer CA, Holland JF (2002) Randomized controlled trial of azacitidine in patients with the myelodysplastic syndrome: a study of the cancer and leukemia group B. J Clin Oncol 20:2429–2440
28. Silverman LR, McKenzie DR, Peterson BL, Holland JF, Backstrom JT, Beach CL, Larson RA (2006) Cancer and Leukemia Group B: Further analysis of trials with azacitidine in patients with myelodysplastic syndrome: studies 8421, 8921, and 9221 by the Cancer and Leukemia Group B. J Clin Oncol 24:3895–3903
29. Heim S (1992) Cytogenetic findings in primary and secondary MDS. Leuk Res 16:43–46
30. Arber DA, Brunning RD, Orazi A et al (2008) Acute myeloid leukaemaia with myelodysplastic-related changes. In: Swerdlow SH, Campo E, Harris NL et al (eds) WHO classification of tumors of haematopoietic and lympohoid tissues (4th edn). Lyon, International Agency for Research on Cancer
31. Harris NL, Jaffe ES, Diebold J, Flandrin G, Muller-Hermelink HK, Vardiman J, Lister TA, Bloomfield CD (1999) World Health Organization classification of neoplastic diseases of the hematopoietic and lymphoid tissues: report of the Clinical Advisory Committee meeting-Airlie House, Virginia, November 1997. J Clin Oncol 17:3835–3849
32. Fenaux P, Mufti GJ, Hellstrom-Lindberg E, Santini V, Gattermann N, Germing U, Sanz G, List AF, Gore S, Seymour JF, Dombret H, Backstrom J, Zimmerman L, McKenzie D, Beach CL, Silverman LR (2010) Azacitidine prolongs overall survival compared with conventional care regimens in elderly patients with low bone marrow blast count acute myeloid leukemia. J Clin Oncol 28:562–569
33. Maurillo L, Venditti A, Spagnoli A, Gaidano G, Ferrero D, Oliva E, Lunghi M, D'Arco AM, Levis A, Pastore D, Di Renzo N, Santagostino A, Pavone V, Buccisano F, Musto P (2012) Azacitidine for the treatment of patients with acute myeloid leukemia: Report of 82 patients enrolled in an Italian compassionate program. Cancer 118:1014–1022
34. Al-Ali HK, Jaekel N, Junghanss C, Maschmeyer G, Krahl R, Cross M, Hoppe G, Niederwieser D (2012) Azacitidine in patients with acute myeloid leukemia medically unfit for or resistant to chemotherapy: a multicenter phase I/II study. Leuk Lymphoma 53:110–117
35. Blum W, Garzon R, Klisovic RB, Schwind S, Walker A, Geyer S, Liu S, Havelange V, Becker H, Schaaf L, Mickle J, Devine H, Kefauver C, Devine SM, Chan KK, Heerema NA, Bloomfield CD, Grever MR, Byrd JC, Villalona-Calero M, Croce CM, Marcucci G (2010) Clinical response and miR-29b predictive significance in older AML patients treated with a 10-day schedule of decitabine. Proc Natl Acad Sci USA 107:7473–7478
36. Lyons RM, Cosgriff TM, Modi SS, Gersh RH, Hainsworth JD, Cohn AL, McIntyre HJ, Fernando IJ, Backstrom JT, Beach CL (2009) Hematologic response to three alternative dosing schedules of azacitidine in patients with myelodysplastic syndromes. J Clin Oncol 27:1850–1856

37. Prebet T, Sun Z, Ketterling RP, Hicks G, Beach CL, Greenberg PL, Paietta EM, Czader M, Gabrilove J, Erba H, Tallman MS, Gore SD (2010) A 10 day schedule of azacitidine induces more complete cytogenetic remissions than the standard schedule in myelodysplasia and acute myeloid leukemia with myelodysplasia-related changes: results of the E1905 US Leukemia Intergroup Study. Blood 116(21):Abst. 4013
38. Marcucci G, Silverman L, Eller M, Lintz L, Beach CL (2005) Bioavailability of azacitidine subcutaneous versus intravenous in patients with the myelodysplastic syndromes. J Clin Pharmacol 45:597–602
39. Martin MG, Walgren RA, Procknow E, Uy GL, Stockerl-Goldstein K, Cashen AF, Westervelt P, Abboud CN, Kreisel F, Augustin K, Dipersio JF, Vij R (2009) A phase II study of 5-day intravenous azacitidine in patients with myelodysplastic syndromes. Am J Hematol 84:560–564
40. Uchida T, Ogawa Y, Kobayashi Y, Ishikawa T, Ohashi H, Hata T, Usui N, Taniwaki M, Ohnishi K, Akiyama H, Ozawa K, Ohyashiki K, Okamoto S, Tomita A, Nakao S, Tobinai K, Ogura M, Ando K, Hotta T (2011) Phase I and II study of azacitidine in Japanese patients with myelodysplastic syndromes. Cancer Sci 102:1680–1686
41. Kaminskas E, Farrell AT, Wang YC, Sridhara R, Pazdur R (2005) FDA drug approval summary: azacitidine (5-azacytidine, Vidaza) for injectable suspension. Oncologist 10:176–182
42. Ziemba A, Hayes E, Freeman BB III, Ye T, Pizzorno G (2011) Development of an oral form of azacytidine: 2'3'5'triacetyl-5-azacytidine. Chemother Res Pract 2011:965826
43. Garcia-Manero G, Stoltz ML, Ward MR, Kantarjian H, Sharma S (2008) A pilot pharmacokinetic study of oral azacitidine. Leukemia 22:1680–1684
44. Garcia-Manero G, Gore SD, Cogle C, Ward R, Shi T, Macbeth KJ, Laille E, Giordano H, Sakoian S, Jabbour E, Kantarjian H, Skikne B (2011) Phase I study of oral azacitidine in myelodysplastic syndromes, chronic myelomonocytic leukemia, and acute myeloid leukemia. J Clin Oncol 29:2521–2527
45. Hollenbach PW, Nguyen AN, Brady H, Williams M, Ning Y, Richard N, Krushel L, Aukerman SL, Heise C, MacBeth KJ (2010) A comparison of azacitidine and decitabine activities in acute myeloid leukemia cell lines. PLoS One 5:e9001
46. Pinto A, Attadia V, Fusco A, Ferrara F, Spada OA, Di Fiore PP (1984) 5-Aza-2′-deoxycytidine induces terminal differentiation of leukemic blasts from patients with acute myeloid leukemias. Blood 64:922–929
47. Petti MC, Mandelli F, Zagonel V, De Gregoris C, Merola MC, Latagliata R, Gattei V, Fazi P, Monfardini S, Pinto A (1993) Pilot study of 5-aza-2′-deoxycytidine (Decitabine) in the treatment of poor prognosis acute myelogenous leukemia patients: preliminary results. Leukemia 7(Suppl 1):36–41
48. Zagonel V, Lo Re G, Marotta G, Babare R, Sardeo G, Gattei V, De Angelis V, Monfardini S, Pinto A (1993) 5-Aza-2′-deoxycytidine (Decitabine) induces trilineage response in unfavourable myelodysplastic syndromes. Leukemia 7(Suppl 1):30–35
49. Willemze R, Archimbaud E, Muus P (1993) Preliminary results with 5-aza-2′-deoxycytidine (DAC)-containing chemotherapy in patients with relapsed or refractory acute leukemia. The EORTC Leukemia Cooperative Group. Leukemia 7(Suppl 1):49–50
50. Kantarjian HM, O'Brien SM, Estey E, Giralt S, Beran M, Rios MB, Keating M, de Vos D, Talpaz M (1997) Decitabine studies in chronic and acute myelogenous leukemia. Leukemia 11(Suppl 1):S35–S36
51. Wijermans PW, Krulder JW, Huijgens PC, Neve P (1997) Continuous infusion of low-dose 5-Aza-2′-deoxycytidine in elderly patients with high-risk myelodysplastic syndrome. Leukemia 11(Suppl 1):S19–S23
52. Wijermans P, Lubbert M, Verhoef G, Bosly A, Ravoet C, Andre M, Ferrant A (2000) Low-dose 5-aza-2′-deoxycytidine, a DNA hypomethylating agent, for the treatment of high-risk myelodysplastic syndrome: a multicenter phase II study in elderly patients. J Clin Oncol 18:956–962
53. Lubbert M, Wijermans P, Kunzmann R, Verhoef G, Bosly A, Ravoet C, Andre M, Ferrant A (2001) Cytogenetic responses in high-risk myelodysplastic syndrome following low-dose

treatment with the DNA methylation inhibitor 5-aza-2′-deoxycytidine. Br J Haematol 114:349–357
54. Issa JP, Garcia-Manero G, Giles FJ, Mannari R, Thomas D, Faderl S, Bayar E, Lyons J, Rosenfeld CS, Cortes J, Kantarjian HM (2004) Phase 1 study of low-dose prolonged exposure schedules of the hypomethylating agent 5-aza-2′-deoxycytidine (decitabine) in hematopoietic malignancies. Blood 103:1635–1640
55. Kantarjian H, Oki Y, Garcia-Manero G, Huang X, O'Brien S, Cortes J, Faderl S, Bueso-Ramos C, Ravandi F, Estrov Z, Ferrajoli A, Wierda W, Shan J, Davis J, Giles F, Saba HI, Issa JP (2007) Results of a randomized study of 3 schedules of low-dose decitabine in higher-risk myelodysplastic syndrome and chronic myelomonocytic leukemia. Blood 109:52–57
56. Steensma DP, Baer MR, Slack JL, Buckstein R, Godley LA, Garcia-Manero G, Albitar M, Larsen JS, Arora S, Cullen MT, Kantarjian H (2009) Multicenter study of decitabine administered daily for 5 days every 4 weeks to adults with myelodysplastic syndromes: the alternative dosing for outpatient treatment (ADOPT) trial. J Clin Oncol 27:3842–3848
57. Blum W, Klisovic RB, Hackanson B, Liu Z, Liu S, Devine H, Vukosavljevic T, Huynh L, Lozanski G, Kefauver C, Plass C, Devine SM, Heerema NA, Murgo A, Chan KK, Grever MR, Byrd JC, Marcucci G (2007) Phase I study of decitabine alone or in combination with valproic acid in acute myeloid leukemia. J Clin Oncol 25:3884–3891
58. Braun T, Itzykson R, Renneville A, de Renzis B, Dreyfus F, Laribi K, Bouabdallah K, Vey N, Toma A, Recher C, Royer B, Joly B, Vekhoff A, Lafon I, Sanhes L, Meurice G, Orear C, Preudhomme C, Gardin C, Ades L, Fontenay M, Fenaux P, Droin N, Solary E (2011) Groupe Francophone des Myelodysplasies: Molecular predictors of response to decitabine in advanced chronic myelomonocytic leukemia: a phase 2 trial. Blood 118:3824–3831
59. Wijermans PW, Ruter B, Baer MR, Slack JL, Saba HI, Lubbert M (2008) Efficacy of decitabine in the treatment of patients with chronic myelomonocytic leukemia (CMML). Leuk Res 32:587–591
60. Costa R, Abdulhaq H, Haq B, Shadduck RK, Latsko J, Zenati M, Atem FD, Rossetti JM, Sahovic EA, Lister J (2011) Activity of azacitidine in chronic myelomonocytic leukemia. Cancer 117:2690–2696
61. Prebet T, Gore SD, Esterni B, Gardin C, Itzykson R, Thepot S, Dreyfus F, Rauzy OB, Recher C, Ades L, Quesnel B, Beach CL, Fenaux P, Vey N (2011) Outcome of high-risk myelodysplastic syndrome after azacitidine treatment failure. J Clin Oncol 29:3322–3327
62. Jabbour E, Garcia-Manero G, Batty N, Shan J, O'Brien S, Cortes J, Ravandi F, Issa JP, Kantarjian H (2010) Outcome of patients with myelodysplastic syndrome after failure of decitabine therapy. Cancer 116:3830–3834
63. Talbert PB, Henikoff S (2010) Histone variants–ancient wrap artists of the epigenome. Nat Rev Mol Cell Biol 11:264–275
64. Lin RJ, Nagy L, Inoue S, Shao W, Miller WH Jr (1998) Evans RM: Role of the histone deacetylase complex in acute promyelocytic leukaemia. Nature 391:811–814
65. Altucci L, Gronemeyer H (2001) The promise of retinoids to fight against cancer. Nat Rev Cancer 1:181–193
66. Gelmetti V, Zhang J, Fanelli M, Minucci S, Pelicci PG, Lazar MA (1998) Aberrant recruitment of the nuclear receptor corepressor-histone deacetylase complex by the acute myeloid leukemia fusion partner ETO. Mol Cell Biol 18:7185–7191
67. Richon VM, Emiliani S, Verdin E, Webb Y, Breslow R, Rifkind RA, Marks PA (1998) A class of hybrid polar inducers of transformed cell differentiation inhibits histone deacetylases. 95:3003–3007
68. Quintas-Cardama A, Santos FP, Garcia-Manero G (2011) Histone deacetylase inhibitors for the treatment of myelodysplastic syndrome and acute myeloid leukemia. Leukemia 25:226–235
69. Cameron EE, Bachman KE, Myohanen S, Herman JG, Baylin SB (1999) Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. Nat Genet 21:103–107

70. Gore SD, Baylin S, Sugar E, Carraway H, Miller CB, Carducci M, Grever M, Galm O, Dauses T, Karp JE, Rudek MA, Zhao M, Smith BD, Manning J, Jiemjit A, Dover G, Mays A, Zwiebel J, Murgo A, Weng LJ, Herman JG (2006) Combined DNA methyltransferase and histone deacetylase inhibition in the treatment of myeloid neoplasms. Cancer Res 66:6361–6369
71. Fandy TE, Herman JG, Kerns P, Jiemjit A, Sugar EA, Choi SH, Yang AS, Aucott T, Dauses T, Odchimar-Reissig R, Licht J, McConnell MJ, Nasrallah C, Kim MK, Zhang W, Sun Y, Murgo A, Espinoza-Delgado I, Oteiza K, Owoeye I, Silverman LR, Gore SD, Carraway HE (2009) Early epigenetic changes and DNA damage do not predict clinical response in an overlapping schedule of 5-azacytidine and entinostat in patients with myeloid malignancies. Blood 114:2764–2773
72. Maslak P, Chanel S, Camacho LH, Soignet S, Pandolfi PP, Guernah I, Warrell R, Nimer S (2006) Pilot study of combination transcriptional modulation therapy with sodium phenylbutyrate and 5-azacytidine in patients with acute myeloid leukemia or myelodysplastic syndrome. Leukemia 20:212–217
73. Garcia-Manero G, Kantarjian HM, Sanchez-Gonzalez B, Yang H, Rosner G, Verstovsek S, Rytting M, Wierda WG, Ravandi F, Koller C, Xiao L, Faderl S, Estrov Z, Cortes J, O'brien S, Estey E, Bueso-Ramos C, Fiorentino J, Jabbour E, Issa JP (2006) Phase 1/2 study of the combination of 5-aza-2′-deoxycytidine with valproic acid in patients with leukemia. Blood 108:3271–3279
74. Silverman LR, Verma A, Odchimar-Reissig R, LeBlanc A, Nejfeld V, Gabrilove JL (2008) A phase I trial of the epigenetic modulators vorinostat, in combination with azacitidine (azaC) in patients with the myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML): a study of the New York Cancer Consortium. Blood 112:3656
75. Garcia-Manero G, Estey E, Jabbour E, Kadia TM, Estrov Z, Cortes J (2010) Phase II study of 5-azacitidine and vorinostat in patients (pts) with newly diagnosed myelodysplastic syndrome (MDS) or acute myelogenous leukemia (AML) not eligible for clinicalt trials because poor performance of presence of other comorbidities. Blood 116:Abstr. 604
76. Garcia-Manero G, Yang AS, Giles F, Faderl S, Ravandi F, Cortes J, Newsome WJ, Issa JP, Patterson TA, Dubay M, Li Z, Kantarjian H, Martell RE (2007) Phase I/II study of MGCD0103, an oral isotype-selective histone deacetylase (HDAC) inhibitor, in combination with 5-Azacitidine in higher-risk myelodysplastic syndrome (MDS) and acute myelogenous leukemia (AML). Blood 110
77. Grant S (2009) New agents for AML and MDS. Best Pract Res Clin Haematol 22:501–507
78. Carraway HE, Sidney Kimmel Comprehensive Cancer Center (2000–2012, Feb 11) A trial to evaluate two schedules of MS275 in combination with 5AC in elderly patients with acute myeloid leukemia (AML). In: ClinicalTrials.gov [Internet]. Bethesda (MD): National Library of Medicine (US). Available from: http://www.clinicaltrials.gov/ct2/show/NCT01305499:NCT01305499
79. Scandura JM, Roboz GJ, Moh M, Morawa E, Brenet F, Bose JR, Villegas L, Gergis US, Mayer SA, Ippoliti CM, Curcio TJ, Ritchie EK, Feldman EJ (2011) Phase 1 study of epigenetic priming with decitabine prior to standard induction chemotherapy for patients with AML. Blood 118:1472–1480
80. De Padua SL, de Lima M, Kantarjian H, Faderl S, Kebriaei P, Giralt S, Davisson J, Garcia-Manero G, Champlin R, Issa JP, Ravandi F (2009) Feasibility of allo-SCT after hypomethylating therapy with decitabine for myelodysplastic syndrome. Bone Marrow Transplant 43:839–843
81. Lubbert M, Bertz H, Ruter B, Marks R, Claus R, Wasch R, Finke J (2009) Non-intensive treatment with low-dose 5-aza-2′-deoxycytidine (DAC) prior to allogeneic blood SCT of older MDS/AML patients. Bone Marrow Transplant 44:585–588
82. Field T, Perkins J, Huang Y, Kharfan-Dabaja MA, Alsina M, Ayala E, Fernandez HF, Janssen W, Lancet J, Perez L, Sullivan D, List A, Anasetti C (2010) 5-Azacitidine for myelodysplasia before allogeneic hematopoietic cell transplantation. Bone Marrow Transplant 45:255–260

83. Gerds AT, Gooley TA, Estey EH, Appelbaum FR, Deeg HJ, Scott BL (2012) Pre-transplant therapy with azacitidine vs induction chemotherapy and posttransplant outcome in patients with MDS. Biol Blood Marrow Transplant; in press. [Epub ahead of print]
84. Lubbert M, Bertz H, Wasch R, Marks R, Ruter B, Claus R, Finke J (2010) Efficacy of a 3-day, low-dose treatment with 5-azacytidine followed by donor lymphocyte infusions in older patients with acute myeloid leukemia or chronic myelomonocytic leukemia relapsed after allografting. Bone Marrow Transplant 45:627–632
85. Czibere A, Bruns I, Kroger N, Platzbecker U, Lind J, Zohren F, Fenk R, Germing U, Schroder T, Graf T, Haas R, Kobbe G (2010) 5-Azacytidine for the treatment of patients with acute myeloid leukemia or myelodysplastic syndrome who relapse after allo-SCT: a retrospective analysis. Bone Marrow Transplant 45:872–876
86. Bolanos-Meade J, Smith BD, Gore SD, McDevitt MA, Luznik L, Fuchs EJ, Jones RJ (2011) 5-Azacytidine as Salvage Treatment in Relapsed Myeloid Tumors After Allogeneic Bone Marrow Transplantation. Biol Blood Marrow Transplant 17:754–758
87. Loh Y (2000) Singapore General Hospital: Study of decitabine induction prior to allogeneic hematopoietic cell transplant in newly diagnosed MDS patients [cited 2012, Feb 11]. In: ClinicalTrials.gov [Internet]. Bethesda (MD): National Library of Medicine (US). http://www.clinicaltrials.gov/ct2/show/NCT01333449:NCT01333449
88. Shen L, Kantarjian H, Guo Y, Lin E, Shan J, Huang X, Berry D, Ahmed S, Zhu W, Pierce S, Kondo Y, Oki Y, Jelinek J, Saba H, Estey E, Issa JP (2010) DNA methylation predicts survival and response to therapy in patients with myelodysplastic syndromes. J Clin Oncol 28:605–613
89. Karpf AR, Lasek AW, Ririe TO, Hanks AN, Grossman D, Jones DA (2004) Limited gene activation in tumor and normal epithelial cells treated with the DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine. Mol Pharmacol 65:18–27
90. Link PA, Baer MR, James SR, Jones DA, Karpf AR (2008) p53-inducible ribonucleotide reductase (p53R2/RRM2B) is a DNA hypomethylation-independent decitabine gene target that correlates with clinical response in myelodysplastic syndrome/acute myelogenous leukemia. Cancer Res 68:9358–9366
91. Langemeijer SM, Kuiper RP, Berends M, Knops R, Aslanyan MG, Massop M, Stevens-Linders E, van Hoogen P, van Kessel AG, Raymakers RA, Kamping EJ, Verhoef GE, Verburgh E, Hagemeijer A, Vandenberghe P, de Witte T, van der Reijden BA, Jansen JH (2009) Acquired mutations in TET2 are common in myelodysplastic syndromes. Nat Genet 41:838–842
92. Kosmider O, Gelsi-Boyer V, Cheok M, Grabar S, Della-Valle V, Picard F, Viguié F, Quesnel B, Beyne-Rauzy O, Solary E, Vey N, Hunault-Berger M, Fenaux P, Mansat-De Mas V, Delabesse E, Guardiola P, Lacombe C, Vainchenker W, Preudhomme C, Dreyfus F, Bernard OA, Birnbaum D, Fontenay M, Groupe Francophone des Myélodysplasies (2009) TET2 mutation is an independent favorable prognostic factor in myelodysplastic syndromes (MDSs). Blood 114:3285–3291
93. Itzykson R, Kosmider O, Cluzeau T, Mansat-De Mas V, Dreyfus F, Beyne-Rauzy O, Quesnel B, Vey N, Gelsi-Boyer V, Raynaud S, Preudhomme C, Adès L, Fenaux P, Fontenay M, Groupe Francophone des Myelodysplasies (GFM) (2011) Impact of TET2 mutations on response rate to azacitidine in myelodysplastic syndromes and low blast count acute myeloid leukemias. Leukemia 25:1147–1152
94. Walter MJ, Ding L, Shen D, Shao J, Grillot M, McLellan M, Fulton R, Schmidt H, Kalicki-Veizer J, O'Laughlin M, Kandoth C, Baty J, Westervelt P, DiPersio JF, Mardis ER, Wilson RK, Ley TJ, Graubert TA (2011) Recurrent DNMT3A mutations in patients with myelodysplastic syndromes. Leukemia 25:1153–1158
95. Ley TJ, Ding L, Walter MJ, McLellan MD, Lamprecht T, Larson DE, Kandoth C, Payton JE, Baty J, Welch J, Harris CC, Lichti CF, Townsend RR, Fulton RS, Dooling DJ, Koboldt DC, Schmidt H, Zhang Q, Osborne JR, Lin L, O'Laughlin M, McMichael JF, Delehaunty KD, McGrath SD, Fulton LA, Magrini VJ, Vickery TL, Hundal J, Cook LL, Conyers JJ, Swift GW, Reed JP, Alldredge PA, Wylie T, Walker J, Kalicki J, Watson MA, Heath S, Shannon

WD, Varghese N, Nagarajan R, Westervelt P, Tomasson MH, Link DC, Graubert TA, DiPersio JF, Mardis ER, Wilson RK (2010) DNMT3A mutations in acute myeloid leukemia. N Engl J Med 363:2424–2433

96. Thol F, Damm F, Lüdeking A, Winschel C, Wagner K, Morgan M, Yun H, Göhring G, Schlegelberger B, Hoelzer D, Lübbert M, Kanz L, Fiedler W, Kirchner H, Heil G, Krauter J, Ganser A, Heuser M (2011) Incidence and prognostic influence of DNMT3A mutations in acute myeloid leukemia. J Clin Oncol 29:2889–2896

Chapter 14
Epigenetic Targeting Therapies to Overcome Chemotherapy Resistance

Curt Balch and Kenneth P. Nephew

Abstract It is now well established that epigenetic aberrations occur early in malignant transformation, raising the possibility of identifying chemopreventive compounds or reliable diagnostic screening using epigenetic biomarkers. Combinatorial therapies effective for the reexpression of tumor suppressors, facilitating resensitization to conventional chemotherapies, hold great promise for the future therapy of cancer. This approach may also perturb cancer stem cells and thus represent an effective means for managing a number of solid tumors. We believe that in the near future, anticancer drug regimens will routinely include epigenetic therapies, possibly in conjunction with inhibitors of "stemness" signal pathways, to effectively reduce the devastating occurrence of cancer chemotherapy resistance.

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Chemo-, radio-, and hormonal therapies have proved invaluable for the management of numerous solid and hematologic cancers. Commonly used chemotherapies include topoisomerase inhibitors, microtubule-targeting agents (for mitotic spindle disruption), and DNA-alkylating agents, while hormonal therapies include antiestrogens (such as tamoxifen) and androgen-ablating drugs [1]. Despite the success of these agents (often early during patient therapy), the majority of patients eventually develop resistance to these interventions, and it is believed that >90% of all cancer deaths result from therapy-refractory, metastatic disease [2, 3]. Resistance to therapy is believed to be multifactorial, involving reduced delivery/uptake, membrane efflux, metabolic inactivation, loss of the therapeutic target, and autocrine/paracrine signaling (involving the local tumor microenvironment). Attenuation of cancer cell death pathways, due to hyperactive growth/survival pathways and/or suppression of cell cycle arrest/apoptosis cascades, is considered a major contributor to the loss of therapeutic sensitivity in cancer [4, 5].

While tumor progression is clearly associated with DNA sequence anomalies (e.g., point mutations, DNA gains or losses within specific loci, and/or translocations), *epigenetic* aberrations are now believed to play an equivalent (or even greater) role [6–8]. Epigenetics is classically defined as the study of heritable changes in gene expression that occur without a change in the DNA sequence. Epigenetic modifications include methylation of C5 of cytosines within CG dyads, numerous posttranslational modifications of histone residues, repositioning of whole (histone octomer) nucleosomes, deposition of histone protein variants, and posttranscriptional regulation of protein translation by microRNAs [8–10].

As noted above, cancer progression is characterized by genetic and epigenetic misregulation of signal transduction cascades (often in association with altered microRNA expression) [11, 12], and it has been hypothesized that the cancer cell phenotype resembles a reversion of adult tissue cells to an embryonic-like state (i.e., loss of differentiation), with immortalization replacing age-related apoptosis and senescence [13, 14]. Analogously, one recent, increasingly accepted carcinogenesis paradigm is that a mature, heterogeneous tumor represents a "caricature" of the normal organ from which it derives, due to the abnormal differentiation of "cancer stem cells" (CSCs) [15]. Normal tissue stem cells are relatively long-lived, due to quiescence or relatively slow cell division and expression of various phenotypes that confer resistance to genotoxic or cytotoxic agents, including enhanced DNA repair, metabolic inactivation and/or expulsion of cytotoxins, oxidative stress protection, and enhanced pro-survival (i.e., antiapoptotic) signaling [16]. While not necessarily derived from normal stem cells [16], CSCs have been shown to possess numerous "stemness" phenotypes, including the aforementioned defense mechanisms against environmental insults, thus facilitating resistance to most conventional anticancer agents [15, 16]. In addition to studies of hematologic malignancies, chemoresistant stem-like cells have now been identified and characterized in several solid tumors, including hepatocellular, colon, breast, glioma, pancreatic, and ovarian cancers [16].

To reverse the multi-/pluripotent phenotypes of progenitor tumor cells, numerous well-known differentiation agents are under investigation as potential cancer therapeutics, including vitamin D, retinoids, arsenic trioxide, and phytochemicals

[18, 19]. It is feasible that successful delivery of differentiating agents to CSCs might reduce malignant stem cell populations and improve conventional therapy responses, in addition to hampering tumor regrowth [8]. Similar to normal development, which is governed by epigenetic modifications that allow tissue-specific gene expression [20], abnormal differentiation states of tumor subpopulations are also largely regulated by atypical epigenetic modifications to DNA/chromatin [21]. The existence of "epigenetic plasticity" (associated with extensive chromatin remodeling) [22] was further exemplified by the recent generation of "induced pluripotent," embryonic stem-like cells from terminally differentiated, adult tissue cells [23, 24]. By contrast, it was also demonstrated that even highly aggressive cancer cells (including melanoma and estrogen receptor-negative breast cancer cells) possess a highly "plastic" phenotype capable of reversion to their respective differentiated, normal tissue phenotypes [25, 26].

In this chapter, we discuss agents capable of reversing cancer-associated, repressive epigenetic modifications. The emphasis of this article is on the possible restoration of drug response pathways/targets that could potentially reverse chemoresistance, a destructive and usually fatal complication of numerous malignancies.

14.1 Preclinical Studies of DNA Hypomethylating and Deacetylase-Inhibiting Agents for Overcoming Drug Resistance

As noted above, cancer is often characterized by a loss of differentiated and tissue-specialized phenotypes, which are maintained by epigenetic modifications that drive lineage- and organ-specific development. Over the past 50 years, the L-1210 acute lymphoblastic and Friend erythroleukemia mouse models have been widely used to screen antileukemic compounds, several of which were found to possess differentiating activity [27, 28]. Several of those differentiating agents were later discovered to be inhibitors of repressive epigenetic modifications and more specifically, histone deacetylase and DNA methyltransferase inhibitors (HDACIs and DNMTIs, respectively) [8, 29, 30].

Preclinical cancer studies of DNA methyltransferase inhibitors (DNMTIs). The two best-characterized DNA methyltransferase inhibitors (DNMTIs) are 5-azacytidine (5-aza-C, Vidaza) and its deoxyribose analog, 5-aza-2′-deoxycytidine (5-aza-dC, decitabine), with both compounds possessing the non-methylatable pyrimidine analog azacytosine [31]. Both DNMTIs, first synthesized and shown as antileukemic in the 1960s (Fig. 14.1), are now FDA-approved for therapy of the hematologic malignancy myelodysplastic syndrome (MDS) [10] (see next section). Following cellular uptake, these cytidine analogs are triphosphorylated and incorporated into the newly synthesized DNA strand during S phase (5-aza-C is also integrated into RNA) [32]. However, a C5-to-N5 substitution in the cytosine six-member heterocyclic ring precludes methyl group acceptance, resulting in covalent and irreversible binding of the DNMT enzyme to the fraudulent base, followed by the eventual cel-

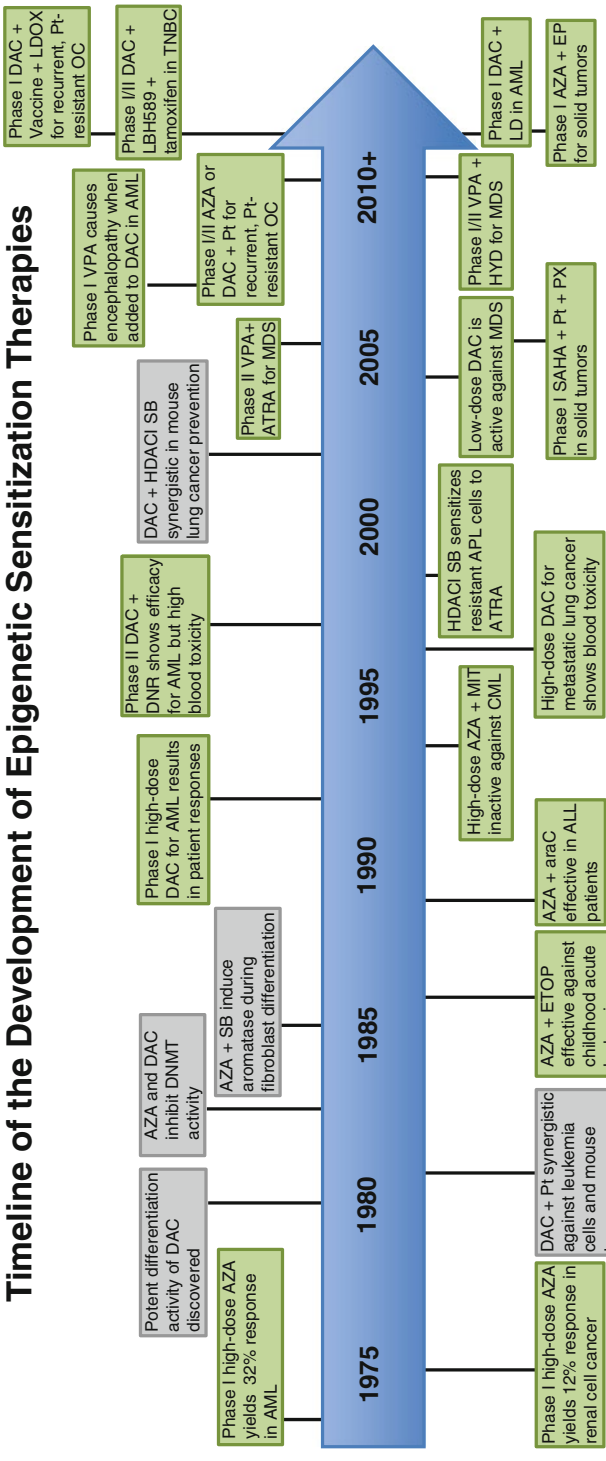

Fig. 14.1 Timeline of notable events supporting epigenetic sensitization to conventional cancer therapies. *Green boxes* indicate clinical studies, and *gray boxes* denote preclinical studies. The timeline chronology begins approximately 10 years after the discovery of DNA methyltransferase inhibitors. The cited studies are discussed in the text. See references [17, 29, 42, 43, 169, 171, 185, 211–222] for details. *AZA* 5-azacytidine or VIDAZA; *DAC* 5-aza-2′-deoxycytidine or decitabine/dacogen; *ALL* acute lymphoblastic leukemia; *AML* acute myeloid leukemia; *ara-C* arabinosylcytosine (cytarabine); *ATRA* all-trans retinoic acid; *CML* chronic myelogenous leukemia; *DNR* daunorubicin; *EP* erlotinib, Tarceva; *ETOP* etoposide; *FDA* Food and Drug Administration; *HDAC* histone deacetylase; *HDACI* histone deacetylase inhibitor; *HYD* hydralazine; *LD* lenalidomide, Revlimid; *LDOX* liposomal doxorubicin; *MDS* myelodyspastic syndrome; *MIT* mitoxatrone; *OC* epithelial ovarian cancer or primary peritoneal carcinoma; *PX* paclitaxel; *Pt* platinum; *SAHA* suberoylanilide hydroxamic acid, Vorinostat, Zolinza; *SB* sodium butyrate; *TNBC* triple negative breast cancer; *Vaccine* NY-ESO-1 protein immunization; *VPA* valproic acid (valproate)

lular depletion of DNMT, via ubiquitin-associated proteasome targeting [8, 10, 33]. Due to their requirement of nascent strand DNA incorporation, the hypomethylating activity of these cytosine analogs is replication-dependent, requiring several cell divisions to complete the demethylation of each DNA strand [34], consistent with successful patient trials typically requiring multiple treatment cycles prior to detectable response ([32, 35] and see following section).

Following their initial syntheses in 1964 [36], 5-aza-C and 5-aza-dC were later found to possess antileukemic activity in mouse disease models, elicit cancer cell differentiation, and enhance response to the chemotherapeutics etoposide and cisplatin [29, 37] (Fig. 14.1). These nucleoside analogs potently hypomethylate a number of tumor suppressor genes (TSGs), resulting in their transcriptional upregulation [6, 38, 39]. Decitabine-mediated DNA demethylation has also been reported to associate with reduced methylation at lysines 9 and 27 of histone H3 (H3K9 and H3K27, respectively), two other repressive chromatin "marks" [40, 41], in addition to enhanced acetylation at H3K9 and H3K14 (two activating chromatin marks). Such "crosstalk" between repressive chromatin modifications is believed to result from coordinated activity of histone and DNA methyltransferase enzymes associated with large, multimeric epigenetic repressive protein complexes.

Since its inception, the cytidine analog 5-aza-C has been extensively studied in cell and animal model systems. Early studies demonstrated potent antileukemic activity in the L1210 mouse model, followed by reports of 5-aza-C efficacy against solid tumors, using various preclinical cancer models (Fig. 14.1) [42, 43]. In medulloblastoma cells, 5-aza-C was also shown to inhibit proliferation, coincident with promoter demethylation and upregulation of a TSG, *KLF4* [44]. More recently, it was shown that intratracheal administration of 5-aza-C, in an orthotopic mouse lung cancer model, exhibited fivefold reduced myelosuppression and threefold enhanced survival, as compared to i.v. administration [45]. While subsequent studies further established 5-aza-C as a differentiating agent, particularly in effecting myogenesis [46–48], other work firmly established its ability to induce TSGs and initiate apoptosis in cancer cells, including those of the liver, colon, and ovary [49–51].

In contrast to 5-aza-C, its deoxyribose analog 5-aza-dC is not incorporated into RNA and is thus more stable and potent (active at submicromolar concentrations), although its activity is similarly attenuated by cytosine deaminases [8]. In a myriad of cell line studies, decitabine was shown to hypomethylate and derepress numerous TSGs, some of the most commonly studied being *p16*, *APC*, *RASSF1A*, *hMLH1*, *PTEN*, and *DAPK* [6, 38, 39]. Several of these (and other) genes encode protein constituents of apoptosis pathways, and thus (like aza-dC) in addition to differentiation, 5-aza-dC can robustly induce apoptosis [52, 53]. Preclinical studies have now firmly established 5-aza-dC activity against hematologic cancers, including acute myeloid leukemia (AML), chronic myeloid leukemia, acute lymphocytic leukemia, and MDS (Fig. 14.1) [54–57], and may also elicit senescence and autophagy [58]. Although clinical studies of 5-aza-dC have yet to demonstrate substantial activity against solid tumors (see below), preclinical studies have convincingly shown proof-of-principle for antitumor efficacy [59–62]. Moreover, in two studies, an indirect apoptotic role was found in that 5-aza-dC hypomethylated and upregulated

microRNA-181, a regulator of *NOTCH4* and *KRAS*, while in liver cancer cells, 5-aza-dC induced the tumor suppressor microRNAs 124 and 203 [63, 64]. As noted above, epigenetic alterations in cancer often hyperactivate specific oncogenic pathways; 5-aza-dC is now known to antagonize several of those pathways, while upregulating tumor suppressive signaling. Examples of oncogenic signal blockade by 5-aza-dC was demonstrated by its upregulation of the endogenous Wnt pathway inhibitor DKK, resulting in significant xenograft tumor growth inhibition [65].

In addition to 5-aza-dC and 5-aza-C, various other compounds have been shown to elicit DNA demethylation. As decitabine is subject to intracellular deamination and aqueous instability (resulting in loss of hypomethylating activity), a more stable dinucleotide, 5-aza-dC-dG (SGI-110, Astex Pharmaceuticals, Cambridge, UK), was shown to resist cytidine deaminase, while also demonstrating potent antigrowth effects against bladder cancer cells and mouse xenografts, with negligible toxicity [66, 67]. Likewise, an elaidic acid analog of 5-aza-C, CP-4200, possessed enhanced stability and much higher tumoricidal activity than the parent compound (aza-C), possibly due to its independence from nucleoside uptake transporters [68]. Using a different (genetic) approach, short inhibitory RNAs against DNMTs 1 and 3b elicited DNA demethylation and gene derepression similar to (or greater than) deoxycytosine analogs [69]. In addition to decitabine, we have also studied another cytidine analog DNMTI, zebularine, demonstrating that this agent hypomethylates TSGs and allows for the chemosensitization of platinum-resistant ovarian cancer cells lines [70]. Other zebularine studies have demonstrated its greater stability than 5-aza-dC, demethylation in tumors *in vivo*, and colon cancer chemoprevention in a widely used mouse model [71, 72]. Like 5-aza-C, however, zebularine is a ribonucleotide and thus its potency is limited by inefficient reduction prior to incorporation into DNA [73]. Toward rectifying that limitation, deoxyzebularine phosphoramidate prodrugs were recently demonstrated as more potent hypomethylating agents in vitro, while also exhibiting antineoplastic activity against pancreatic cancer cell lines [74].

Several non-nucleoside compounds have also demonstrated DNA-hypomethylating activity. These include two previously FDA-approved agents, the antihypertensive hydralazine and the antiarrhythmic procainamide [75]. However, these compounds were found much less potent than 5-aza-dC [75, 76]. A mushroom-derived antibiotic, Verticullin A, likewise displayed DNMTI activity against SW620 colon cancer cells, upregulating several genes concordant with demethylation of their respective promoters, while also resensitizing those cells to the apoptosis-inducing, "death receptor" ligand TRAIL [77]. More recently, various "rationally designed," non-nucleoside DNMT inhibitors (thus influencing enzyme activity without DNA incorporation) have also demonstrated potent downregulation of methyltransferase activity. Two of these, SGI-1027 and RG108, facilitated reexpression of silenced TSGs, while also negatively affecting growth of colon and hepatocellular carcinoma cells [78–81]. Likewise, various high-throughput screens, using various reporter assays and virtual "docking" computational approaches, are now in widespread use for the identification of non-nucleoside DNA methyltransferase [82, 83]. These approaches will almost certainly lead to the identification of novel DNA methylation inhibitors.

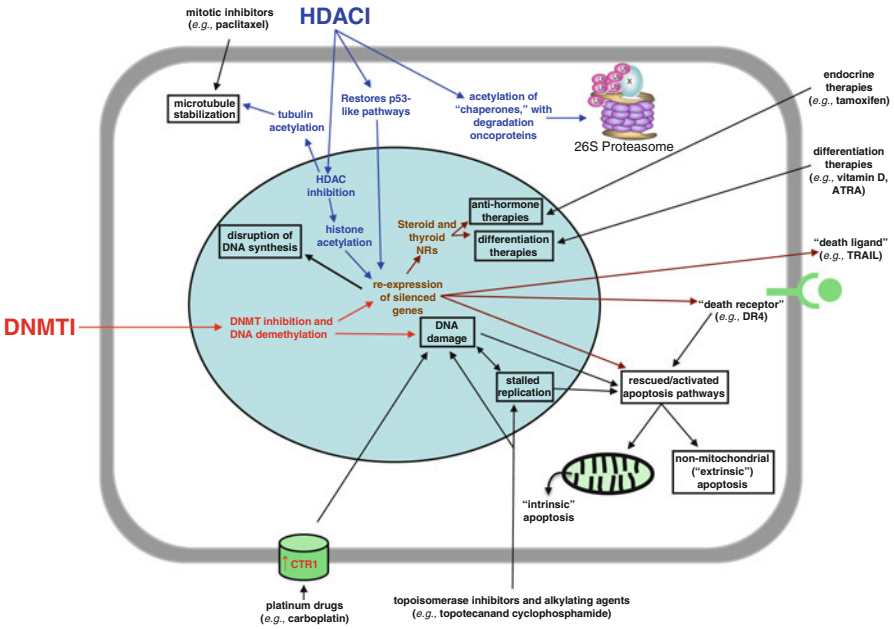

Fig. 14.2 Therapy response signals potentially affected by HDACIs and DNMTIs. Possible therapy-sensitization mechanisms by HDACIs and/or DNMTs. *Red* text denotes proteins/pathways impacted predominantly by DNMTIs, *blue* text indicates HDACI targets, and *brown* text designates possible alteration by either agent (and/or DNMTI/HDACI co-augmentation). *Black boxes* indicate pathway intersections where therapy sensitization may occur following DNMT/HDACI treatment. *ATRA* all-trans retinoic acid; *CTR1* copper transporter-1; *DNMTI* DNA methyltransferase inhibitor; *DR4* death receptor-4; *HDAC* histone deacetylase; *HDACI* histone deacetylase inhibitor; *TRAIL* TNF-related apoptosis-inducing ligand

While DNMTIs have shown success as monotherapies for hematologic cancers, accumulating evidence suggests they will be most effective when combined with conventional or targeted chemotherapies, likely via chemosensitization of resistant tumor subpopulations [8, 22, 84]. Such chemosensitization is hypothesized to result from DNMTI-mediated derepression of gene members of drug response pathways or inhibition of pro-survival pathways [8, 9, 85]. As shown in Fig. 14.2, multiple preclinical studies have now demonstrated that DNMTIs can resensitize resistant malignancies to numerous chemotherapeutics, via upregulation of pro-apoptosis pathways (both extrinsic and intrinsic), while also inhibiting oncogenic signaling cascades such as Wnt, PI3K/Akt, hedgehog, and Notch [65, 86–89]. In two early studies of the L1210 mouse leukemia models, cytoxicity of 5-aza-C was augmented by coadministration with another nucleoside analog, cytarabine; the hypothesized mechanism of action of this combination was inhibition of DNA synthesis [90]. Likewise, 5-aza-C antileukemic activity was also enhanced by a cytidine deaminase inhibitor [91]. More recently, in a study of aggressive prostate cancer, 5-aza-C caused potent but well-tolerated resensitization of tumor xenografts to docetaxel and cisplatin, concomitant with upregulation of a number of TSGs [92].

Like 5-aza-C, chemosensitization by 5-aza-dC is now well established. In one early study, 5-aza-dC combined with the topoisomerase-1 inhibitor topotecan, was synergistically cytotoxic to mouse colorectal adenocarcinomas [93]. Later, it was demonstrated that 5-aza-dC could resensitize platinum-resistant ovarian cancer cells and mouse xenografts to cisplatin, due to promoter demethylation and reexpression of the mismatch repair enzyme gene *hMLH1* [94, 95]. In two colon cancer studies, 5-aza-dC was found to be synergistically tumoricidal when combined with 5-fluorouracil (an antimetabolite) and the antineoplastic hormone irinotecan [96, 97]. Likewise, a study of colon cancer cells revealed that 5-aza-dC treatment resulted in upregulation of ten interferon pathway-associated genes, likely via induction of IFN-alpha2a and activation of STATs 1, 2, and 3 [98]. In endocrine cancers, DNMTIs have also been demonstrated to sensitize cancer cells to antihormonal therapies. For example, 5-aza-dC was shown to upregulate the DNA-methylation-repressed TSG *PTEN*, an inhibitor of the PI3K/Akt pathway, suppressing the growth of tamoxifen-resistant breast cancer cell xenografts and restoring responsiveness to antiestrogens [99]. The latter finding is further supported by a correlation of epigenetic aberrations and PI3K/Akt oncogenic signaling in breast cancer cells; those aberrations were reversible by a 5-aza-dC/PI3K inhibitor combination, which also cooperatively inhibited the growth of mouse xenografts [100]. Restoration of antiestrogen sensitivity in breast cancer is believed to be due (at least in part) to reexpression of the estrogen receptors alpha and/or beta [101, 102]. Similarly, in prostate cancer, androgen receptor silencing has been linked to both histone deacetylation and DNA methylation [103, 104]. In other prostate cancer studies, 5-aza-dC could sensitize both androgen-dependent and -independent prostate cancer cells to paclitaxel, while both DNMTIs and HDACIs cooperatively upregulated estrogen receptor-beta and delayed androgen independence in a common mouse model [105–107].

Preclinical cancer studies of histone deacetylase inhibitors (HDACIs). As histone deacetylation is another epigenetic modification repressive of TSGs, histone deacetylase inhibitors (HDACIs) also represent promising antineoplastics. Interestingly, the first HDAC inhibitor was the common organic solvent dimethylsulfoxide (DMSO), as discovered by Charlotte Friend to elicit differentiation of erythroleukemia cells [108]. Following that discovery, numerous other hybrid polar compounds were synthesized and similarly screened for differentiating activity, but whose mechanism of action (deacetylase inhibition, resulting in enhanced protein acetylation) remained unknown for over 20 years [109]. Numerous HDACIs, which antagonize the action of zinc-dependent histone deacetylases by chelation of the metal cation, have been shown to induce differentiation and apoptosis in tumor, but not normal, cells (reviewed in [110]). One proposed mechanism for cancer cell-specific HDACI toxicity is the induction of cell cycle checkpoints [111]; one such effect (G2 arrest followed by apoptosis) was also demonstrated in platinum-resistant ovarian cancer cells [112]. Of note, while HDACIs potently induce histone hyperacetylation, their effects on non-histone protein acetylation (including transcription factors, molecular chaperones, cargo transporters, and cytoskeletal proteins) may play an even greater role in their antineoplastic activity [110, 113]. In ovarian cancer in particular, several HDACIs induced cytodifferentiation and apop-

tosis of cultured cells and mouse xenografts [112, 114–117]. Newer studies suggest that HDACI repression of telomerase (*hTERT*) represents another anticancer mechanism of action (reviewed in [118]). Alternative non-epigenetic, HDACI antineoplastic effects include oncoprotein destabilization by acetylation of "chaperone" proteins (suggesting synergism with HSP inhibitors), diminished processing of "aggresomes" of misfolded proteins (suggesting synergism with proteasome inhibitors), acetylation of transcription factors, and reconstitution of p53-like tumor suppressive pathways (reviewed in [30, 110, 119], and see Fig. 14.2).

Similar to DNMTIs, preclinical studies have shown HDACIs to be most effective in combination with standard therapies, suggesting HDACI upregulation of drug response (apoptotic) or cellular differentiation pathways. In ovarian cancer preclinical studies, vorinostat alone was found effective against paclitaxel-resistant ovarian cancer cells; however, its antitumor activity was far greater in combination with paclitaxel [120–122]. Other HDACIs have similarly sensitized ovarian cancer cells to retinoids and the widely used chemotherapy cisplatin [115, 123, 124]. Similarly, our group demonstrated that a rationally designed HDACI, AR42, possessed greater cisplatin-resensitizing activity than vorinostat in chemoresistant ovarian cancer cells and mouse xenografts, enhancing both epithelial differentiation and apoptotic potential [125]. One specific example supporting HDACI-associated differentiation in therapy sensitization was that cholangiocarcinoma cells treated with the HDACI valproate upregulated numerous genes associated with differentiation, during sensitization to gemcitabine [126]. In similar studies, the HDACI Trichostatin A augmented UV-induced apoptosis over threefold in colon cancer RKO cells [127], and also sensitized osteosarcoma cells to a potentially antineoplastic, natural product geninstein [128].

Several HDACIs have also been demonstrated to upregulate "death receptor" apoptosis pathways, allowing resensitization of resistant cancer cells to death receptor ligands (Fig. 14.2). One report showed the HDACI MS-275 (entitostat) to resensitize aggressive MDA-MB-468 breast cancer cells to the death ligand TRAIL, both in cell culture and in mouse xenografts, while downregulating genes associated with the metastasis-related epithelial-to-mesenchymal transition [129]. In addition, HDACI-associated TRAIL sensitization (via reexpression of caspase-8) was markedly augmented by combination with interferon-gamma in meduloblastoma cells [130]. TRAIL sensitization by the HDACI valproate was also demonstrated in pancreatic cancer cell lines, via inhibition of HDAC2 and the restoration of extrinsic apoptosis pathways [131], while in hepatocellular carcinoma cell lines, the HDACIs valproic acid and ITF2357 both effected sensitization to TRAIL [132].

HDACIs have also shown activity against hormone-resistant neoplasms, including breast, uterine, and prostate cancers. Similar to DNMTI/antiestrogen studies HDACIs enhanced tamoxifen induction of both autophagy and apoptosis in tamoxifen-resistant breast cancer cells; that effect was further enhanced by inhibitors of autophagy [133]. In endometrial cancer studies, TSA/paclitaxel-combined treatment of mice bearing cancer cell tumor xenografts reduced tumor masses by >50% [134]. Moreover, another xenograft study showed that the HDACI apicidin reduced tumor size and repressed the angiogenesis-mediating oncoprotein VEGF [135]. Interestingly, it appears that in endometrial cancer, HDACIs may exert antigrowth

effects through repression of estrogen receptor-target genes, coincident with induction of genes targeted by the glucocorticoid receptor [136].

Preclinical studies of DNMTI/HDACI combinations. While HDACIs and DNMTIs have demonstrated clinical activity as single agent therapies for hematopoietic malignancies, DNA methylation and histone deacetylation also cooperatively inhibit gene transcription (often in multiple-repressor protein complexes), and relief of both silencing mechanisms may be necessary for maximal gene derepression [8, 137]. In ovarian cancer cells, a DNMTI/HDACI combination synergistically upregulated the pro-apoptotic gene *TMS1*/*ASC*, in contrast to either epigenetic agent alone [138], while a 5-aza-dC/vorinostat regimen induced various imprinted genes and also inhibited tumor xenograft growth [139]. Similarly, 5-aza-C combined with the HDACI Trichostatin A facilitated derepression of the progesterone receptor-B gene in endometrial cancer cells [140], while 5-aza-C plus entitostat cooperatively upregulated several pro-apoptosis genes and reduced tumor xenograft sizes by >75% in a mouse lung cancer model [141]. A newer preclinical study showed 5-aza-dC combined with the HDACI valproate was cancer-chemopreventive in a mouse medulloblastoma/rhabdosarcoma model, while each agent alone was not [142]. Interestingly, one compound, UVI5008, was found to be a "triple epigenetic inhibitor," concordantly inhibiting zinc-dependent HDACs, the DNA methyltransferase DNMT3A, and another family of HDACs that require a NAD^+ cofactor (rather than zinc), the sirtuins [143]. In that study, UV15008 potently induced apoptosis in breast cancer cells/xenografts via ROS production and activation of death receptor (i.e., extrinsic), mitochondria-independent, apoptosis [144].

It has also been reported that HDACIs and DNMTIs may actually mimic the epigenetic effects of one another. For example, it has been reported that several HDACIs can demethylate DNA, including Trichostatin A, valproate, and MS-275 (entitostat, SNDX-275) [145–148], possibly via transcriptional downregulation of DNMT-coding genes, as demonstrated in a study of human endometrial cancer cells [149]. Analogously, 5-aza-dC was also found to effect gene-specific, but not global, histone acetylation [150, 151]. However, a phase I study of AML or MDS patients examining 5-aza-C (5–14 days) followed by phenylbutyrate (5 days) demonstrated that 5-aza-C treatment alone resulted in histone acetylation in peripheral blood cells; phenylbutyrate, however, did not prevent remethylation of the cyclin-dependent kinase inhibitor gene *p15* (*CDKN2B*) [152]. Even so, these reciprocal epigenetic modifications, between HDACIs and DNMTIs, appear to be quite rare and context-dependent in nature.

While DNMTI/HDACI combinations often result in greater gene alterations than each agent in isolation, pairing of these epigenetic therapies will likely be even more effective in coordination with conventional cancer therapies [8–10]. For example, while caspase-8 gene reexpression in small cell lung cancer cells required a DNMTI/HDACI combination (thus restoring a functional apoptosis pathway), the induction of apoptosis still required the death receptor ligand TRAIL [153]. Similarly, combined treatment of decitabine and belinostat demonstrated significantly greater cisplatin sensitization of platinum-resistant ovarian cancer cell xenografts, in tumor-bearing mice, than either epigenetic therapy alone [154].

14.2 Clinical Studies of DNA Hypomethylating Agents and HDAC Inhibitors for Overcoming Drug Resistance

Four epigenetic derepressive agents are now FDA approved for two hematologic malignancies, MDS treatment with DNMTIs 5-aza-C (Vidaza) and 5-aza-dC (decitabine), and cutaneous T-cell lymphoma therapy using the HDACIs vorinostat and romidepsin [8, 9, 155]. While other hematologic malignancies will likely gain approval for monotherapy DNMTIs and HDACIs, including peripheral T-cell lymphoma and Hodgkin's disease, single-agent clinical studies of various solid tumors have proved fairly disappointing. For the latter, epigenetic drugs will likely prove most beneficial when combined with long-established approaches such as conventional cytotoxic chemotherapies, endocrine therapies, differentiation therapy, and radiotherapy [156, 157].

Studies of DNA methyltransferase inhibitors. In addition to incorporation into DNA, the ribonucleoside analog 5-aza-C is also incorporated into several RNA species, resulting in greater toxicity and lower stability than 5-aza-dC. Consequently, a more recent clinical studies have focused on 5-aza-dC (decitabine), although 5-aza-C remains widely used. An early Vidaza study of patients with acute leukemia, administered at 37–81 total mg/m^2, given over 30–60 h, resulted in some clinical benefit in 89% of patients, although substantial hematologic toxicity was observed in all patients [158]. A separate trial of 21 elderly patients with high-risk MDS, treatment with decitabine at 50 mg/m^2/day for three consecutive days, yielded a response rate of 54% (15 of 21), although significant myelotoxicity caused the death of 5/21 (17%) patients [159]. Another MDS phase I study, using an overall similar drug exposure (45 mg/m^2 b.i.d. for 3 days), yielded an overall response rate of 49%, but similarly resulted in moderate-to-severe toxicity (predominantly myelodepression), resulting in the death in 7% of the enrolled patients [160].

To possibly ameliorate the high toxicity and limited benefit of extended decitabine infusions (previously using regimens approaching its maximum tolerated dose), lower dose schedules were examined. In phase I/II sickle cell anemia studies of hydroxyurea-resistant patients, low-dose (0.3 mg/kg), repetitive doses (5 days/week for 2 weeks) of decitabine were found sufficient for demethylation and reexpression of fetal hemoglobin with little or no neutropenia [161, 162]. Such low-dose treatments were largely based on a mouse embryonic fibroblast study showing myotube differentiation and hypomethylation at low decitabine doses (1–5 μM), with cytotoxicity and increased methylation at higher (>5 μM) doses [29]. Subsequently, one MDS clinical trial examined a variety of repetitive low decitabine doses, with 1-h administration daily over longer durations (10–20 days) [163]. The results of that landmark study demonstrated that 15 mg/m^2 decitabine, administered over ten consecutive days, resulted in a response rate of 83% and was well tolerated, as compared to previous studies using >5-fold higher doses [163]. That pioneering work resulted in the widespread adoption of low-dose hypomethylating agents, both as monotherapies and in combination with other agents.

While single-agent decitabine demonstrated significant efficacy for MDS and other hematologic malignancies, solid tumor studies have been fairly disappointing, motivating studies of 5-aza-dC in combination with other conventional agents. Early combination studies, however, demonstrated minimal-to-moderate activity, with substantial toxicity. In a phase II study of non-small cell lung cancer, a maximum tolerated decitabine dose of 67 mg/m^2, given concurrently with 33 mg/m^2 cisplatin over a 2-h period for 3 consecutive days of a 21-day cycle, resulted in no objective responses and significant hematologic toxicity [164]. Similarly, a phase II trial of squamous cell cervical cancer, with decitabine administered continuously at 50 mg/m^2/day for 3 days, concurrent with 30 mg/m^2 cisplatin, resulted in eight partial and five stable disease responses; however, unacceptable toxicity was observed, resulting in one patient death [165]. However, based on the low-dose MDS efficacy study by Issa et al., newer trials have examined lower doses of decitabine in various combined regimens. One recent phase I/II combinatorial ovarian cancer study, of decitabine paired with carboplatin, demonstrated no significant improvement over carboplatin alone [166, 167]. By contrast, a separate phase IIa clinical trial of 5-aza-cytidine (Vidaza) and carboplatin resulted in one complete, three partial, and ten stable disease responses (of 29 total patients), with a 7.5-month average duration of response [168]. Likewise, our group recently completed a phase I trial of low-dose decitabine (five consecutive-day regimen), in combination with carboplatin in platinum-resistant ovarian cancer patients, revealing acceptable tolerability of the regimen [169]. Biological activity in vivo was also demonstrated, as assessed by hypomethylation of genome-wide repetitive elements (in peripheral blood cells) and specific ovarian cancer-associated genes (in plasma, ascites, or tumor) [169], resulting in one complete, six stable, and four (6-month) disease progression-free responses [169]. The successful phase II component of that study was recently described [170], and the results are promising. Other clinical studies combining 5-aza-dC with the EGFR antagonist erlotinib showed responses in 4 of 11 patients with advanced tumors [171]. However, a neuroblastoma trial of 5-aza-dC combined with cyclophosphamide or doxorubicin showed toxicity at the 5-aza-dC doses required for disease response [172]. In a 13-patient AML phase I study, decitabine combined with arsenic trioxide and/or ascorbic acid resulted in one complete remission and five patients with stable disease [173]. While chemosensitization by DNMTIs is believed to largely result from the restoration of apoptosis pathways, one recent phase II study of refractory solid tumors and lymphomas showed patient response to correlate with both DNA hypomethylation and expression of the copper transporter CTR1, a protein that facilitates platinum drug uptake [174] (Fig. 14.2).

Studies of histone deacetylase inhibitors. Like DNMTIs, despite successful studies of hematologic malignancies, solid tumor clinical trials of monotherapeutic HDACIs suggest similarly limited clinical activity. In ovarian cancer, two monotherapeutic phase I/II trials of the HDACIs vorinostat and belinostat proved tolerable but showed only moderate clinical activity [175, 176]. One recent phase II trial of the HDACI romidepsin in androgen-independent prostate cancer, although well tolerated, likewise showed minimal antineoplastic activity [177]. Another belinostat trial for metastatic renal cancer also yielded no patient responses [178]. Consequently,

it is now widely believed that these agents will be most effective in combination with conventional chemotherapies ([8, 9, 34] and see following sections).

For ovarian cancer, two recent ovarian cancer trials pairing belinostat with paclitaxel/carboplatin, and vorinostat with carboplatin, demonstrated safety and moderate clinical activity [179, 180], while planned clinical trials include HDACIs in combination with inhibitors of the DNA repair enzyme PARP or inhibitors of the embryonic signal mediator Hedgehog [181, 182]. Another phase II study of the HDACI vorinostat combined with the antiestrogen tamoxifen, in hormone-refractory breast cancer patients, yielded a clinical benefit rate (response or stable disease for over 24 weeks) of 40%, although toxicity necessitated dose adjustment in several patients [183]. Similarly, a 12-patient phase I trial combining the HDACI panobinostat with the angiogenesis inhibitor bevacizumab resulted in three partial responses and seven cases of stable disease [184].

Clinical studies of combined DNA methyltransferase and histone deacetylase inhibitors. DNMTI/HDACI combinations have also now been established to exert additive/synergistic effects on gene expression in vitro. However, success in clinical trials has been, similar to single-agent regimens, largely restricted to hematologic malignancies. For example, a phase II study of the DNMTI/HDACI combination of hydralazine and valproate for MDS showed an overall response rate of 50% [185]. Most solid tumor studies, however, have shown less efficacy. Nonetheless, one phase I clinical trial combining the HDACI valproic acid and the DNMTI azacytidine for various solid tumors demonstrated safety, in vivo biological activity, and stable disease in 25% of the enrolled patients, although no partial or complete responses were observed [186]. Likewise, a recent phase I study of 5-aza-dC/vorinostat combination resulted in 29% of non-Hodgkin's lymphoma and various solid tumors [187]. By analogy, a recent phase I/II trial of an azacytidine/entitostat combination in non-small cell lung cancer yielded major objective responses in 4 of 19 patients, and demethylation of a four-gene panel correlated with improved progression-free and overall survival [188].

Based on the above mentioned in vivo findings, it is speculated that chromatin depressive agents (singly or combined) alone may be only marginally efficient for eradicating cancer cells, thus motivating studies of their combination with conventional therapeutics [6, 8, 94]. For example, while apoptosis pathway function may be restored by epigenetic derepression, it is possible that epigenetic drugs remain inadequate as cancer cell stressors capable of provoking programmed cell death. In one phase III ovarian cancer trial (NCT00533299), the DNMTI hydralazine is being combined with the HDACI valproic acid, with or without the topoisomerase inhibitor topotecan, while a previous phase II trial of the same combination (hydralazine/valproate), coincident with four different chemotherapy regimens, yielded three partial and four stable disease responses (as assessed by the ovarian cancer marker CA-125) [189]. In various leukemias, a phase I trial of 5-aza-dC combined with valproic acid demonstrated acceptable patient tolerability and an objective response rate of 22% [190], while a melanoma trial combining 5-aza-dC and intravenous bolus interleukin-2 was well-tolerated and yielded a 31% objective response rate [191].

5-aza-C is also being examined in a phase I/II ovarian cancer trial (NCT00529022) in combination with valproic acid and carboplatin.

In addition to reactivation of TSGs (and possible chemotherapy response cascades), DNMTIs and HDACIs have also been found to induce various cancer/testis antigens (CTAs, components of the "tumor recognition complex") [34]. CTA proteins, expressed in male germ cells but normally silenced in adult tissues, are expressed in various malignancies as antigenic peptides copresented with HLA Class I/II molecules and thus may represent immunotherapy targets [192]. However, as CTA expression is often variable, due to epigenetic repression, more consistent reexpression can be achieved by DNMT and/or HDAC inhibitors [191]. Consequently, an ongoing phase I ovarian cancer trial (NCT00887796) is investigating decitabine combined with liposomal doxorubicin and peptide vaccination for the CTA NY-ESO-1, while two other trials (NCT00701298, NCT00886457, for unspecified cancers) are combining decitabine with interferon-α2b. These trials were based on the preclinical studies by Karpf et al. [98, 193] mentioned earlier. Thus, in addition to tumor suppressor reactivation, epigenetic therapies may also hold promise in immunotherapy.

14.3 Future Directions for the Use of Epigenetic Therapies for Overcoming Chemotherapy Resistance

One current focus within cancer epigenetic research is the design of specific inhibitors of enzymes facilitating other epigenetic repressive modifications, including the gene-repressive histone methyltransferases (HMTs) EZH2, which trimethylates histone H3, lysine 27 (H3K27me3), and DOT1L, which trimethylates H3K79 [194, 195]. Consistent with epigenetic gene repression in cancer, one DOT1L inhibitor, EPZ004777, showed activity against mixed lineage leukemia cells [196]. Similarly, one EZH2 inhibitor, DZNep, an *S*-adenosylmethionine (SAM) analog that also inhibits methylation of H4K20, resulting in upregulation of numerous previously silenced TSGs [194, 197]. DZNep has also shown anticancer activity against mouse prostate tumors and breast cancer, AML, and neuroblastoma cells [194, 197–199]. Similar to DNMTIs, DZNep induction of apoptosis was also augmented by HDACIs [200, 201], and recent studies of DZNep suggest possible negative effects toward CSCs [199, 202]. High-throughput approaches continue to identify various novel epigenetic therapies, including inhibitors of the Jarid family of H3K4me3 histone demethylases, the repressive HMT G9a (which trimethylates H3K9), isoform-specific HDACs, and various histone acetyltransferases [203–207]. In addition, tumor-suppressive microRNAs have been successfully delivered to tumors in mouse models of liver (miR-26a), colon (miRs-145 and -33a), and prostate cancers, using adeno-associated viruses, polyethylenimine conjugation, and rhabdomyosarcoma (miRs-1 and −206) [208–210]. Taken together it is likely that these emerging epigenetic therapeutics could be used for the much anticipated therapeutic approach of

"personalized medicine," based not only on patients' genomic/gene expression profiles, but also on their epigenetic profiles.

14.4 Summary/Conclusions

It is now well established that epigenetics is a principle mediator of mammalian development. To successfully carry out tissue/organ differentiation, genomic DNA expression is precisely regulated by a host of epigenetic modifications. It is thus not surprising that aberrant chromatin modifications result in defective differentiation states, a hallmark of cancer cells. It has also been recently shown that even highly aggressive cancer cells can revert to their original, tissue-specific differentiation state, and that epigenetic therapies may facilitate this phenomenon. Consequently, chromatin-altering agents hold promise for the treatment of numerous malignant diseases, particular when complemented with other (traditional or pathway-targeted) antineoplastic therapies.

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References

1. Gralow J, Ozols RF, Bajorin DF, Cheson BD, Sandler HM, Winer EP, Bonner J, Demetri GD, Curran W Jr, Ganz PA, Kramer BS, Kris MG, Markman M, Mayer RJ, Raghavan D, Ramsey S, Reaman GH, Sawaya R, Schuchter LM, Sweetenham JW, Vahdat LT, Davidson NE, Schilsky RL, Lichter AS (2008) Clinical cancer advances 2007: major research advances in cancer treatment, prevention, and screening—a report from the American Society of Clinical Oncology. J Clin Oncol 26(2):313–325
2. Jemal A, Siegel R, Xu J, Ward E (2010) Cancer statistics, 2010. CA Cancer J Clin 60(5):277–300
3. Longley DB, Johnston PG (2005) Molecular mechanisms of drug resistance. J Pathol 205(2):275–292
4. Raguz S, Yague E (2008) Resistance to chemotherapy: new treatments and novel insights into an old problem. Br J Cancer 99(3):387–391
5. Tredan O, Galmarini CM, Patel K, Tannock IF (2007) Drug resistance and the solid tumor microenvironment. J Natl Cancer Inst 99(19):1441–1454
6. Balch C, Huang TH, Brown R, Nephew KP (2004) The epigenetics of ovarian cancer drug resistance and resensitization. Am J Obstet Gynecol 191(5):1552–1572
7. Barton CA, Clark SJ, Hacker NF, O'Brien PM (2008) Epigenetic markers of ovarian cancer. Adv Exp Med Biol 622:35–51
8. Jones PA, Baylin SB (2007) The epigenomics of cancer. Cell 128(4):683–692
9. Esteller M (2008) Epigenetics in cancer. N Engl J Med 358(11):1148–1159

10. Rodriguez-Paredes M, Esteller M (2011) Cancer epigenetics reaches mainstream oncology. Nat Med 17(3):330–339
11. Lu L, Katsaros D, de la Longrais IA, Sochirca O, Yu H (2007) Hypermethylation of let-7a-3 in epithelial ovarian cancer is associated with low insulin-like growth factor-II expression and favorable prognosis. Cancer Res 67(21):10117–10122
12. Wiley A, Katsaros D, Chen H, Rigault de la Longrais IA, Beeghly A, Puopolo M, Singal R, Zhang Y, Amoako A, Zelterman D, Yu H (2006) Aberrant promoter methylation of multiple genes in malignant ovarian tumors and in ovarian tumors with low malignant potential. Cancer 107(2):299–308
13. Daley GQ (2008) Common themes of dedifferentiation in somatic cell reprogramming and cancer. Cold Spring Harb Symp Quant Biol 73:171–174
14. Dimri GP (2005) What has senescence got to do with cancer? Cancer Cell 7(6):505–512
15. Dalerba P, Cho RW, Clarke MF (2007) Cancer stem cells: models and concepts. Annu Rev Med 58:267–284
16. Jordan CT (2009) Cancer stem cells: controversial or just misunderstood? Cell Stem Cell 4(3):203–205
17. Von Hoff DD, Slavik M, Muggia FM (1976) 5-Azacytidine. A new anticancer drug with effectiveness in acute myelogenous leukemia. Ann Intern Med 85(2):237–245
18. Delva L, Zelent A, Naoe T, Fenaux P, Waxman S, Degos L, Chomienne C (2007) Meeting report: the 11th International Conference on Differentiation Therapy and Innovative Therapeutics in Oncology. Cancer Res 67(22):10635–10637
19. Ma WW, Adjei AA (2009) Novel agents on the horizon for cancer therapy. CA Cancer J Clin 59(2):111–137
20. Vincent A, Van Seuningen I (2009) Epigenetics, stem cells and epithelial cell fate. Differentiation 78(2–3):99–107
21. Scaffidi P, Misteli T (2010) Cancer epigenetics: from disruption of differentiation programs to the emergence of cancer stem cells. Cold Spring Harb Symp Quant Biol 75:251–258
22. Lotem J, Sachs L (2006) Epigenetics and the plasticity of differentiation in normal and cancer stem cells. Oncogene 25(59):7663–7672
23. Djuric U, Ellis J (2010) Epigenetics of induced pluripotency, the seven-headed dragon. Stem Cell Res Ther 1(1):3
24. Hochedlinger K, Plath K (2009) Epigenetic reprogramming and induced pluripotency. Development 136(4):509–523
25. Costa FF, Seftor EA, Bischof JM, Kirschmann DA, Strizzi L, Arndt K, de Fatima Bonaldo M, Soares MB, Hendrix MJ (2009) Epigenetically reprogramming metastatic tumor cells with an embryonic microenvironment. Epigenomics 1(2):387–398
26. Hendrix MJ, Seftor EA, Seftor RE, Kasemeier-Kulesa J, Kulesa PM, Postovit LM (2007) Reprogramming metastatic tumour cells with embryonic microenvironments. Nat Rev Cancer 7(4):246–255
27. Goldin A, Sandberg JS, Henderson ES, Newman JW, Frei E III, Holland JF (1971) The chemotherapy of human and animal acute leukemia. Cancer Chemother Pharmacol 55(4):309–505
28. Ney PA, D'Andrea AD (2000) Friend erythroleukemia revisited. Blood 96(12):3675–3680
29. Jones PA, Taylor SM (1980) Cellular differentiation, cytidine analogs and DNA methylation. Cell 20(1):85–93
30. Lane AA, Chabner BA (2009) Histone deacetylase inhibitors in cancer therapy. J Clin Oncol 27(32):5459–5468
31. Yang X, Lay F, Han H, Jones PA (2010) Targeting DNA methylation for epigenetic therapy. Trends Pharmacol Sci 31(11):536–546
32. Issa JP (2007) DNA methylation as a therapeutic target in cancer. Clin Cancer Res 13(6):1634–1637
33. Ewald B, Sampath D, Plunkett W (2008) Nucleoside analogs: molecular mechanisms signaling cell death. Oncogene 27(50):6522–6537
34. Yoo CB, Jones PA (2006) Epigenetic therapy of cancer: past, present and future. Nat Rev Drug Discov 5(1):37–50

35. Jabbour E, Issa JP, Garcia-Manero G, Kantarjian H (2008) Evolution of decitabine development: accomplishments, ongoing investigations, and future strategies. Cancer 112(11):2341–2351
36. Piskala A, Sorm F (1964) Nucleic acids components and the analogues. LI. Synthesis of 1-glycosyl derivatives of 5-azauracil and 5-azacytosine. Collect Czech Chem Commun 29:2060–2076
37. Shutt RH, Krueger RG (1972) The effect of actinomycin D and 5-azacytidine on macromolecular synthesis in murine myeloma tumor cells. J Immunol 108(3):819–830
38. Takai N, Kawamata N, Walsh CS, Gery S, Desmond JC, Whittaker S, Said JW, Popoviciu LM, Jones PA, Miyakawa I, Koeffler HP (2005) Discovery of epigenetically masked tumor suppressor genes in endometrial cancer. Mol Cancer Res 3(5):261–269
39. Sasaki M, Kaneuchi M, Fujimoto S, Tanaka Y, Dahiya R (2003) Hypermethylation can selectively silence multiple promoters of steroid receptors in cancers. Mol Cell Endocrinol 202(1–2):201–207
40. Nguyen CT, Weisenberger DJ, Velicescu M, Gonzales FA, Lin JC, Liang G, Jones PA (2002) Histone H3-lysine 9 methylation is associated with aberrant gene silencing in cancer cells and is rapidly reversed by 5-aza-2′-deoxycytidine. Cancer Res 62(22):6456–6461
41. Abbosh PH, Montgomery JS, Starkey JA, Novotny M, Zuhowski EG, Egorin MJ, Moseman AP, Golas A, Brannon KM, Balch C, Huang TH, Nephew KP (2006) Dominant-negative histone H3 lysine 27 mutant derepresses silenced tumor suppressor genes and reverses the drug-resistant phenotype in cancer cells. Cancer Res 66(11):5582–5591
42. Vesely J (1982) Synergistic effect of cis-dichlorodiammineplatinum and 5-aza-2′-deoxycytidine on mouse leukemic cells in vivo and in vitro. Int J Cancer 29(1):81–85
43. Taylor SM, Jones PA (1982) Mechanism of action of eukaryotic DNA methyltransferase. Use of 5-azacytosine-containing DNA. J Mol Biol 162(3):679–692
44. Nakahara Y, Northcott PA, Li M, Kongkham PN, Smith C, Yan H, Croul S, Ra YS, Eberhart C, Huang A, Bigner D, Grajkowska W, Van Meter T, Rutka JT, Taylor MD (2010) Genetic and epigenetic inactivation of Kruppel-like factor 4 in medulloblastoma. Neoplasia 12(1):20–27
45. Mahesh S, Saxena A, Qiu X, Perez-Soler R, Zou Y (2010) Intratracheally administered 5-azacytidine is effective against orthotopic human lung cancer xenograft models and devoid of important systemic toxicity. Clin Lung Cancer 11(6):405–411
46. Walker C, Shay JW (1984) 5-Azacytidine induced myogenesis in a differentiation defective cell line. Differentiation 25(3):259–263
47. Liu L, Harrington M, Jones PA (1986) Characterization of myogenic cell lines derived by 5-azacytidine treatment. Dev Biol 117(2):331–336
48. Hustad CM, Jones PA (1991) Effect of myogenic determination on tumorigenicity of chemically transformed 10T1/2 cells. Mol Carcinog 4(2):153–161
49. Schneider-Stock R, Diab-Assef M, Rohrbeck A, Foltzer-Jourdainne C, Boltze C, Hartig R, Schonfeld P, Roessner A, Gali-Muhtasib H (2005) 5-Aza-cytidine is a potent inhibitor of DNA methyltransferase 3a and induces apoptosis in HCT-116 colon cancer cells via Gadd45- and p53-dependent mechanisms. J Pharmacol Exp Ther 312(2):525–536
50. Wang XM, Wang X, Li J, Evers BM (1998) Effects of 5-azacytidine and butyrate on differentiation and apoptosis of hepatic cancer cell lines. Ann Surg 227(6):922–931
51. Burrows JF, Chanduloy S, McIlhatton MA, Nagar H, Yeates K, Donaghy P, Price J, Godwin AK, Johnston PG, Russell SE (2003) Altered expression of the septin gene, SEPT9, in ovarian neoplasia. J Pathol 201(4):581–588
52. Balch C, Montgomery JS, Paik HI, Kim S, Huang TH, Nephew KP (2005) New anti-cancer strategies: epigenetic therapies and biomarkers. Front Biosci 10:1897–1931
53. Momparler RL (2005) Epigenetic therapy of cancer with 5-aza-2′-deoxycytidine (decitabine). Semin Oncol 32(5):443–451
54. Wilson VL, Jones PA, Momparler RL (1983) Inhibition of DNA methylation in L1210 leukemic cells by 5-aza-2′-deoxycytidine as a possible mechanism of chemotherapeutic action. Cancer Res 43(8):3493–3496

55. Momparler RL, Bouchard J, Samson J (1985) Induction of differentiation and inhibition of DNA methylation in HL-60 myeloid leukemic cells by 5-AZA-2′-deoxycytidine. Leuk Res 9(11):1361–1366
56. Limonta M, Colombo T, Damia G, Catapano CV, Conter V, Gervasoni M, Masera G, Liso V, Specchia G, Giudici G et al (1993) Cytotoxic activity and mechanism of action of 5-Aza-2′-deoxycytidine in human CML cells. Leuk Res 17(11):977–982
57. Corn PG, Kuerbitz SJ, van Noesel MM, Esteller M, Compitello N, Baylin SB, Herman JG (1999) Transcriptional silencing of the p73 gene in acute lymphoblastic leukemia and Burkitt's lymphoma is associated with 5′ CpG island methylation. Cancer Res 59(14):3352–3356
58. Schnekenburger M, Grandjenette C, Ghelfi J, Karius T, Foliguet B, Dicato M, Diederich M (2011) Sustained exposure to the DNA demethylating agent, 2′-deoxy-5-azacytidine, leads to apoptotic cell death in chronic myeloid leukemia by promoting differentiation, senescence, and autophagy. Biochem Pharmacol 81(3):364–378
59. Obata T, Toyota M, Satoh A, Sasaki Y, Ogi K, Akino K, Suzuki H, Murai M, Kikuchi T, Mita H, Itoh F, Issa JP, Tokino T, Imai K (2003) Identification of HRK as a target of epigenetic inactivation in colorectal and gastric cancer. Clin Cancer Res 9(17):6410–6418
60. Alcazar O, Achberger S, Aldrich W, Hu Z, Negrotto S, Saunthararajah Y, Triozzi P (2012) Epigenetic regulation by decitabine of melanoma differentiation in vitro and in vivo. Int J Cancer 131(1):18–29
61. Chen W, Gao N, Shen Y, Cen JN (2010) Hypermethylation downregulates Runx3 gene expression and its restoration suppresses gastric epithelial cell growth by inducing p27 and caspase3 in human gastric cancer. J Gastroenterol Hepatol 25(4):823–831
62. Tseng RC, Lee SH, Hsu HS, Chen BH, Tsai WC, Tzao C, Wang YC (2010) SLIT2 attenuation during lung cancer progression deregulates beta-catenin and E-cadherin and associates with poor prognosis. Cancer Res 70(2):543–551
63. Furuta M, Kozaki KI, Tanaka S, Arii S, Imoto I, Inazawa J (2010) miR-124 and miR-203 are epigenetically silenced tumor-suppressive microRNAs in hepatocellular carcinoma. Carcinogenesis 31(5):766–776
64. Hashimoto Y, Akiyama Y, Otsubo T, Shimada S, Yuasa Y (2010) Involvement of epigenetically silenced microRNA-181c in gastric carcinogenesis. Carcinogenesis 31(5):777–784
65. Aguilera O, Fraga MF, Ballestar E, Paz MF, Herranz M, Espada J, Garcia JM, Munoz A, Esteller M, Gonzalez-Sancho JM (2006) Epigenetic inactivation of the Wnt antagonist DICKKOPF-1 (DKK-1) gene in human colorectal cancer. Oncogene 25(29):4116–4121
66. Chuang JC, Warner SL, Vollmer D, Vankayalapati H, Redkar S, Bearss DJ, Qiu X, Yoo CB, Jones PA (2010) S110, a 5-Aza-2′-deoxycytidine-containing dinucleotide, is an effective DNA methylation inhibitor in vivo and can reduce tumor growth. Mol Cancer Ther 9(5):1443–1450
67. Yoo CB, Jeong S, Egger G, Liang G, Phiasivongsa P, Tang C, Redkar S, Jones PA (2007) Delivery of 5-aza-2′-deoxycytidine to cells using oligodeoxynucleotides. Cancer Res 67(13):6400–6408
68. Brueckner B, Rius M, Markelova MR, Fichtner I, Hals PA, Sandvold ML, Lyko F (2010) Delivery of 5-azacytidine to human cancer cells by elaidic acid esterification increases therapeutic drug efficacy. Mol Cancer Ther 9(5):1256–1264
69. Leu YW, Rahmatpanah F, Shi H, Wei SH, Liu JC, Yan PS, Huang TH (2003) Double RNA interference of DNMT3b and DNMT1 enhances DNA demethylation and gene reactivation. Cancer Res 63(19):6110–6115
70. Balch C, Yan P, Craft T, Young S, Skalnik DG, Huang TH, Nephew KP (2005) Antimitogenic and chemosensitizing effects of the methylation inhibitor zebularine in ovarian cancer. Mol Cancer Ther 4(10):1505–1514
71. Yoo CB, Chuang JC, Byun HM, Egger G, Yang AS, Dubeau L, Long T, Laird PW, Marquez VE, Jones PA (2008) Long-term epigenetic therapy with oral zebularine has minimal side effects and prevents intestinal tumors in mice. Cancer Prev Res 1(4):233–240
72. Cheng JC, Matsen CB, Gonzales FA, Ye W, Greer S, Marquez VE, Jones PA, Selker EU (2003) Inhibition of DNA methylation and reactivation of silenced genes by zebularine. J Natl Cancer Inst 95(5):399–409

73. Cheng JC, Yoo CB, Weisenberger DJ, Chuang J, Wozniak C, Liang G, Marquez VE, Greer S, Orntoft TF, Thykjaer T, Jones PA (2004) Preferential response of cancer cells to zebularine. Cancer Cell 6(2):151–158
74. Yoo CB, Valente R, Congiatu C, Gavazza F, Angel A, Siddiqui MA, Jones PA, McGuigan C, Marquez VE (2008) Activation of p16 gene silenced by DNA methylation in cancer cells by phosphoramidate derivatives of 2′-deoxyzebularine. J Med Chem 51(23):7593–7601
75. Segura-Pacheco B, Trejo-Becerril C, Perez-Cardenas E, Taja-Chayeb L, Mariscal I, Chavez A, Acuna C, Salazar AM, Lizano M, Duenas-Gonzalez A (2003) Reactivation of tumor suppressor genes by the cardiovascular drugs hydralazine and procainamide and their potential use in cancer therapy. Clin Cancer Res 9(5):1596–1603
76. Chuang JC, Yoo CB, Kwan JM, Li TW, Liang G, Yang AS, Jones PA (2005) Comparison of biological effects of non-nucleoside DNA methylation inhibitors versus 5-aza-2′-deoxycytidine. Mol Cancer Ther 4(10):1515–1520
77. Liu F, Liu Q, Yang D, Bollag WB, Robertson K, Wu P, Liu K (2011) Verticillin A overcomes apoptosis resistance in human colon carcinoma through DNA methylation-dependent upregulation of BNIP3. Cancer Res 71(21):6807–6816
78. Brueckner B, Garcia Boy R, Siedlecki P, Musch T, Kliem HC, Zielenkiewicz P, Suhai S, Wiessler M, Lyko F (2005) Epigenetic reactivation of tumor suppressor genes by a novel small-molecule inhibitor of human DNA methyltransferases. Cancer Res 65(14):6305–6311
79. Datta J, Ghoshal K, Denny WA, Gamage SA, Brooke DG, Phiasivongsa P, Redkar S, Jacob ST (2009) A new class of quinoline-based DNA hypomethylating agents reactivates tumor suppressor genes by blocking DNA methyltransferase 1 activity and inducing its degradation. Cancer Res 69(10):4277–4285
80. Medina-Franco JL, Caulfield T (2011) Advances in the computational development of DNA methyltransferase inhibitors. Drug Discov Today 16:418–425
81. Siedlecki P, Garcia Boy R, Musch T, Brueckner B, Suhai S, Lyko F, Zielenkiewicz P (2006) Discovery of two novel, small-molecule inhibitors of DNA methylation. J Med Chem 49(2):678–683
82. Medina-Franco JL, Caulfield T (2011) Advances in the computational development of DNA methyltransferase inhibitors. Drug Discov Today 16(9–10):418–425
83. Castellano S, Kuck D, Viviano M, Yoo J, Lopez-Vallejo F, Conti P, Tamborini L, Pinto A, Medina-Franco JL, Sbardella G (2011) Synthesis and biochemical evaluation of delta(2)-isoxazoline derivatives as DNA methyltransferase 1 inhibitors. J Med Chem 54(21):7663–7677
84. Balch C, Nephew KP (2010) The role of chromatin, microRNAs, and tumor stem cells in ovarian cancer. Cancer Biomark 8(4):203–221
85. Wood TE, Dalili S, Simpson CD, Sukhai MA, Hurren R, Anyiwe K, Mao X, Suarez Saiz F, Gronda M, Eberhard Y, MacLean N, Ketela T, Reed JC, Moffat J, Minden MD, Batey RA, Schimmer AD (2010) Selective inhibition of histone deacetylases sensitizes malignant cells to death receptor ligands. Mol Cancer Ther 9(1):246–256
86. Hirata H, Hinoda Y, Nakajima K, Kawamoto K, Kikuno N, Ueno K, Yamamura S, Zaman MS, Khatri G, Chen Y, Saini S, Majid S, Deng G, Ishii N, Dahiya R (2011) Wnt antagonist DKK1 acts as a tumor suppressor gene that induces apoptosis and inhibits proliferation in human renal cell carcinoma. Int J Cancer 128(8):1793–1803
87. Liu T, Zhang X, So CK, Wang S, Wang P, Yan L, Myers R, Chen Z, Patterson AP, Yang CS, Chen X (2007) Regulation of Cdx2 expression by promoter methylation, and effects of Cdx2 transfection on morphology and gene expression of human esophageal epithelial cells. Carcinogenesis 28(2):488–496
88. Xu J, Zhou JY, Tainsky MA, Wu GS (2007) Evidence that tumor necrosis factor-related apoptosis-inducing ligand induction by 5-Aza-2′-deoxycytidine sensitizes human breast cancer cells to adriamycin. Cancer Res 67(3):1203–1211
89. Chun SG, Zhou W, Yee NS (2009) Combined targeting of histone deacetylases and hedgehog signaling enhances cytoxicity in pancreatic cancer. Cancer Biol Ther 8(14):1328–1339
90. Neil GL, Berger AE, Bhuyan BK, DeSante DC (1976) Combination chemotherapy of L1210 leukemia with 1-beta-D-arabinofuranosylcytosine and 5-azacytidine. Cancer Res 36(3):1114–1120

91. Neil GL, Moxley TE, Kuentzel SL, Manak RC, Hanka LJ (1975) Enhancement by tetrahydrouridine (NSC-112907) of the oral activity of 5-azacytidine (NSC-102816) in L1210 leukemic mice. Cancer Chemother Pharmacol 59(3):459–465
92. Festuccia C, Gravina GL, D'Alessandro AM, Muzi P, Millimaggi D, Dolo V, Ricevuto E, Vicentini C, Bologna M (2009) Azacitidine improves antitumor effects of docetaxel and cisplatin in aggressive prostate cancer models. Endocr Relat Cancer 16(2):401–413
93. Anzai H, Frost P, Abbruzzese JL (1992) Synergistic cytotoxicity with 2′-deoxy-5-azacytidine and topotecan in vitro and in vivo. Cancer Res 52(8):2180–2185
94. Balch C, Montgomery JS, Paik HI, Kim S, Kim S, Huang TH, Nephew KP (2005) New anticancer strategies: epigenetic therapies and biomarkers. Front Biosci 10:1897–1931
95. Plumb JA, Strathdee G, Sludden J, Kaye SB, Brown R (2000) Reversal of drug resistance in human tumor xenografts by 2′-deoxy-5-azacytidine-induced demethylation of the hMLH1 gene promoter. Cancer Res 60(21):6039–6044
96. Morita S, Iida S, Kato K, Takagi Y, Uetake H, Sugihara K (2006) The synergistic effect of 5-aza-2′-deoxycytidine and 5-fluorouracil on drug-resistant tumors. Oncology 71(5–6):437–445
97. Ishiguro M, Iida S, Uetake H, Morita S, Makino H, Kato K, Takagi Y, Enomoto M, Sugihara K (2007) Effect of combined therapy with low-dose 5-aza-2′-deoxycytidine and irinotecan on colon cancer cell line HCT-15. Ann Surg Oncol 14(5):1752–1762
98. Karpf AR, Peterson PW, Rawlins JT, Dalley BK, Yang Q, Albertsen H, Jones DA (1999) Inhibition of DNA methyltransferase stimulates the expression of signal transducer and activator of transcription 1, 2, and 3 genes in colon tumor cells. Proc Natl Acad Sci USA 96(24):14007–14012
99. Phuong NT, Kim SK, Lim SC, Kim HS, Kim TH, Lee KY, Ahn SG, Yoon JH, Kang KW (2011) Role of PTEN promoter methylation in tamoxifen-resistant breast cancer cells. Breast Cancer Res Treat 130(1):73–83
100. Zuo T, Liu TM, Lan X, Weng YI, Shen R, Gu F, Huang YW, Liyanarachchi S, Deatherage DE, Hsu PY, Taslim C, Ramaswamy B, Shapiro CL, Lin HJ, Cheng AS, Jin VX, Huang TH (2011) Epigenetic silencing mediated through activated PI3K/AKT signaling in breast cancer. Cancer Res 71(5):1752–1762
101. Stearns V, Zhou Q, Davidson NE (2007) Epigenetic regulation as a new target for breast cancer therapy. Cancer Invest 25(8):659–665
102. Sharma D, Saxena NK, Davidson NE, Vertino PM (2006) Restoration of tamoxifen sensitivity in estrogen receptor-negative breast cancer cells: tamoxifen-bound reactivated ER recruits distinctive corepressor complexes. Cancer Res 66(12):6370–6378
103. Gao L, Alumkal J (2010) Epigenetic regulation of androgen receptor signaling in prostate cancer. Epigenetics 5(2):100–104
104. Nelson WG, Yegnasubramanian S, Agoston AT, Bastian PJ, Lee BH, Nakayama M, De Marzo AM (2007) Abnormal DNA methylation, epigenetics, and prostate cancer. Front Biosci 12:4254–4266
105. Shang D, Liu Y, Liu Q, Zhang F, Feng L, Lv W, Tian Y (2009) Synergy of 5-aza-2′-deoxycytidine (DAC) and paclitaxel in both androgen-dependent and -independent prostate cancer cell lines. Cancer Lett 278(1):82–87
106. Zorn CS, Wojno KJ, McCabe MT, Kuefer R, Gschwend JE, Day ML (2007) 5-aza-2′-deoxycytidine delays androgen-independent disease and improves survival in the transgenic adenocarcinoma of the mouse prostate mouse model of prostate cancer. Clin Cancer Res 13(7):2136–2143
107. Walton TJ, Li G, Seth R, McArdle SE, Bishop MC, Rees RC (2008) DNA demethylation and histone deacetylation inhibition co-operate to re-express estrogen receptor beta and induce apoptosis in prostate cancer cell-lines. Prostate 68(2):210–222
108. Friend C, Scher W, Holland JG, Sato T (1971) Hemoglobin synthesis in murine virus-induced leukemic cells in vitro: stimulation of erythroid differentiation by dimethyl sulfoxide. Proc Natl Acad Sci USA 68(2):378–382
109. Marks PA, Breslow R (2007) Dimethyl sulfoxide to vorinostat: development of this histone deacetylase inhibitor as an anticancer drug. Nat Biotechnol 25(1):84–90

110. Minucci S, Pelicci PG (2006) Histone deacetylase inhibitors and the promise of epigenetic (and more) treatments for cancer. Nat Rev Cancer 6(1):38–51
111. Qiu L, Burgess A, Fairlie DP, Leonard H, Parsons PG, Gabrielli BG (2000) Histone deacetylase inhibitors trigger a G2 checkpoint in normal cells that is defective in tumor cells. Mol Biol Cell 11(6):2069–2083
112. Strait KA, Warnick CT, Ford CD, Dabbas B, Hammond EH, Ilstrup SJ (2005) Histone deacetylase inhibitors induce G2-checkpoint arrest and apoptosis in cisplatinum-resistant ovarian cancer cells associated with overexpression of the Bcl-2-related protein Bad. Mol Cancer Ther 4(4):603–611
113. Choudhary C, Kumar C, Gnad F, Nielsen ML, Rehman M, Walther TC, Olsen JV, Mann M (2009) Lysine acetylation targets protein complexes and co-regulates major cellular functions. Science 325(5942):834–840
114. Plumb JA, Finn PW, Williams RJ, Bandara MJ, Romero MR, Watkins CJ, La Thangue NB, Brown R (2003) Pharmacodynamic response and inhibition of growth of human tumor xenografts by the novel histone deacetylase inhibitor PXD101. Mol Cancer Ther 2(8):721–728
115. Qian X, LaRochelle WJ, Ara G, Wu F, Petersen KD, Thougaard A, Sehested M, Lichenstein HS, Jeffers M (2006) Activity of PXD101, a histone deacetylase inhibitor, in preclinical ovarian cancer studies. Mol Cancer Ther 5(8):2086–2095
116. Uchida H, Maruyama T, Nagashima T, Asada H, Yoshimura Y (2005) Histone deacetylase inhibitors induce differentiation of human endometrial adenocarcinoma cells through upregulation of glycodelin. Endocrinology 146(12):5365–5373
117. Takai N, Desmond JC, Kumagai T, Gui D, Said JW, Whittaker S, Miyakawa I, Koeffler HP (2004) Histone deacetylase inhibitors have a profound antigrowth activity in endometrial cancer cells. Clin Cancer Res 10(3):1141–1149
118. Rahman R, Grundy R (2011) Histone deacetylase inhibition as an anticancer telomerase-targeting strategy. Int J Cancer 129(12):2765–2774
119. Xu WS, Parmigiani RB, Marks PA (2007) Histone deacetylase inhibitors: molecular mechanisms of action. Oncogene 26(37):5541–5552
120. Cooper AL, Greenberg VL, Lancaster PS, van Nagell JR Jr, Zimmer SG, Modesitt SC (2007) In vitro and in vivo histone deacetylase inhibitor therapy with suberoylanilide hydroxamic acid (SAHA) and paclitaxel in ovarian cancer. Gynecol Oncol 104:596–601
121. Dietrich CS III, Greenberg VL, DeSimone CP, Modesitt SC, van Nagell JR, Craven R, Zimmer SG (2010) Suberoylanilide hydroxamic acid (SAHA) potentiates paclitaxel-induced apoptosis in ovarian cancer cell lines. Gynecol Oncol 116(1):126–130
122. Sonnemann J, Gange J, Pilz S, Stotzer C, Ohlinger R, Belau A, Lorenz G, Beck JF (2006) Comparative evaluation of the treatment efficacy of suberoylanilide hydroxamic acid (SAHA) and paclitaxel in ovarian cancer cell lines and primary ovarian cancer cells from patients. BMC Cancer 6:183
123. Zuco V, Benedetti V, De Cesare M, Zunino F (2010) Sensitization of ovarian carcinoma cells to the atypical retinoid ST1926 by the histone deacetylase inhibitor, RC307: enhanced DNA damage response. Int J Cancer 126(5):1246–1255
124. Son DS, Wilson AJ, Parl AK, Khabele D (2010) The effects of the histone deacetylase inhibitor romidepsin (FK228) are enhanced by aspirin (ASA) in COX-1 positive ovarian cancer cells through augmentation of p21. Cancer Biol Ther 9(11):928–935
125. Yang YT, Balch C, Kulp SK, Mand MR, Nephew KP, Chen CS (2009) A rationally designed histone deacetylase inhibitor with distinct antitumor activity against ovarian cancer. Neoplasia 11(6):552–563; 553 p following 563
126. Iwahashi S, Shimada M, Utsunomiya T, Morine Y, Imura S, Ikemoto T, Mori H, Hanaoka J, Saito Y (2011) Histone deacetylase inhibitor enhances the anti-tumor effect of gemcitabine: a special reference to gene-expression microarray analysis. Oncol Rep 26(5):1057–1062
127. Kim MS, Baek JH, Chakravarty D, Sidransky D, Carrier F (2005) Sensitization to UV-induced apoptosis by the histone deacetylase inhibitor trichostatin A (TSA). Exp Cell Res 306(1):94–102
128. Roh MS, Kim CW, Park BS, Kim GC, Jeong JH, Kwon HC, Suh DJ, Cho KH, Yee SB, Yoo YH (2004) Mechanism of histone deacetylase inhibitor Trichostatin A induced apoptosis in human osteosarcoma cells. Apoptosis 9(5):583–589

129. Srivastava RK, Kurzrock R, Shankar S (2010) MS-275 sensitizes TRAIL-resistant breast cancer cells, inhibits angiogenesis and metastasis, and reverses epithelial-mesenchymal transition in vivo. Mol Cancer Ther 9(12):3254–3266
130. Hacker S, Dittrich A, Mohr A, Schweitzer T, Rutkowski S, Krauss J, Debatin KM, Fulda S (2009) Histone deacetylase inhibitors cooperate with IFN-gamma to restore caspase-8 expression and overcome TRAIL resistance in cancers with silencing of caspase-8. Oncogene 28(35):3097–3110
131. Schuler S, Fritsche P, Diersch S, Arlt A, Schmid RM, Saur D, Schneider G (2010) HDAC2 attenuates TRAIL-induced apoptosis of pancreatic cancer cells. Mol Cancer Ther 9:80
132. Pathil A, Armeanu S, Venturelli S, Mascagni P, Weiss TS, Gregor M, Lauer UM, Bitzer M (2006) HDAC inhibitor treatment of hepatoma cells induces both TRAIL-independent apoptosis and restoration of sensitivity to TRAIL. Hepatology 43(3):425–434
133. Thomas S, Thurn KT, Bicaku E, Marchion DC, Munster PN (2011) Addition of a histone deacetylase inhibitor redirects tamoxifen-treated breast cancer cells into apoptosis, which is opposed by the induction of autophagy. Breast Cancer Res Treat 130(2):437–447
134. Dowdy SC, Jiang S, Zhou XC, Hou X, Jin F, Podratz KC, Jiang SW (2006) Histone deacetylase inhibitors and paclitaxel cause synergistic effects on apoptosis and microtubule stabilization in papillary serous endometrial cancer cells. Mol Cancer Ther 5(11):2767–2776
135. Ahn MY, Chung HY, Choi WS, Lee BM, Yoon S, Kim HS (2010) Anti-tumor effect of apicidin on Ishikawa human endometrial cancer cells both in vitro and in vivo by blocking histone deacetylase 3 and 4. Int J Oncol 36(1):125–131
136. Bali P, Pranpat M, Swaby R, Fiskus W, Yamaguchi H, Balasis M, Rocha K, Wang HG, Richon V, Bhalla K (2005) Activity of suberoylanilide hydroxamic Acid against human breast cancer cells with amplification of her-2. Clin Cancer Res 11(17):6382–6389
137. Morey L, Brenner C, Fazi F, Villa R, Gutierrez A, Buschbeck M, Nervi C, Minucci S, Fuks F, Di Croce L (2008) MBD3, a component of the NuRD complex, facilitates chromatin alteration and deposition of epigenetic marks. Mol Cell Biol 28(19):5912–5923
138. Terasawa K, Sagae S, Toyota M, Tsukada K, Ogi K, Satoh A, Mita H, Imai K, Tokino T, Kudo R (2004) Epigenetic inactivation of TMS1/ASC in ovarian cancer. Clin Cancer Res 10(6):2000–2006
139. Chen MY, Liao WS, Lu Z, Bornmann WG, Hennessey V, Washington MN, Rosner GL, Yu Y, Ahmed AA, Bast RC Jr (2011) Decitabine and suberoylanilide hydroxamic acid (SAHA) inhibit growth of ovarian cancer cell lines and xenografts while inducing expression of imprinted tumor suppressor genes, apoptosis, G2/M arrest, and autophagy. Cancer 117(19):4424–4438
140. Xiong Y, Dowdy SC, Gonzalez Bosquet J, Zhao Y, Eberhardt NL, Podratz KC, Jiang SW (2005) Epigenetic-mediated upregulation of progesterone receptor B gene in endometrial cancer cell lines. Gynecol Oncol 99(1):135–141
141. Belinsky SA, Grimes MJ, Picchi MA, Mitchell HD, Stidley CA, Tesfaigzi Y, Channell MM, Liu Y, Casero RA Jr, Baylin SB, Reed MD, Tellez CS, March TH (2011) Combination therapy with vidaza and entinostat suppresses tumor growth and reprograms the epigenome in an orthotopic lung cancer model. Cancer Res 71(2):454–462
142. Ecke I, Petry F, Rosenberger A, Tauber S, Monkemeyer S, Hess I, Dullin C, Kimmina S, Pirngruber J, Johnsen SA, Uhmann A, Nitzki F, Wojnowski L, Schulz-Schaeffer W, Witt O, Hahn H (2009) Antitumor effects of a combined 5-aza-2′deoxycytidine and valproic acid treatment on rhabdomyosarcoma and medulloblastoma in Ptch mutant mice. Cancer Res 69(3):887–895
143. Herranz D, Serrano M (2010) SIRT1: recent lessons from mouse models. Nat Rev Cancer 10(12):819–823
144. Nebbioso A, Pereira R, Khanwalkar H, Matarese F, Garcia-Rodriguez J, Miceli M, Logie C, Kedinger V, Ferrara F, Stunnenberg HG, de Lera AR, Gronemeyer H, Altucci L (2011) Death receptor pathway activation and increase of ROS production by the triple epigenetic inhibitor, UVI5008. Mol Cancer Ther 10(12):2394–2404

145. Milutinovic S, D'Alessio AC, Detich N, Szyf M (2007) Valproate induces widespread epigenetic reprogramming which involves demethylation of specific genes. Carcinogenesis 28(3):560–571
146. Dong E, Guidotti A, Grayson DR, Costa E (2007) Histone hyperacetylation induces demethylation of reelin and 67-kDa glutamic acid decarboxylase promoters. Proc Natl Acad Sci USA 104(11):4676–4681
147. Arzenani MK, Zade AE, Ming Y, Vijverberg SJ, Zhang Z, Khan Z, Sadique S, Kallenbach L, Hu L, Vukojevic V, Ekstrom TJ (2011) Genomic DNA hypomethylation by histone deacetylase inhibition implicates DNMT1 nuclear dynamics. Mol Cell Biol 31(19):4119–4128
148. Ou JN, Torrisani J, Unterberger A, Provencal N, Shikimi K, Karimi M, Ekstrom TJ, Szyf M (2007) Histone deacetylase inhibitor Trichostatin A induces global and gene-specific DNA demethylation in human cancer cell lines. Biochem Pharmacol 73(9):1297–1307
149. Xiong Y, Dowdy SC, Podratz KC, Jin F, Attewell JR, Eberhardt NL, Jiang SW (2005) Histone deacetylase inhibitors decrease DNA methyltransferase-3B messenger RNA stability and down-regulate de novo DNA methyltransferase activity in human endometrial cells. Cancer Res 65(7):2684–2689
150. Scott SA, Dong WF, Ichinohasama R, Hirsch C, Sheridan D, Sanche SE, Geyer CR, Decoteau JF (2006) 5-Aza-2′-deoxycytidine (decitabine) can relieve p21WAF1 repression in human acute myeloid leukemia by a mechanism involving release of histone deacetylase 1 (HDAC1) without requiring p21WAF1 promoter demethylation. Leuk Res 30(1):69–76
151. Egger G, Aparicio AM, Escobar SG, Jones PA (2007) Inhibition of histone deacetylation does not block resilencing of p16 after 5-aza-2′-deoxycytidine treatment. Cancer Res 67(1):346–353
152. Gore SD, Baylin S, Sugar E, Carraway H, Miller CB, Carducci M, Grever M, Galm O, Dauses T, Karp JE, Rudek MA, Zhao M, Smith BD, Manning J, Jiemjit A, Dover G, Mays A, Zwiebel J, Murgo A, Weng LJ, Herman JG (2006) Combined DNA methyltransferase and histone deacetylase inhibition in the treatment of myeloid neoplasms. Cancer Res 66(12):6361–6369
153. Kaminskyy VO, Surova OV, Vaculova A, Zhivotovsky B (2011) Combined inhibition of DNA methyltransferase and histone deacetylase restores caspase-8 expression and sensitizes SCLC cells to TRAIL. Carcinogenesis 32(10):1450–1458
154. Steele N, Finn P, Brown R, Plumb JA (2009) Combined inhibition of DNA methylation and histone acetylation enhances gene re-expression and drug sensitivity in vivo. Br J Cancer 100(5):758–763
155. Matei DE, Nephew KP (2010) Epigenetic therapies for chemoresensitization of epithelial ovarian cancer. Gynecol Oncol 116(2):195–201
156. Kristensen LS, Nielsen HM, Hansen LL (2009) Epigenetics and cancer treatment. Eur J Pharmacol 625(1–3):131–142
157. Sigalotti L, Fratta E, Coral S, Cortini E, Covre A, Nicolay HJ, Anzalone L, Pezzani L, Di Giacomo AM, Fonsatti E, Colizzi F, Altomonte M, Calabro L, Maio M (2007) Epigenetic drugs as pleiotropic agents in cancer treatment: biomolecular aspects and clinical applications. J Cell Physiol 212(2):330–344
158. Momparler RL, Rivard GE, Gyger M (1985) Clinical trial on 5-aza-2′-deoxycytidine in patients with acute leukemia. Pharmacol Ther 30(3):277–286
159. Wijermans PW, Krulder JW, Huijgens PC, Neve P (1997) Continuous infusion of low-dose 5-Aza-2′-deoxycytidine in elderly patients with high-risk myelodysplastic syndrome. Leukemia 11(suppl 1):S19–S23
160. Wijermans P, Lubbert M, Verhoef G, Bosly A, Ravoet C, Andre M, Ferrant A (2000) Low-dose 5-aza-2′-deoxycytidine, a DNA hypomethylating agent, for the treatment of high-risk myelodysplastic syndrome: a multicenter phase II study in elderly patients. J Clin Oncol 18(5):956–962
161. DeSimone J, Koshy M, Dorn L, Lavelle D, Bressler L, Molokie R, Talischy N (2002) Maintenance of elevated fetal hemoglobin levels by decitabine during dose interval treatment of sickle cell anemia. Blood 99(11):3905–3908

162. Koshy M, Dorn L, Bressler L, Molokie R, Lavelle D, Talischy N, Hoffman R, van Overveld W, DeSimone J (2000) 2-deoxy 5-azacytidine and fetal hemoglobin induction in sickle cell anemia. Blood 96(7):2379–2384
163. Issa JP, Garcia-Manero G, Giles FJ, Mannari R, Thomas D, Faderl S, Bayar E, Lyons J, Rosenfeld CS, Cortes J, Kantarjian HM (2004) Phase 1 study of low-dose prolonged exposure schedules of the hypomethylating agent 5-aza-2′-deoxycytidine (decitabine) in hematopoietic malignancies. Blood 103(5):1635–1640
164. Invest New DrugsSchwartsmann G, Schunemann H, Gorini CN, Filho AF, Garbino C, Sabini G, Muse I, DiLeone L, Mans DR (2000) A phase I trial of cisplatin plus decitabine, a new DNA-hypomethylating agent, in patients with advanced solid tumors and a follow-up early phase II evaluation in patients with inoperable non-small cell lung cancer. Invest New Drugs 18(1):83–91
165. Pohlmann P, DiLeone LP, Cancella AI, Caldas AP, Dal Lago L, Campos O Jr, Monego E, Rivoire W, Schwartsmann G (2002) Phase II trial of cisplatin plus decitabine, a new DNA hypomethylating agent, in patients with advanced squamous cell carcinoma of the cervix. Am J Clin Oncol 25(5):496–501
166. Appleton K, Mackay HJ, Judson I, Plumb JA, McCormick C, Strathdee G, Lee C, Barrett S, Reade S, Jadayel D, Tang A, Bellenger K, Mackay L, Setanoians A, Schatzlein A, Twelves C, Kaye SB, Brown R (2007) Phase I and pharmacodynamic trial of the DNA methyltransferase inhibitor decitabine and carboplatin in solid tumors. J Clin Oncol 25(29):4603–4609
167. Glasspool RM, Gore M, Rustin G, McNeish I, Wilson R, Pledge S, Paul J, Mackean M, Halford S, Kaye S (2009) Randomized phase II study of in combination with carboplatin compared with carboplatin alone in patients with recurrent advanced ovarian cancer. J Clin Oncol 26(15S (May 20 suppl)):Abstract 5562
168. Fu S, Hu W, Iyer R, Kavanagh JJ, Coleman RL, Levenback CF, Sood AK, Wolf JK, Gershenson DM, Markman M, Hennessy BT, Kurzrock R, Bast RC Jr (2011) Phase 1b-2a study to reverse platinum resistance through use of a hypomethylating agent, azacitidine, in patients with platinum-resistant or platinum-refractory epithelial ovarian cancer. Cancer 117(8):1661–1669
169. Fang F, Balch C, Schilder J, Breen T, Zhang S, Shen C, Li L, Kulesavage C, Snyder AJ, Nephew KP, Matei DE (2010) A phase 1 and pharmacodynamic study of decitabine in combination with carboplatin in patients with recurrent, platinum-resistant, epithelial ovarian cancer. Cancer 116(17):4043–4053
170. Matei D, Fang F, Shen C, Schilder J, Arnold A, Zeng Y, Berry WA, Huang T, Nephew KP (2012). Epigenetic resensitization to platinum in ovarian cancer. Cancer Res 72(9): 2197–2205
171. Bauman J, Verschraegen C, Belinsky S, Muller C, Rutledge T, Fekrazad M, Ravindranathan M, Lee SJ, Jones D (2012) A phase I study of 5-azacytidine and erlotinib in advanced solid tumor malignancies. Cancer Chemother Pharmacol 69(2):547–554
172. George RE, Lahti JM, Adamson PC, Zhu K, Finkelstein D, Ingle AM, Reid JM, Krailo M, Neuberg D, Blaney SM, Diller L (2010) Phase I study of decitabine with doxorubicin and cyclophosphamide in children with neuroblastoma and other solid tumors: a Children's Oncology Group study. Pediatr Blood Cancer 55(4):629–638
173. Welch JS, Klco JM, Gao F, Procknow E, Uy GL, Stockerl-Goldstein KE, Abboud CN, Westervelt P, DiPersio JF, Hassan A, Cashen AF, Vij R (2011) Combination decitabine, arsenic trioxide, and ascorbic acid for the treatment of myelodysplastic syndrome and acute myeloid leukemia: a phase I study. Am J Hematol 86(9):796–800
174. Stewart DJ, Issa JP, Kurzrock R, Nunez MI, Jelinek J, Hong D, Oki Y, Guo Z, Gupta S, Wistuba II (2009) Decitabine effect on tumor global DNA methylation and other parameters in a phase I trial in refractory solid tumors and lymphomas. Clin Cancer Res 15(11): 3881–3888
175. Modesitt SC, Sill M, Hoffman JS, Bender DP (2008) A phase II study of vorinostat in the treatment of persistent or recurrent epithelial ovarian or primary peritoneal carcinoma: a Gynecologic Oncology Group study. Gynecol Oncol 109(2):182–186

176. Mackay HJ, Hirte H, Colgan T, Covens A, MacAlpine K, Grenci P, Wang L, Mason J, Pham PA, Tsao MS, Pan J, Zwiebel J, Oza AM (2010) Phase II trial of the histone deacetylase inhibitor belinostat in women with platinum resistant epithelial ovarian cancer and micropapillary (LMP) ovarian tumours. Eur J Cancer 46(9):1573–1579
177. Molife LR, Attard G, Fong PC, Karavasilis V, Reid AH, Patterson S, Riggs CE Jr, Higano C, Stadler WM, McCulloch W, Dearnaley D, Parker C, de Bono JS (2010) Phase II, two-stage, single-arm trial of the histone deacetylase inhibitor (HDACi) romidepsin in metastatic castration-resistant prostate cancer (CRPC). Ann Oncol 21(1):109–113
178. Hainsworth JD, Infante JR, Spigel DR, Arrowsmith ER, Boccia RV, Burris HA (2011) A phase II trial of panobinostat, a histone deacetylase inhibitor, in the treatment of patients with refractory metastatic renal cell carcinoma. Cancer Invest 29(7):451–455
179. Takai N, Narahara H (2010) Histone deacetylase inhibitor therapy in epithelial ovarian cancer. J Oncol 2010:458431
180. Thurn KT, Thomas S, Moore A, Munster PN (2011) Rational therapeutic combinations with histone deacetylase inhibitors for the treatment of cancer. Future Oncol 7(2):263–283
181. Rodon J, Iniesta MD, Papadopoulos K (2009) Development of PARP inhibitors in oncology. Expert Opin Investig Drugs 18(1):31–43
182. Teicher BA (2010) Combinations of PARP, hedgehog and HDAC inhibitors with standard drugs. Curr Opin Pharmacol 10(4):397–404
183. Munster PN, Thurn KT, Thomas S, Raha P, Lacevic M, Miller A, Melisko M, Ismail-Khan R, Rugo H, Moasser M, Minton SE (2011) A phase II study of the histone deacetylase inhibitor vorinostat combined with tamoxifen for the treatment of patients with hormone therapy-resistant breast cancer. Br J Cancer 104(12):1828–1835
184. Drappatz J, Lee EQ, Hammond S, Grimm SA, Norden AD, Beroukhim R, Gerard M, Schiff D, Chi AS, Batchelor TT, Doherty LM, Ciampa AS, Lafrankie DC, Ruland S, Snodgrass SM, Raizer JJ, Wen PY (2012) Phase I study of panobinostat in combination with bevacizumab for recurrent high-grade glioma. J Neurooncol 107(1):133–138
185. Candelaria M, Herrera A, Labardini J, Gonzalez-Fierro A, Trejo-Becerril C, Taja-Chayeb L, Perez-Cardenas E, de la Cruz-Hernandez E, Arias-Bofill D, Vidal S, Cervera E, Duenas-Gonzalez A (2011) Hydralazine and magnesium valproate as epigenetic treatment for myelodysplastic syndrome. Preliminary results of a phase-II trial. Ann Hematol 90(4):379–387
186. Braiteh F, Soriano AO, Garcia-Manero G, Hong D, Johnson MM, Silva Lde P, Yang H, Alexander S, Wolff J, Kurzrock R (2008) Phase I study of epigenetic modulation with 5-azacytidine and valproic acid in patients with advanced cancers. Clin Cancer Res 14(19):6296–6301
187. Stathis A, Hotte SJ, Chen EX, Hirte HW, Oza AM, Moretto P, Webster S, Laughlin A, Stayner LA, McGill S, Wang L, Zhang WJ, Espinoza-Delgado I, Holleran JL, Egorin MJ, Siu LL (2011) Phase I study of decitabine in combination with vorinostat in patients with advanced solid tumors and non-Hodgkin's lymphomas. Clin Cancer Res 17(6):1582–1590
188. Juergens RA, Wrangle J, Vendetti FP, Murphy SC, Zhao M, Coleman B, Sebree R, Rodgers K, Hooker CM, Franco N, Lee BH, Tsai S, Delgado IE, Rudek MA, Belinsky SA, Herman JG, Baylin SB, Brock MV, Rudin CM (2011) Combination epigenetic therapy has efficacy in patients with refractory advanced non-small cell lung cancer. Cancer Discov 1:598–607
189. Candelaria M, Gallardo-Rincon D, Arce C, Cetina L, Aguilar-Ponce JL, Arrieta O, Gonzalez-Fierro A, Chavez-Blanco A, de la Cruz-Hernandez E, Camargo MF, Trejo-Becerril C, Perez-Cardenas E, Perez-Plasencia C, Taja-Chayeb L, Wegman-Ostrosky T, Revilla-Vazquez A, Duenas-Gonzalez A (2007) A phase II study of epigenetic therapy with hydralazine and magnesium valproate to overcome chemotherapy resistance in refractory solid tumors. Ann Oncol 18(9):1529–1538
190. Garcia-Manero G, Kantarjian HM, Sanchez-Gonzalez B, Yang H, Rosner G, Verstovsek S, Rytting M, Wierda WG, Ravandi F, Koller C, Xiao L, Faderl S, Estrov Z, Cortes J, O'Brien S, Estey E, Bueso-Ramos C, Fiorentino J, Jabbour E, Issa JP (2006) Phase 1/2 study of the combination of 5-aza-2′-deoxycytidine with valproic acid in patients with leukemia. Blood 108(10):3271–3279
191. Gollob JA, Sciambi CJ, Peterson BL, Richmond T, Thoreson M, Moran K, Dressman HK, Jelinek J, Issa JP (2006) Phase I trial of sequential low-dose 5-aza-2′-deoxycytidine plus

high-dose intravenous bolus interleukin-2 in patients with melanoma or renal cell carcinoma. Clin Cancer Res 12(15):4619–4627
192. Simpson AJ, Caballero OL, Jungbluth A, Chen YT, Old LJ (2005) Cancer/testis antigens, gametogenesis and cancer. Nat Rev Cancer 5(8):615–625
193. Karpf AR (2006) A potential role for epigenetic modulatory drugs in the enhancement of cancer/germ-line antigen vaccine efficacy. Epigenetics 1(3):116–120
194. Tan J, Yang X, Zhuang L, Jiang X, Chen W, Lee PL, Karuturi RK, Tan PB, Liu ET, Yu Q (2007) Pharmacologic disruption of Polycomb-repressive complex 2-mediated gene repression selectively induces apoptosis in cancer cells. Genes Dev 21(9):1050–1063
195. Yao Y, Chen P, Diao J, Cheng G, Deng L, Anglin JL, Prasad BV, Song Y (2011) Selective inhibitors of histone methyltransferase DOT1L: design, synthesis, and crystallographic studies. J Am Chem Soc 133(42):16746–16749
196. Daigle SR, Olhava EJ, Therkelsen CA, Majer CR, Sneeringer CJ, Song J, Johnston LD, Scott MP, Smith JJ, Xiao Y, Jin L, Kuntz KW, Chesworth R, Moyer MP, Bernt KM, Tseng JC, Kung AL, Armstrong SA, Copeland RA, Richon VM, Pollock RM (2011) Selective killing of mixed lineage leukemia cells by a potent small-molecule DOT1L inhibitor. Cancer Cell 20(1):53–65
197. Wang C, Liu Z, Woo CW, Li Z, Wang L, Wei JS, Marquez VE, Bates SE, Jin Q, Khan J, Ge K, Thiele CJ (2012) EZH2 mediates epigenetic silencing of neuroblastoma suppressor genes CASZ1, CLU, RUNX3 and NGFR. Cancer Res 72(1):315–324
198. Zhou J, Bi C, Cheong LL, Mahara S, Liu SC, Tay KG, Koh TL, Yu Q, Chng WJ (2011) The histone methyltransferase inhibitor, DZNep, up-regulates TXNIP, increases ROS production, and targets leukemia cells in AML. Blood 118(10):2830–2839
199. Crea F, Hurt EM, Mathews LA, Cabarcas SM, Sun L, Marquez VE, Danesi R, Farrar WL (2011) Pharmacologic disruption of Polycomb Repressive Complex 2 inhibits tumorigenicity and tumor progression in prostate cancer. Mol Cancer 10:40
200. Fiskus W, Wang Y, Sreekumar A, Buckley KM, Shi H, Jillella A, Ustun C, Rao R, Fernandez P, Chen J, Balusu R, Koul S, Atadja P, Marquez VE, Bhalla KN (2009) Combined epigenetic therapy with the histone methyltransferase EZH2 inhibitor 3-deazaneplanocin A and the histone deacetylase inhibitor panobinostat against human AML cells. Blood 114(13):2733–2743
201. Hayden A, Johnson PW, Packham G, Crabb SJ (2011) S-adenosylhomocysteine hydrolase inhibition by 3-deazaneplanocin A analogues induces anti-cancer effects in breast cancer cell lines and synergy with both histone deacetylase and HER2 inhibition. Breast Cancer Res Treat 127(1):109–119
202. Suva ML, Riggi N, Janiszewska M, Radovanovic I, Provero P, Stehle JC, Baumer K, Le Bitoux MA, Marino D, Cironi L, Marquez VE, Clement V, Stamenkovic I (2009) EZH2 is essential for glioblastoma cancer stem cell maintenance. Cancer Res 69(24):9211–9218
203. Quinn AM, Allali-Hassani A, Vedadi M, Simeonov A (2010) A chemiluminescence-based method for identification of histone lysine methyltransferase inhibitors. Mol Biosyst 6(5):782–788
204. King ON, Li XS, Sakurai M, Kawamura A, Rose NR, Ng SS, Quinn AM, Rai G, Mott BT, Beswick P, Klose RJ, Oppermann U, Jadhav A, Heightman TD, Maloney DJ, Schofield CJ, Simeonov A (2010) Quantitative high-throughput screening identifies 8-hydroxyquinolines as cell-active histone demethylase inhibitors. PLoS One 5(11):e15535
205. Tang W, Luo T, Greenberg EF, Bradner JE, Schreiber SL (2011) Discovery of histone deacetylase 8 selective inhibitors. Bioorg Med Chem Lett 21(9):2601–2605
206. Vedadi M, Barsyte-Lovejoy D, Liu F, Rival-Gervier S, Allali-Hassani A, Labrie V, Wigle TJ, Dimaggio PA, Wasney GA, Siarheyeva A, Dong A, Tempel W, Wang SC, Chen X, Chau I, Mangano TJ, Huang XP, Simpson CD, Pattenden SG, Norris JL, Kireev DB, Tripathy A, Edwards A, Roth BL, Janzen WP, Garcia BA, Petronis A, Ellis J, Brown PJ, Frye SV, Arrowsmith CH, Jin J (2011) A chemical probe selectively inhibits G9a and GLP methyltransferase activity in cells. Nat Chem Biol 7(8):566–574
207. Cole PA (2008) Chemical probes for histone-modifying enzymes. Nat Chem Biol 4(10): 590–597

208. Kota J, Chivukula RR, O'Donnell KA, Wentzel EA, Montgomery CL, Hwang HW, Chang TC, Vivekanandan P, Torbenson M, Clark KR, Mendell JR, Mendell JT (2009) Therapeutic microRNA delivery suppresses tumorigenesis in a murine liver cancer model. Cell 137(6):1005–1017
209. Ibrahim AF, Weirauch U, Thomas M, Grunweller A, Hartmann RK, Aigner A (2011) MicroRNA replacement therapy for miR-145 and miR-33a is efficacious in a model of colon carcinoma. Cancer Res 71(15):5214–5224
210. Taulli R, Bersani F, Foglizzo V, Linari A, Vigna E, Ladanyi M, Tuschl T, Ponzetto C (2009) The muscle-specific microRNA miR-206 blocks human rhabdomyosarcoma growth in xenotransplanted mice by promoting myogenic differentiation. J Clin Invest 119(8):2366–2378
211. Avramis VI, Mecum RA, Nyce J, Steele DA, Holcenberg JS (1989) Pharmacodynamic and DNA methylation studies of high-dose 1-beta-D-arabinofuranosyl cytosine before and after in vivo 5-azacytidine treatment in pediatric patients with refractory acute lymphocytic leukemia. Cancer Chemother Pharmacol 24(4):203–210
212. Belinsky SA, Klinge DM, Stidley CA, Issa JP, Herman JG, March TH, Baylin SB (2003) Inhibition of DNA methylation and histone deacetylation prevents murine lung cancer. Cancer Res 63(21):7089–7093
213. Goldberg J, Gryn J, Raza A, Bennett J, Browman G, Bryant J, Grunwald H, Larson R, Vogler R, Preisler H (1993) Mitoxantrone and 5-azacytidine for refractory/relapsed ANLL or CML in blast crisis: a leukemia intergroup study. Am J Hematol 43(4):286–290
214. Hakami N, Look AT, Steuber PC, Krischer J, Castleberry R, Harris R, Ravindranath Y, Vietti TJ (1987) Combined etoposide and 5-azacitidine in children and adolescents with refractory or relapsed acute nonlymphocytic leukemia: a Pediatric Oncology Group Study. J Clin Oncol 5(7):1022–1025
215. Huang Y, Nayak S, Jankowitz R, Davidson NE, Oesterreich S (2011) Epigenetics in breast cancer: what's new? Breast Cancer Res 13(6):225
216. Kim MS, Blake M, Baek JH, Kohlhagen G, Pommier Y, Carrier F (2003) Inhibition of histone deacetylase increases cytotoxicity to anticancer drugs targeting DNA. Cancer Res 63(21):7291–7300
217. Leshin M (1985) 5-Azacytidine and sodium butyrate induce expression of aromatase in fibroblasts from chickens carrying the henny feathering trait but not from wild-type chickens. Proc Natl Acad Sci USA 82(9):3005–3009
218. Liu WH, Yung BY (1998) Mortalization of human promyelocytic leukemia HL-60 cells to be more susceptible to sodium butyrate-induced apoptosis and inhibition of telomerase activity by down-regulation of nucleophosmin/B23. Oncogene 17(23):3055–3064
219. Momparler RL, Bouffard DY, Momparler LF, Dionne J, Belanger K, Ayoub J (1997) Pilot phase I-II study on 5-aza-2′-deoxycytidine (Decitabine) in patients with metastatic lung cancer. Anticancer Drugs 8(4):358–368
220. Pollyea DA, Kohrt HE, Gallegos L, Figueroa ME, Abdel-Wahab O, Zhang B, Bhattacharya S, Zehnder J, Liedtke M, Gotlib JR, Coutre S, Berube C, Melnick A, Levine R, Mitchell BS, Medeiros BC (2012) Safety, efficacy and biological predictors of response to sequential azacitidine and lenalidomide for elderly patients with acute myeloid leukemia. Leukemia 26(5):893–901
221. Schwartsmann G, Fernandes MS, Schaan MD, Moschen M, Gerhardt LM, Di Leone L, Loitzembauer B, Kalakun L (1997) Decitabine (5-Aza-2′-deoxycytidine; DAC) plus daunorubicin as a first line treatment in patients with acute myeloid leukemia: preliminary observations. Leukemia 11(suppl 1):S28–S31
222. Willemze R, Archimbaud E, Muus P (1993) Preliminary results with 5-aza-2′-deoxycytidine (DAC)-containing chemotherapy in patients with relapsed or refractory acute leukemia. The EORTC Leukemia Cooperative Group. Leukemia 7(suppl 1):49–50

Chapter 15
Methods for Cancer Epigenome Analysis

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Abstract Accurate detection of epimutations in tumor cells is crucial for understanding the molecular pathogenesis of cancer. Alterations in DNA methylation in cancer are functionally important and clinically relevant, but even this well-studied area is continually re-evaluated in light of unanticipated results, such as the strong association between aberrant DNA methylation in adult tumors and polycomb group profiles in embryonic stem cells, cancer-associated genetic mutations in epigenetic regulators such as *DNMT3A* and *TET* family genes, and the discovery of altered 5-hydroxymethylcytosine, a product of TET proteins acting on 5-methylcytosine, in human tumors with TET mutations. The abundance and distribution of covalent histone modifications in primary cancer tissues relative to normal cells is an important but largely uncharted area, although there is good evidence for a mechanistic role of cancer-specific alterations in histone modifications in tumor etiology, drug response, and tumor progression. Meanwhile, the discovery of new epigenetic marks continues, and there are many useful methods for epigenome analysis applicable to primary tumor samples, in addition to cancer cell lines. For DNA methylation and hydroxymethylation, next-generation sequencing allows increasingly inexpensive and quantitative whole-genome profiling. Similarly, the refinement and maturation of chromatin immunoprecipitation with next-generation sequencing (ChIP-seq) has made possible genome-wide mapping of histone modifications, open chromatin, and transcription factor binding sites. Computational tools have been developed apace with these epigenome methods to better enable accurate interpretation of the profiling data.

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Abbreviations

5MC	5-methylcytosine
5HMC	5-hydroxymethylcytosine
ChIP-seq	Chromatin immunoprecipitation-sequencing
MBD	Methyl binding domain
MeDIP	Methyl DNA immunoprecipitation
MRE	Methyl-sensitive restriction enzyme
RRBS	Reduced representation bisulfite sequencing

15.1 Introduction

DNA methylation is required for genome function through its roles in maintenance of chromatin structure, chromosome stability, and transcription [1–4]. 5-methylcytosine (5MC) is found at a subset of 5′-CpG-3′ dinucleotides and is also sometimes observed at CpNpG, notably in embryonic stem cells [5–7] but also in adult tissues [8]. The modified DNA base 5-hydroxymethylcytosine (5HMC) is also present in mammalian genomes, albeit at a much lower levels compared to 5MC [9, 10]. TET proteins catalyze the hydroxylation of 5MC to generate 5HMC, and can act further on 5HMC to yield 5-formylcytosine and carboxylcytosine [10–12].

The N-terminal tails of histone proteins are modified by acetylation, methylation, phosphorylation, ubiquitylation, crotonylation [13], and other covalent modifications. At some histone residues, such as histone H3 lysine 4 (H3K4), methylation can be mono-, di-, or tri-methyl. Furthermore, multiple types of modifications can exist on a single histone molecule. In addition to DNA methylation and histone modifications, there are other interrelated, potentially epigenetic mechanisms including specific deposition of histone variants, noncoding RNAs, chromatin remodeling, and nuclear organization, which are not discussed here. Current epigenomic methods, especially those making use of next-generation sequencing, provide powerful tools to map 5MC, 5HMC, and histone modifications at high resolution across the genome. However, there are many considerations for selecting the most suitable method, including ease of use, cost, resolution, specificity, quantitation, and availability of computational methods to analyze the data. We describe current epigenomic methods below, focusing primarily on genome-scale mapping methods that use next-generation sequencing.

15.2 Methods for Measurement of DNA Methylation and Hydroxymethylation

There are three main approaches to detect 5MC and 5HMC. Methyl-sensitive restriction enzymes (MRE) cut DNA based on methylation status of cytosines within their recognition sequences (Fig. 15.1a). A second approach includes

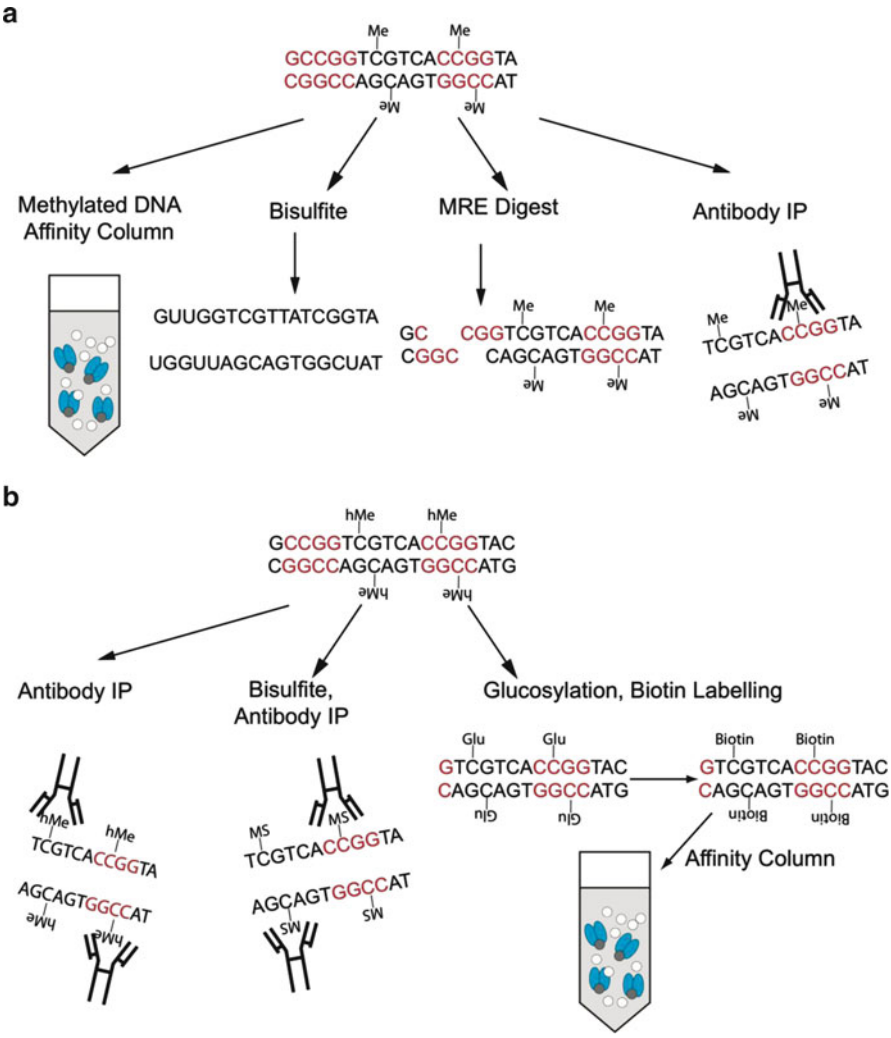

Fig. 15.1 A summary of methods for direct detection of cytosine methylation and hydroxymethylation. (**a**) Methylated DNA can be detected with methyl-sensitive restriction enzymes (MRE), the use of antibodies specific for 5-methylcytosine (5MC), by binding to affinity columns that contain methylated DNA binding domains or by the conversion of DNA with sodium bisulfite. It is important to note that some MRE are also sensitive to hydroxymethylation. (**b**) Several methods have been developed to detect 5 hydroxymethylcytosine (5HMC). These include the addition of a biotin tag to 5HMC through glucosylation and subsequent chemical steps which is followed by an affinity pulldown of the biotin tag, the use of antibodies specific for 5HMC and conversion of 5HMC to 5-cytosine methylenesulfonate (MS) which is then immunoprecipitated with an antibody specific to 5CMS. *Me* methylated cytosine; *hMe* hydroxymethylated cytosine; *Glu* glucosylated cytosine

differential chemical conversion or enzymatic modification of cytosine according to methylation/hydroxymethylation status, such as sodium bisulfite conversion and 5HMC-specific glucosylation. Third, enrichment methods include methyl DNA immunoprecipitation (MeDIP), hydroxyMeDIP (hMeDIP), and methyl binding domain (MBD) affinity purification that are used to enrich for methylated or hydroxymethylated regions. These approaches can be applied to investigate a single locus, hundreds of thousands of loci, or to all mappable sites genome-wide.

15.2.1 Overview of DNA Methylation Reagents

MRE have been used widely for precise, reliable, and inexpensive methylation detection. MRE only assay CpGs within their recognition sites but when multiple non-redundant and frequent-cutting MRE are used in parallel, this limitation is less problematic. There are approximately 50 unique MRE, though only a few have a methylation-insensitive isoschizomer. MRE can resolve the methylation status regionally or at individual CpGs, depending on the platform used following MRE digestion. Some MRE are inhibited by methylation or hydroxymethylation, for example, *Hpa*II [10]. The reliability of MRE enables their straightforward application to next-generation sequencing (MRE-seq) allowing analysis of greater than one million CpGs.

Antibodies against 5MC and 5HMC, and columns containing methylated DNA-binding proteins (domains of MBD2 or MeCP2 alone, or MBD2b combination with MBD3L) allow enrichment for 5MC/5HMC independent of DNA sequence (Fig. 15.1a, b) [14–17]. Enrichment is greater for regions with higher methylated CpG content relative to fully methylated regions with lower CpG content. These reagents are simple to use and many are commercially available. The lower-limit of resolution is determined initially by the size range of DNA prior to enrichment, generally 100–300 bp, and subsequently by the platform used to assess the enrichment, commonly oligonucleotide arrays and next-generation sequencing.

Chemicals including sodium bisulfite and hydrazine react differentially with unmethylated vs. methylated cytosine and allow DNA methylation mapping at single base resolution (Fig. 15.1a) [18–20]. Of these, sodium bisulfite is the most commonly used as it results in a positive display of methylation, among other advantages. Sodium bisulfite initiates conversion of cytosine to uracil, which is replaced by thymine during PCR amplification. In contrast, methylated cytosines are non-reactive, and remain as cytosine after bisulfite treatment. Sequencing of individual clones of the PCR product allows assessment of methylation status of contiguous CpGs derived from a single genomic DNA fragment. Bisulfite has many advantages, including single CpG resolution, detection of strand and allele-specific methylation, and detection of non-CpG cytosine methylation. Unlike other methylation-detection reagents, bisulfite provides estimates of absolute rather than relative DNA methylation levels, depending on the platform used. The reduced sequence complexity of the genome following bisulfite treatment complicates its

application to oligonucleotide arrays [21], but is not a major issue when a sequencing platform is used. Hydroxymethylated cytosines are resistant to conversion to uracil and are indistinguishable from 5MC in bisulfite sequencing. The reaction of 5HMC with bisulfite yields cytosine methylenesulfonate, which can be specifically detected with an affinity method [22]. Alternatively, the hydroxyl group of 5HMC can be enzymatically glucosylated and biotin labeled to detect 5HMC [22, 23].

15.2.2 Methyl-Sensitive Restriction Enzyme Methods

The HTF (*Hpa*II tiny fragments) enrichment by ligation-mediated PCR, or HELP assay, uses the methyl-sensitive *Hpa*II along with its methylation-insensitive isoschizomer *Msp*I to identify unmethylated CpG sites within the sequence 5′-CCGG-3′ [24]. Genomic DNA digested separately with each enzyme is size-selected to capture small DNA fragments. Custom adaptors complementary to digest ends are ligated and the adaptor-ligated molecules are amplified by PCR. The amplification products can be analyzed using a variety of platforms, including next-generation sequencing on the Illumina platform (HELP-seq) [25]. Methyl-seq is a second Illumina sequencing-based assay that uses *Hpa*II/*Msp*I [26]. Similar to HELP, the protocol involves separate *Hpa*II and *Msp*I digests, adaptor ligation, and Illumina sequencing. Approximately 65% of the CpG islands (CGIs) in the human genome are sampled using Methyl-seq. MRE methods are generally biased to CGIs, which constitute 1–2% of the genome and 7% of all CpGs in the genome. Methyl-seq is similarly biased, though non-CGI sites account for ~61% of the regions assayed, including a variety of genomic sequences such as promoters, exons, introns, and intergenic regions.

Ball et al. reported a third variation of MRE-seq, using *Hpa*II/*Msp*I digestion with Illumina sequencing to analyze DNA methylation in the PGP1 EBV-transformed B-lymphocyte cell line [27]. This approach, termed methyl-sensitive cut counting (MSCC), assayed ~1.4 million unique *Hpa*II sites. Using MSCC and a complementary method, bisulfite padlock probe sequencing (BSPP) to assay the methylation status of approximately 10,000 CpGs, highly expressed genes were found to be associated with high gene-body methylation and low promoter methylation. MSCC read counts were linearly related to BSPP percent methylation at 381 CpG sites that were assayed with both methods, suggesting that MSCC allows relative quantification of methylation levels.

DNA methylation has also been assessed through traditional Sanger sequencing combined with MRE in digital karyotyping [28, 29]. Using a combination of MRE that recognize 6–8 bp sites and methylation insensitive restriction enzymes, a library of short sequence tags is generated. The number of tags sequenced reflects the level of methylation at each recognition site, with lower tag counts representing greater methylation levels. In this method, the number of sites analyzed depends on the MRE used—use of *Asc*I, for example, can generate over 5,000 unique tags that correspond to >4,000 genes.

These sequencing-based methods demonstrate the utility of MRE for analysis of DNA methylation. The single CpG resolution and ability to assay a significant portion of the methylome with next-generation sequencing, including most CGIs, makes this a powerful, accurate, and straightforward way to assess methylation across the genome. When used alone, the MRE-seq methods enable relative rather than absolute methylation levels to be estimated. An integrative method [30, 31] that combines MRE-seq in parallel with MeDIP-seq to increase resolution, CpG coverage, and accuracy in quantitation is discussed below.

15.2.3 *Mcr*BC and CHARM

The methylation-dependent restriction enzyme *Mcr*BC recognizes methylated DNA and cuts near its recognition sequence. *Mcr*BC recognizes $R^mC(N)_{55–103}R^mC$ and cuts once between each pair of half-sites, close to one half-site or the other. The cuts can be distributed over several base pairs and approximately 30 base pairs distant from the methylated base, generating a distribution of DNA ends rather than precisely defined DNA ends. *Mcr*BC is useful to size-separate methylated DNA from unmethylated DNA, since the unmethylated DNA remains high-molecular weight after digestion. *Mcr*BC was initially applied to microarrays [32].

The "comprehensive high-throughput arrays for relative methylation" (CHARM) method is an array-based technique for methylation profiling using *Mcr*BC [33]. To improve specificity and sensitivity, probes were optimized based on location and CpG density on custom arrays. Because neighboring CpG sites tend to have a highly correlated methylation status, neighboring probe signals are averaged to reduce background noise without loss of sensitivity or specificity, though modestly reducing resolution. By comparing CHARM to MeDIP or *Hpa*II on arrays, Irizarry et al. showed that *Mcr*BC yields better methylome coverage than *Hpa*II and less bias for CpG density than MeDIP. Using CHARM, aberrant DNA methylation was found in colon cancer at sequences up to 2 kb flanking CGIs, referred to as CGI shores [34]. These data demonstrate the utility of *Mcr*BC-based methylation detection, and the new biological insights afforded by the CHARM method.

15.2.4 Methyl DNA Immunoprecipitation

In addition to MRE and *Mcr*BC, methylation can be assessed by immunoprecipitation of methylated DNA with a monoclonal antibody against 5-methylcytidine (MeDIP) [14]. This antibody does not recognize 5HMC [35], which can be specifically immunoprecipitated with an anti-5HMC antibody [36–39]. A major advantage of MeDIP-based detection is that it is not limited to a specific restriction site and theoretically any fragment with a methylated cytosine is immunoprecipitated. One approach involves the coupling of MeDIP with DNA

microarrays to obtain relative methylation levels at the loci represented on the array [14, 40–44].

MeDIP combined with next-generation sequencing (MeDIP-seq) can be used to interrogate the majority of mappable CpG and non-CpG cytosines in the genome. In a step forward from array-based methods, MeDIP-seq allows analysis of monoallelic methylation and methylation in a significant number of repeat sequences. Most protocols generate a MeDIP sequencing library by sonicating DNA followed by end-repair, adaptor ligation, immunoprecipitation with the anti-methylcytidine antibody and PCR amplification. The methylation-enriched library is sequenced and the reads are mapped back to a reference genome. A specific genomic region shows higher read density when methylated in one sample compared to when the same region is unmethylated in another sample, although read density between different regions is affected by the density of methylated CpGs, DNA copy number, and potentially other factors (discussed in Robinson et al. [45, 46]). These considerations are also important for MBD affinity-based approaches. MeDIP-seq has been applied to a variety of sample types from multiple organisms including human cancer [30, 31, 47–53].

Several computational methods have been specifically designed for analyzing MeDIP data while addressing local density of methylated CpGs. MEDME (modeling experimental data with MeDIP enrichment) is a combination of analytical and experimental methodologies that improve the interpretation of MeDIP-chip data, and addresses the non-linear relationship between enrichment signal and CpG density that is particular to MeDIP-chip [54]. A second analytical method for MeDIP-chip and also MeDIP–seq data called Bayesian tool for methylation analysis (BATMAN) uses a CpG density-derived coupling factor to quantify methylation levels across a range of CpG densities [47]. MEDIPS is a third approach that, like BATMAN, uses a CpG density coupling factor and in addition provides a framework for evaluating quality control parameters, estimating absolute methylation and comparing samples to detect regions of statistically significant differential methylation [51]. MeDIP-chip and MeDIP-seq are lower resolution compared to bisulfite-based methods. On the other hand, MeDIP-seq provides comprehensive methylome coverage at a fraction of the cost of shotgun bisulfite sequencing. Experimental and computational advances should enable increased resolution and quantitation of methylation levels using MeDIP-seq alone or in combination with MRE-seq.

15.2.5 Affinity-Based Enrichment Using Methyl Binding Domains

The Methylated CpG Island Recovery Assay (MIRA) is an alternative to MeDIP for selecting/enriching for methylated DNA, particularly at CGIs [15–17]. MIRA involves size fractionation of DNA, either by sonication or with *Mse*I which recognizes 5′-TTAA, a site that is typically found outside of CGIs. After digestion, adaptors are ligated to the DNA followed by selective binding of methylated fragments

on a column with full-length MBD2b and MBD3L1 proteins. MBD2b is a methyl-binding protein that exhibits a high affinity for methylated DNA relative to unmethylated DNA [15]. MBD3L1 lacks a methyl-CpG binding domain but can interact with MBD2b and improves enrichment of methylated DNA [15]. The methylated DNA eluted from the column is amplified by PCR, fluorescently labeled and hybridized to a microarray.

There are several similar approaches that combine affinity enrichment with Illumina sequencing. In MethylCap-seq, the MBD of MeCP2 is used to capture methylated DNA fragments after sonication [52, 55]. Binding occurs at low salt concentration and then a step-wise elution of captured DNA is performed by increasing the salt concentration, allowing collection of fractions with differing methylated CpG density, with highly methylated, CpG-dense fragments eluting at the higher salt concentrations. The eluates can be sequenced separately or pooled. The MBD2 MBD alone can be used for enrichment followed by Illumina sequencing, called MBD-isolated Genome Sequencing (MiGS) [56]. In this protocol, a single elution is performed. MBD2 enrichment with serial elution in increasing salt has been called MBD-seq [31, 57] or MBDCap-seq [45].

Several studies have directly compared MeDIP-seq with MBD affinity-based sequencing. Harris et al. found that MeDIP-seq and MBD-seq were 99% concordant using binary methylation calls in 200 bp windows or 1,000 bp windows [31]. MeDIP-seq enriched more at regions of low methylated CpG density compared to MBD-seq. Also, MeDIP-seq appeared to detect non-CpG methylation (i.e., at CpNpG) but MBD-seq did not, as predicted. Bock et al. compared MeDIP-seq with MethylCap-seq and observed similar levels of accuracy in quantifying methylation when comparing each to Infinium 27 K data. In both of these studies, MeDIP-seq and MBD affinity-based sequencing performed well in comparison with bisulfite next-generation sequencing.

15.2.6 Integrative MeDIP- and MRE-seq

MeDIP-seq and other affinity-based methods provide a positive display of methylated loci, and the absence of signal usually represents unmethylated loci, but also could be a result of regions that are difficult to PCR amplify or sequence, or insufficient sequencing depth. A method that combines MeDIP-seq with MRE-seq leverages their complementarity [30, 31, 58]. Independent MeDIP-seq and MRE-seq libraries are generated from the same DNA sample and sequenced separately. For MRE-seq, three to five parallel digests are performed using the MRE *Hpa*II, *Aci*I, *Hin*61, *Bsh*1236I, and *Hpy*CH4IV; the digests are size-selected and combined into a single library. Because the restriction sites from these enzymes are non-overlapping, each additional enzyme greatly increases coverage of unique CpG sites. At a moderate sequencing depth integrated MeDIP- and 3 enzyme MRE-seq together interrogate either uniquely or as multimapping sites ~22 million of the ~29 million

CpGs in the haploid human genome [31]. The integrative method is useful for detecting intermediate methylation, including regions of allelic methylation that overlap with monoallelic histone modifications and monoallelic gene expression [31]. This illustrates another significant advantage of sequencing-based epigenome analyses—the ability to assign an epigenetic state to a given genetic allele. For extensive DNA methylation profiles of human cells and tissues, see http://vizhub.wustl.edu/.

15.2.7 Indirect Methylation Detection with Demethylating Agents and Expression Arrays

Genetic or chemical inhibition of DNA methylation followed by expression array analysis can identify genes that may have been silenced by DNA methylation [59–63]. siRNA or shRNA can be used to knock down the DNA methyltransferases, or cell lines can be treated with demethylating agents such as 5-aza-2′deoxycytidine (5-aza) alone, or 5-aza in combination with histone deacetylase inhibitors. 5-aza is a cytidine analog that is incorporated into DNA and covalently binds and inhibits DNA methyltransferase, resulting in passive demethylation. 5-aza treatment results in activation of genes that were silenced by DNA methylation, provided that the appropriate transcription factors are present. However, interpretation of this indirect assessment of methylation is complicated by the fact that genes lacking promoter methylation may also exhibit an increase in expression following 5-aza treatment [64]. Presumably this results from demethylation at other loci within the same gene or in genes upstream that are required for its expression, though direct effects on unmethylated regulatory elements cannot be ruled out. Furthermore, this approach is best applied to cells grown in culture such as cell lines or early passage primary cells [65], as 5-aza requires replication to induce passive demethylation. The application of this approach to cultured tumor cells is complicated by epigenetic silencing that results from long-term culturing, rather than cancer or cell type-specificity.

15.2.8 Reduced Representation Bisulfite Sequencing

Bisulfite treatment converts unmethylated cytosines to uracil but methylcytosine and hydroxymethylcytosine are resistant to conversion. When followed by cloning and Sanger sequencing, this approach yields quantitative, allelic, contiguous, and base resolution of cytosine methylation. However, the shotgun bisulfite approach has been quite expensive for mammalian methylomes. It is important to note that hydroxymethylcytosine and methylcytosine cannot be distinguished by bisulfite sequencing as both block conversion.

To retain the advantages of methylation detection by bisulfite while reducing the cost of shotgun bisulfite sequencing, Meissner et al. developed a technique that interrogates DNA fragments from a reduced representation of the bisulfite-treated genome [66–68]. The reduction comes from DNA digestion with methylation-insensitive restriction enzyme *Msp*I and fragment size selection. After digestion, the ends of the DNA are filled-in with dGTP and methylated dCTP, followed by the addition of an A overhang to enable adaptor ligation. The adaptors used for this assay are methylated at cytosine residues to prevent conversion during bisulfite treatment. The adaptor-ligated DNA is then size selected on a gel and two fractions are excised—the sizes of which depend on the organism. For mouse DNA, approximately 300,000 *Msp*I fragments that span 40–220 bp are analyzed, which corresponds to nearly 1.4 million CpG sites analyzed at the nucleotide level [67]. These fragments are then bisulfite treated, PCR amplified, and size selected again to generate a sequencing library. Several factors must be considered with this approach. First, the choice of a restriction enzyme to fractionate the DNA will bias the portion of the genome that is represented. A second consideration is the process of mapping reads of bisulfite-converted DNA to the genome. Several mapping algorithms for "bisulfite genomes" have been developed [67, 69–71]. Compared to other sequencing methods, reduced representation bisulfite sequencing (RRBS) provides an efficient way to generate absolute quantification of methylation of more than one million CpG sites at single base pair resolution. Methylation at non-CpG cytosines can also be assessed by RRBS [8]. RRBS has been successfully applied to nanogram quantities of genomic DNA [72] and to large numbers of human cell and tissue types (http://vizhub.wustl.edu/).

15.2.9 Shotgun Bisulfite Sequencing

Shotgun sequencing of bisulfite-treated DNA has been successfully applied to several organisms, including humans [7, 69, 70, 73–78] and provides comprehensive, single cytosine quantification of methylation level when sequence coverage is sufficiently deep. A single-CpG-resolution shotgun bisulfite experiment on human DNA requires hundreds of millions of sequencing reads, with the exact number varying depending on the desired sequencing depth and on read lengths [78]. Many regions >200 bp in the mammalian genome do not contain CpGs and thus a large number of sequence reads may be uninformative, at least for CpG methylation. Prior selection of sequences, for example, through sequence capture methodology, or enrichment of methylated DNA or unmethylated DNA followed by shotgun sequencing could increase the efficiency and decrease the cost of this approach. Bisulfite sequencing that first employs selective "reduction" of the genome (e.g., RRBS) is far less expensive. Nevertheless, the cost of sequencing full DNA methylomes has decreased 20-fold since the first human methylome [7]. Shotgun bisulfite methylomes have been generated for a breast cancer cell line and primary human

mammary epithelial cells [79] and primary colorectal cancer and adjacent normal colon tissue [80].

RRBS and shotgun bisulfite sequencing require algorithms that are tailored to mapping the sequence reads from bisulfite-treated DNA back onto the genome. Several algorithms have been developed for this computationally intensive problem [67, 69–71, 81, 82]. The reduction in base complexity from the bisulfite conversion and the fact that a CpG can be methylated or unmethylated are issues that are addressable though complex when aligning bisulfite reads. Due to the bisulfite conversion process, the forward and reverse strands of DNA are no longer complementary and the sequence reads therefore are aligned to four different bisulfite-converted genomes: forward BS, forward BS reverse complement, reverse BS, reverse BS reverse complement). Thus, for this mapping there is increased search space along with a reduction of sequence complexity, requiring significant computation time for the read mapping [31].

15.2.10 Other Bisulfite Methods

Illumina Infinium methylation assays are mid-range platforms using bisulfite conversion and bead arrays to quantify methylation levels at individual CpGs. The HumanMethylation27 and HumanMethylation450 formats interrogate 27,578 and >450,000 CpGs, respectively. Bead-bound oligonucleotides corresponding to the methylated and unmethylated states of a single CpG site are hybridized to bisulfite-converted DNA and differentially labeled with Cy3 or Cy5. The methylation level is determined by the ratio of Cy3 and Cy5 fluorescence on the bead array. The HumanMethylation27 BeadChip interrogates 12 samples at a time and includes probes from 1,000 cancer-related genes and from putative promoters of 110 miRNA, among others. While there are on average 2 CpG sites assayed per gene for the majority of genes, 150 genes known to exhibit aberrant tumor-specific methylation are assayed at 5–10 CpGs each. The vast majority of 27 K probes are located in promoters. The 450 K platform expands the genomic regions that are assayed by Infinium. Genes are broadly profiled, with probes in the promoter, 5′ UTR, first exon, gene body, and 3′ UTR. Ninety nine percent of CGIs have probes, and the CGI shores, 2 kb regions flanking CGIs, and regions flanking shores, called "shelves," are also examined for most CGIs. Like the 27 K assay, a single 450 BeadChip can assay 12 samples. Both versions require 500 ng of DNA prior to bisulfite conversion. These methods do not assess multiple closely apposed CpGs individually, and such regions are generally avoided in the assay development. This bias is likely to impact biological insights drawn from this data.

Another bisulfite-based method, the Sequenom EpiTyper assay, utilizes MALDI-TOF mass spectrometry to analyze RNA cleavage fragments derived from post-bisulfite PCR products that contain a promoter to drive transcription [83, 84]. This unique assay allows high-throughput quantitative methylation analysis at hundreds

of loci, usually at single CpG resolution, and is quite useful for candidate loci in hundreds of samples, or as a follow-up to genome-wide profiling.

BSPPs are molecular inversion probes designed to target and capture specific CpG sites from bisulfite-converted DNA [27, 85]. The strategy is similar to RRBS in that a subset of CpG sites are analyzed by bisulfite sequencing to reduce the genomic space that must be covered, but with the advantage that particular CpGs can be assayed, instead of only those located within a set of restriction fragments. Tens of thousands of BSPPs can be amplified in single reaction and sequenced on the Illumina platform. Deng et al. were able to assay ~66,000 CpG sites, primarily in CGIs [85]. A prominent advantage of this technology is that it is customizable and can target a specific set of CpG sites of interest to the investigator.

15.3 Detection of 5-Hydroxymethylcytosine

5HMC is abundant in mammalian genomes. The tissue-specificity, genomic distribution, and functional significance of 5HMC are under investigation. Pre-existing 5MC is hydroxylated by the TET family of dioxygenases (TET1, TET2, and TET3) to yield 5HMC [10, 86]. TET proteins can further modify 5HMC resulting in formylmethylcytosine, carboxymethylcytosine, and possibly through steps mediated by base excision repair, unmodified cytosine [11, 12]. *TET1* is an *MLL* translocation partner in acute myeloid leukemia [87, 88] and *TET2* mutations occur in myeloid malignancies associated with decreased 5HMC [89], suggesting that dysregulation of 5HMC plays a role in cancer.

Detecting and quantifying 5HMC is challenging because many reagents used for detecting 5MC do not distinguish 5HMC from 5MC. Like 5MC, 5HMC is resistant to C-to-U transition following bisulfite treatment [90], and these bases are indistinguishable by bisulfite cloning and sequencing or other bisulfite-based methods. In addition, 5HMC reacts with bisulfite to yield cytosine 5-methylenesulfonate (CMS) and DNA with dense CMS is inefficiently amplified during PCR due to *Taq* polymerase stalling at CMS sites [90]. As a result, quantification of hydroxymethylation in regions of dense 5HMC, if they exist in some biological contexts, may be underestimated with bisulfite-based methods. MRE-based methods also do not distinguish 5MC from 5HMC, depending on the enzymes used, such as *Hpa*II, which is inhibited by 5MC or 5HMC in its recognition sequence [10]. Finally, affinity-based 5MC methods (MeDIP-seq, MBD-seq, etc.) are specific to 5MC and do not detect 5HMC directly, but could indirectly enrich for regions with 5HMC when it occurs on the same DNA fragment as 5MC [35].

Global quantification of 5HMC levels (measuring the relative or absolute amount of 5HMC present within a DNA sample) can be assayed by thin layer chromatography (TLC) [9, 10] and high-performance liquid chromatography-mass spectrometry (HPLC-MS) [9, 91]. Recently, a profusion of 5HMC mapping techniques have also been developed, many of which can be employed for genome-wide analysis.

15.3.1 5HMC Glucosylation Methods

There are several methods based on in vitro glucosylation of 5HMC in DNA that can be used for global quantification or mapping of 5HMC. These methods use bacteriophage T4 beta-glucosyltransferase (BGT) to catalyze the addition of a glucose moiety to the hydroxyl group of 5HMC. For global quantification, a radiolabeled substrate (uridine 5′-diphosphate-[^{3}H]-glucose) is used in the BGT-catalyzed reaction. The amount of labeled substrate incorporated is compared to standards, allowing absolute quantification [92]. A mapping method called GLIB (glucosylation, periodate oxidation, biotinylation) combines glucosylation by BGT with subsequent chemical reactions, resulting in the addition of two biotin molecules to each 5HMC [22]. The biotin-tagged 5HMC DNA is then pulled down with streptavidin and sequenced on the Helicos single molecule platform. GLIB has high sensitivity, with 90% recovery of DNA fragments containing a single 5HMC molecule. Song et al. present a second mapping method, in which a chemically engineered glucose containing an azide group is transferred to 5HMC by BGT [23]. The azide group is then chemically tagged with biotin and affinity enriched, with global quantification performed using avidin-horseradish peroxidase and genome-wide mapping through Illumina sequencing. Finally, a method has been developed utilizing the restriction endonuclease *Msp*I, which cuts C^{m}CGG and C^{hm}CGG, but not C^{gluc}CGG sites. Locus-specific 5HMC can be estimated using *Msp*I digestion on BGT-modified DNA followed by quantitative PCR across the cleavage site [36, 93].

15.3.2 5HMC Affinity Enrichment Methods

There are two enrichment methods for 5HMC based on antibodies that detect 5HMC itself or 5-cytosine methylenesulfonate (CMS), the product of reacting 5HMC with sodium bisulfite. The 5HMC antibody with sequencing approach, hMeDIP-seq [36–39], is similar to MeDIP-seq, and informatic tools originally developed for MeDIP-seq data have been employed in hMeDIP-seq. Monoclonal and polyclonal anti-5HMC antibodies are commercially available, but their 5HMC-density dependence [22, 89], along with the relatively low genomic abundance of 5HMC in some tissues, might result in inefficient pulldown of 5HMC-sparse regions. The anti-CMS antibody sequencing approach was developed as a more sensitive, less density-dependent alternative to hMeDIP-seq [22]. CMS pulldown had lower background and decreased density dependence compared to commercial anti-5HMC antibodies. CMS-enriched libraries were sequenced on the Illumina platform. Since Illumina library construction protocols usually require at least one PCR step, the tendency of *Taq* polymerase to stall at regions of dense CMS could be problematic.

The rapid development of methods for the detection and quantification of 5HMC has paralleled the exciting pace of discovery of the distribution and potential

functional roles of this "sixth base." Computational tools that are specific for hMeDIP-seq and CMS-pulldown have not been reported yet. For hMeDIP-seq, tools developed for MeDIP-seq, such as MEDIPS [51] have been adapted [38]. Stroud et al. used SICER, which was originally developed for analyzing chromatin immunoprecipitation-sequencing (ChIP-seq) data for diffusely distributed histone modifications, to define regions of 5HMC enrichment [39]. The next generation of genome-wide mapping methods for 5HMC may involve direct detection of the modified base by single molecule sequencing [23, 94].

15.4 Chromatin Immunoprecipitation-Sequencing

Alterations in histone modification patterns and transcription factor binding impact gene expression and have been implicated in tumorigenesis, cancer cell stemness, metastasis, and drug resistance [95–98]. Chromatin immunoprecipitation coupled with next-generation sequencing (ChIP-seq) has become the gold standard to study histone modifications and transcription factor binding genome-wide. It provides higher resolution, improved signal-to-noise ratios, and when using indexed libraries, it is less expensive than coupling ChIP with microarrays (ChIP-chip) [99]. Fresh or fresh frozen tissue or cells are either kept native (N-ChIP) [100] or formaldehyde cross-linked to preserve weaker DNA–protein interactions (X-ChIP) [101], followed by cell lysis (Fig. 15.2). N-ChIP is primarily used for histone modifications, where the DNA histone interactions are inherently strong [99]. Antibody specificity and immunoprecipitation are more efficient with N-ChIP as epitopes can be disrupted by formaldehyde [100], however, N-ChIP cannot be applied to proteins with lower DNA binding affinities such as transcription factors. Cross-linking ameliorates this problem, and minimizes stochastic nucleosome movement that can occur during N-ChIP [100], however, it also may fix transient non-functional interactions and reacts at lysines which may create biases. Native or cross-linked chromatin is then fragmented by sonication or micrococcal nuclease (MNase) digestion. Both methods impart bias in downstream sequencing [102]. MNase creates higher resolution, primarily mononucleosome (~146 bp) fragments, but is less efficient at cutting between G and C bases, creating greater fragmentation bias [103, 104]. In contrast, sonication provides decreased resolution (200–600 bp) but is more uniform [99]. Fragmented chromatin is immunoprecipitated with an antibody that specifically recognizes the epitope of interest. The success of ChIP reactions is dependent on antibody quality. Polyclonal antibodies are advantageous for X-ChIP experiments, as they reduce the chance of cross-linking destroying antibody interactions [101], but may have increased cross-reactivity. Relative enrichment of ChIP DNA is assayed via qPCR. Enrichment varies greatly with the protein of interest, antibody quality, and positive and negative control regions of the genome that are used. To minimize the number of reads contributing to background noise, it is common to require greater enrichment in ChIP-seq (5–50-fold) when compared to single locus ChIP-PCR [102]. Purified ChIP DNA sequencing libraries are constructed by end

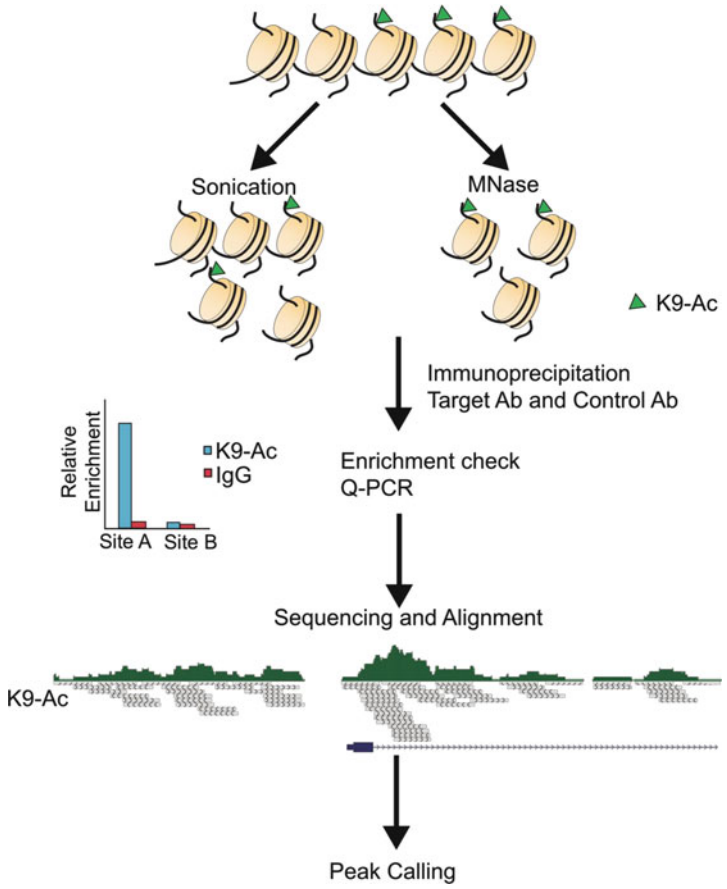

Fig. 15.2 Overview of chromatin immunoprecipitation-sequencing. DNA is fractionated via sonication (~200–600 bp) or with micrococcal nuclease (~146 bp). The fractionated DNA is then immunoprecipitated (IP) with a target antibody and an isotype control antibody. The efficiency of the immunoprecipitation is assayed by quantitative PCR, testing regions that are known to be bound (site A, positive control) or not bound (site B, negative control). The enriched DNA is then used to generate a DNA sequencing library, which is sequenced and reads are aligned to the appropriate genome. Each read is depicted as a *grey line*, the read densities are displayed above in *green* and a gene is shown in *blue*. Finally, the aligned reads are used to generate peaks that mark regions of statistically significant enrichment of reads for the IP of the histone mark or chromatin protein of interest

repair, A base addition, adapter ligation, PCR amplification and size selection. Additional bias may occur during library construction and PCR amplification, as both GC-rich and GC-poor regions are underrepresented [99, 102]. The total number of sequence reads required depends on the quality of ChIP enrichment, the expected number of peaks and peak size, but sequencing multiple-indexed ChIP libraries in a single lane is common practice.

15.4.1 ChIP-seq Data Analysis

Transforming the millions of sequencing reads generated by ChIP-seq into biologically interpretable data is a computationally demanding, multi-step process for which a variety of tools have been developed. While many tools address the same problem, each tool is different and can impact the final result. The first and most resource-intensive step is aligning the sequence reads to the genome. Most sequencing platforms come with alignment pipelines, however, third-party aligners are commonly used, such as MAQ [105], Bowtie [106], BWA [107], SOAP [108, 109], and PASH [110]. These packages differ by alignment algorithm, as well as how multi-aligning reads and gapped vs. un-gapped alignments are handled, resulting in differences in sensitivity and specificity. For most cancer samples a gapped aligner is preferred to allow for the variety of genetic aberrations accumulated in the tumor. Aligned reads are then analyzed to find enriched areas or "peaks" in the genome, for which a number of "peak calling" algorithms have been created [99, 111]. Though the exact method varies between programs, most shift tags based on chromatin fragment size to accumulate tags near the true binding site and increase peak resolution [111]. Regions of statistical enrichment of IP tags relative to a background control are calculated. The most commonly used control is input DNA isolated from the same chromatin batch as the ChIP [99]. This reduces false positives introduced from fragmentation and mappability biases, and controls for genetic differences such as copy number alterations that affect read density. Finally, peaks are filtered based on uneven distributions of sense and antisense tag accumulation [111]. Most current peak callers identify focal enrichments such as transcription factor binding sites, however, some have been developed for broader marks like histone modifications associated with heterochromatin [112–114]. Many groups are actively researching ways to reduce noise and increase true positives.

15.4.2 Application of ChIP-seq to Cancer Epigenomes

The network of transcription regulatory factor interactions and their effects on gene expression in cancer are under investigation. ChIP-seq was initially used to profile T-cells, and since then a main focus has been on embryonic stem cells and cell lines [115–117]. Recently, distinct chromatin states or "signatures" comprised of combinatorial histone marks have been linked to specific functional genomic elements by integrating multiple ChIP-seq data across human cell lines [118–120]. The combinatorial histone signatures identified in these studies have not been investigated in the context of tumor progression. Multidimensional epigenomic profiles of tumors also provide a novel means of sub-type classification, identifying prognostic markers, and insight into tumor cell of origin. ChIP-seq will also help the annotation and functional characterization of non-genic susceptibility loci, as has been recently performed in prostate cancer [121] and in GWAS studies [120]. New techniques are being developed to perform ChIP-seq on a small number of cells, creating an

opportunity to better analyze intratumoral heterogeneity of epigenomic patterns [122, 123]. Finally, chromosome conformation capture (3C) technology [124] and its high-throughput derivatives (4C [125], 5C [126], Hi-C [127], ChIP-Loop [128, 129], ChIA-PET [130]) detect distal DNA–DNA interactions (e.g., promoter-enhancer), but can also be used to identify complex genomic rearrangements in cancers [131]. Coupling ChIP with 3C technologies followed by sequencing will likely be a powerful way to study how both epigenetic patterns and associated structural interactions change during the process of tumorigenesis.

15.5 Future Directions

Recent unanticipated data offer new understanding of, and stimulate new investigations into aberrant epigenetic patterns in cancer. First, promoters with polycomb-mediated histone modifications in ES cells are among those commonly aberrantly hypermethylated in adult tumors [132–134]. Second, cancer-associated mutations occur in the DNA methyltransferase *DNMT3A* [135, 136], suggesting another possible origin of DNA methylation abnormalities, though this remains to be determined. Similarly, the occurrence of *TET1* translocation [87, 88] and *TET2* mutations in cancer points to an etiologic role for these epigenetic regulators and their marks. Finally, human tissues harbor abundant 5HMC, a product of TET proteins acting on 5MC, while cancers with TET mutations tend to have reduced 5HMC.

The future of cancer epigenomic methods will be shaped by two technological trends. First, the rapid pace of advances in next-generation sequencing will continue to improve 5MC/5HMC, histone modification, and chromatin conformation mapping. Genome-wide epigenomic experiments will become increasingly inexpensive and accessible, though paralleled with needs for increased computational power and data storage. Second, direct single molecule sequencing that distinguishes between modified bases without bisulfite conversion could revolutionize mapping of 5MC and 5HMC. For example, in single molecule real-time (SMRT) sequencing, fluorescently labeled nucleotides are incorporated by DNA polymerase on complementary DNA strands. Real-time monitoring of the kinetics of this process can identify both unmodified and modified bases, including N6-methyladenine, 5MC, and 5HMC [94]. SMRT sequencing has also been combined with selective glucosylation and cleavable biotin labeling of 5HMC to improve detection kinetics [23]. Similarly, the direct detection of modified bases via inexpensively produced nanopores, if they become amenable to high-throughput, could be technologically transformative [137].

References

1. Trasler JM, Trasler DG, Bestor TH, Li E, Ghibu F (1996) DNA methyltransferase in normal and Dnmtn/Dnmtn mouse embryos. Dev Dyn 206(3):239–247. doi:10.1002/(SICI)1097-0177(199607)206:3<239::AID-AJA2>3.0.CO;2-J [pii] 10.1002/(SICI)1097-0177(199607)206:3<239::AID-AJA2>3.0.CO;2-J

2. Chen RZ, Pettersson U, Beard C, Jackson-Grusby L, Jaenisch R (1998) DNA hypomethylation leads to elevated mutation rates. Nature 395(6697):89–93
3. Maraschio P, Zuffardi O, Dalla Fior T, Tiepolo L (1988) Immunodeficiency, centromeric heterochromatin instability of chromosomes 1, 9, and 16, and facial anomalies: the ICF syndrome. J Med Genet 25(3):173–180
4. Xu GL, Bestor TH, Bourc'his D, Hsieh CL, Tommerup N, Bugge M, Hulten M, Qu X, Russo JJ, Viegas-Pequignot E (1999) Chromosome instability and immunodeficiency syndrome caused by mutations in a DNA methyltransferase gene. Nature 402(6758):187–191. doi:10.1038/46052
5. Clark SJ, Harrison J, Frommer M (1995) CpNpG methylation in mammalian cells. Nat Genet 10(1):20–27. doi:10.1038/ng0595-20
6. Ramsahoye BH, Biniszkiewicz D, Lyko F, Clark V, Bird AP, Jaenisch R (2000) Non-CpG methylation is prevalent in embryonic stem cells and may be mediated by DNA methyltransferase 3a. Proc Natl Acad Sci USA 97(10):5237–5242. doi:97/10/5237 [pii]
7. Lister R, Pelizzola M, Dowen RH, Hawkins RD, Hon G, Tonti-Filippini J, Nery JR, Lee L, Ye Z, Ngo QM, Edsall L, Antosiewicz-Bourget J, Stewart R, Ruotti V, Millar AH, Thomson JA, Ren B, Ecker JR (2009) Human DNA methylomes at base resolution show widespread epigenomic differences. Nature 462(7271):315–322. doi:nature08514 [pii] 10.1038/nature08514
8. Ziller MJ, Muller F, Liao J, Zhang Y, Gu H, Bock C, Boyle P, Epstein CB, Bernstein BE, Lengauer T, Gnirke A, Meissner A (2011) Genomic distribution and inter-sample variation of non-CpG methylation across human cell types. PLoS Genet 7(12):e1002389. doi:10.1371/journal.pgen.1002389 PGENETICS-D-11-00694 [pii]
9. Kriaucionis S, Heintz N (2009) The nuclear DNA base 5-hydroxymethylcytosine is present in Purkinje neurons and the brain. Science 324(5929):929–930. doi:1169786 [pii] 10.1126/science.1169786
10. Tahiliani M, Koh KP, Shen Y, Pastor WA, Bandukwala H, Brudno Y, Agarwal S, Iyer LM, Liu DR, Aravind L, Rao A (2009) Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. Science 324(5929):930–935. doi:1170116 [pii] 10.1126/science.1170116
11. Ito S, Shen L, Dai Q, Wu SC, Collins LB, Swenberg JA, He C, Zhang Y (2011) Tet proteins can convert 5-methylcytosine to 5-formylcytosine and 5-carboxylcytosine. Science 333(6047):1300–1303. doi:science.1210597 [pii] 10.1126/science.1210597
12. He YF, Li BZ, Li Z, Liu P, Wang Y, Tang Q, Ding J, Jia Y, Chen Z, Li L, Sun Y, Li X, Dai Q, Song CX, Zhang K, He C, Xu GL (2011) Tet-mediated formation of 5-carboxylcytosine and its excision by TDG in mammalian DNA. Science 333(6047):1303–1307. doi:science.1210944 [pii] 10.1126/science.1210944
13. Tan M, Luo H, Lee S, Jin F, Yang JS, Montellier E, Buchou T, Cheng Z, Rousseaux S, Rajagopal N, Lu Z, Ye Z, Zhu Q, Wysocka J, Ye Y, Khochbin S, Ren B, Zhao Y (2011) Identification of 67 histone marks and histone lysine crotonylation as a new type of histone modification. Cell 146(6):1016–1028. doi:S0092-8674(11)00891-9 [pii] 10.1016/j.cell.2011.08.008
14. Weber M, Davies JJ, Wittig D, Oakeley EJ, Haase M, Lam WL, Schubeler D (2005) Chromosome-wide and promoter-specific analyses identify sites of differential DNA methylation in normal and transformed human cells. Nat Genet 37(8):853–862. doi:ng1598 [pii] 10.1038/ng1598
15. Rauch T, Li H, Wu X, Pfeifer GP (2006) MIRA-assisted microarray analysis, a new technology for the determination of DNA methylation patterns, identifies frequent methylation of homeodomain-containing genes in lung cancer cells. Cancer Res 66(16):7939–7947. doi:66/16/7939 [pii] 10.1158/0008-5472.CAN-06-1888
16. Rauch T, Wang Z, Zhang X, Zhong X, Wu X, Lau SK, Kernstine KH, Riggs AD, Pfeifer GP (2007) Homeobox gene methylation in lung cancer studied by genome-wide analysis with a microarray-based methylated CpG island recovery assay. Proc Natl Acad Sci USA 104(13):5527–5532. doi:0701059104 [pii] 10.1073/pnas.0701059104

17. Rauch TA, Wu X, Zhong X, Riggs AD, Pfeifer GP (2009) A human B cell methylome at 100-base pair resolution. Proc Natl Acad Sci USA 106(3):671–678. doi:0812399106 [pii] 10.1073/pnas.0812399106
18. Frommer M, McDonald LE, Millar DS, Collis CM, Watt F, Grigg GW, Molloy PL, Paul CL (1992) A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. Proc Natl Acad Sci USA 89(5):1827–1831
19. Pfeifer GP, Riggs AD (1996) Genomic sequencing by ligation-mediated PCR. Mol Biotechnol 5(3):281–288
20. Clark SJ, Statham A, Stirzaker C, Molloy PL, Frommer M (2006) DNA methylation: bisulphite modification and analysis. Nat Protoc 1(5):2353–2364. doi:nprot.2006.324 [pii] 10.1038/nprot.2006.324
21. Gitan RS, Shi H, Chen CM, Yan PS, Huang TH (2002) Methylation-specific oligonucleotide microarray: a new potential for high-throughput methylation analysis. Genome Res 12(1):158–164. doi:10.1101/gr.202801
22. Pastor WA, Pape UJ, Huang Y, Henderson HR, Lister R, Ko M, McLoughlin EM, Brudno Y, Mahapatra S, Kapranov P, Tahiliani M, Daley GQ, Liu XS, Ecker JR, Milos PM, Agarwal S, Rao A (2011) Genome-wide mapping of 5-hydroxymethylcytosine in embryonic stem cells. Nature 473(7347):394–397. doi:nature10102 [pii] 10.1038/nature10102
23. Song CX, Szulwach KE, Fu Y, Dai Q, Yi C, Li X, Li Y, Chen CH, Zhang W, Jian X, Wang J, Zhang L, Looney TJ, Zhang B, Godley LA, Hicks LM, Lahn BT, Jin P, He C (2011) Selective chemical labeling reveals the genome-wide distribution of 5-hydroxymethylcytosine. Nat Biotechnol 29(1):68–72. doi:nbt.1732 [pii] 10.1038/nbt.1732
24. Khulan B, Thompson RF, Ye K, Fazzari MJ, Suzuki M, Stasiek E, Figueroa ME, Glass JL, Chen Q, Montagna C, Hatchwell E, Selzer RR, Richmond TA, Green RD, Melnick A, Greally JM (2006) Comparative isoschizomer profiling of cytosine methylation: the HELP assay. Genome Res 16(8):1046–1055. doi:gr.5273806 [pii] 10.1101/gr.5273806
25. Oda M, Glass JL, Thompson RF, Mo Y, Olivier EN, Figueroa ME, Selzer RR, Richmond TA, Zhang X, Dannenberg L, Green RD, Melnick A, Hatchwell E, Bouhassira EE, Verma A, Suzuki M, Greally JM (2009) High-resolution genome-wide cytosine methylation profiling with simultaneous copy number analysis and optimization for limited cell numbers. Nucleic Acids Res 37(12):3829–3839. doi:gkp260 [pii] 10.1093/nar/gkp260
26. Brunner AL, Johnson DS, Kim SW, Valouev A, Reddy TE, Neff NF, Anton E, Medina C, Nguyen L, Chiao E, Oyolu CB, Schroth GP, Absher DM, Baker JC, Myers RM (2009) Distinct DNA methylation patterns characterize differentiated human embryonic stem cells and developing human fetal liver. Genome Res 19(6):1044–1056. doi:gr.088773.108 [pii] 10.1101/gr.088773.108
27. Ball MP, Li JB, Gao Y, Lee JH, LeProust EM, Park IH, Xie B, Daley GQ, Church GM (2009) Targeted and genome-scale strategies reveal gene-body methylation signatures in human cells. Nat Biotechnol 27(4):361–368. doi:nbt.1533 [pii] 10.1038/nbt.1533
28. Hu M, Yao J, Cai L, Bachman KE, van den Brule F, Velculescu V, Polyak K (2005) Distinct epigenetic changes in the stromal cells of breast cancers. Nat Genet 37(8):899–905. doi:ng1596 [pii] 10.1038/ng1596
29. Bloushtain-Qimron N, Yao J, Snyder EL, Shipitsin M, Campbell LL, Mani SA, Hu M, Chen H, Ustyansky V, Antosiewicz JE, Argani P, Halushka MK, Thomson JA, Pharoah P, Porgador A, Sukumar S, Parsons R, Richardson AL, Stampfer MR, Gelman RS, Nikolskaya T, Nikolsky Y, Polyak K (2008) Cell type-specific DNA methylation patterns in the human breast. Proc Natl Acad Sci USA 105(37):14076–14081. doi:0805206105 [pii] 10.1073/pnas.0805206105
30. Maunakea AK, Nagarajan RP, Bilenky M, Ballinger TJ, D'Souza C, Fouse SD, Johnson BE, Hong C, Nielsen C, Zhao Y, Turecki G, Delaney A, Varhol R, Thiessen N, Shchors K, Heine VM, Rowitch DH, Xing X, Fiore C, Schillebeeckx M, Jones SJ, Haussler D, Marra MA, Hirst M, Wang T, Costello JF (2010) Conserved role of intragenic DNA methylation in regulating alternative promoters. Nature 466(7303):253–257. doi:nature09165 [pii] 10.1038/nature09165

31. Harris RA, Wang T, Coarfa C, Nagarajan RP, Hong C, Downey SL, Johnson BE, Fouse SD, Delaney A, Zhao Y, Olshen A, Ballinger T, Zhou X, Forsberg KJ, Gu J, Echipare L, O'Geen H, Lister R, Pelizzola M, Xi Y, Epstein CB, Bernstein BE, Hawkins RD, Ren B, Chung WY, Gu H, Bock C, Gnirke A, Zhang MQ, Haussler D, Ecker JR, Li W, Farnham PJ, Waterland RA, Meissner A, Marra MA, Hirst M, Milosavljevic A, Costello JF (2010) Comparison of sequencing-based methods to profile DNA methylation and identification of monoallelic epigenetic modifications. Nat Biotechnol 28(10):1097–1105. doi:nbt.1682 [pii] 10.1038/nbt.1682
32. Rabinowicz PD, Schutz K, Dedhia N, Yordan C, Parnell LD, Stein L, McCombie WR, Martienssen RA (1999) Differential methylation of genes and retrotransposons facilitates shotgun sequencing of the maize genome. Nat Genet 23(3):305–308. doi:10.1038/15479
33. Irizarry RA, Ladd-Acosta C, Carvalho B, Wu H, Brandenburg SA, Jeddeloh JA, Wen B, Feinberg AP (2008) Comprehensive high-throughput arrays for relative methylation (CHARM). Genome Res 18(5):780–790. doi:gr.7301508 [pii] 10.1101/gr.7301508
34. Irizarry RA, Ladd-Acosta C, Wen B, Wu Z, Montano C, Onyango P, Cui H, Gabo K, Rongione M, Webster M, Ji H, Potash JB, Sabunciyan S, Feinberg AP (2009) The human colon cancer methylome shows similar hypo- and hypermethylation at conserved tissue-specific CpG island shores. Nat Genet 41(2):178–186. doi:ng.298 [pii] 10.1038/ng.298
35. Jin SG, Kadam S, Pfeifer GP (2010) Examination of the specificity of DNA methylation profiling techniques towards 5-methylcytosine and 5-hydroxymethylcytosine. Nucleic Acids Res 38(11):e125. doi:gkq223 [pii] 10.1093/nar/gkq223
36. Ficz G, Branco MR, Seisenberger S, Santos F, Krueger F, Hore TA, Marques CJ, Andrews S, Reik W (2011) Dynamic regulation of 5-hydroxymethylcytosine in mouse ES cells and during differentiation. Nature 473(7347):398–402. doi:nature10008 [pii] 10.1038/nature10008
37. Xu Y, Wu F, Tan L, Kong L, Xiong L, Deng J, Barbera AJ, Zheng L, Zhang H, Huang S, Min J, Nicholson T, Chen T, Xu G, Shi Y, Zhang K, Shi YG (2011) Genome-wide regulation of 5hmC, 5mC, and gene expression by Tet1 hydroxylase in mouse embryonic stem cells. Mol Cell 42(4):451–464. doi:S1097-2765(11)00283-8 [pii] 10.1016/j.molcel.2011.04.005
38. Williams K, Christensen J, Pedersen MT, Johansen JV, Cloos PA, Rappsilber J, Helin K (2011) TET1 and hydroxymethylcytosine in transcription and DNA methylation fidelity. Nature 473(7347):343–348. doi:nature10066 [pii] 10.1038/nature10066
39. Stroud H, Feng S, Morey Kinney S, Pradhan S, Jacobsen SE (2011) 5-Hydroxymethylcytosine is associated with enhancers and gene bodies in human embryonic stem cells. Genome Biol 12(6):R54. doi:gb-2011-12-6-r54 [pii] 10.1186/gb-2011-12-6-r54
40. Keshet I, Schlesinger Y, Farkash S, Rand E, Hecht M, Segal E, Pikarski E, Young RA, Niveleau A, Cedar H, Simon I (2006) Evidence for an instructive mechanism of de novo methylation in cancer cells. Nat Genet 38(2):149–153. doi:ng1719 [pii] 10.1038/ng1719
41. Fouse SD, Shen Y, Pellegrini M, Cole S, Meissner A, Van Neste L, Jaenisch R, Fan G (2008) Promoter CpG methylation contributes to ES cell gene regulation in parallel with Oct4/Nanog, PcG complex, and histone H3K4/K27 trimethylation. Cell Stem Cell 2(2):160–169. doi:S1934-5909(07)00327-X [pii] 10.1016/j.stem.2007.12.011
42. Shen Y, Matsuno Y, Fouse SD, Rao N, Root S, Xu R, Pellegrini M, Riggs AD, Fan G (2008) X-inactivation in female human embryonic stem cells is in a nonrandom pattern and prone to epigenetic alterations. Proc Natl Acad Sci USA 105(12):4709–4714. doi:0712018105 [pii] 10.1073/pnas.0712018105
43. Zhang X, Yazaki J, Sundaresan A, Cokus S, Chan SW, Chen H, Henderson IR, Shinn P, Pellegrini M, Jacobsen SE, Ecker JR (2006) Genome-wide high-resolution mapping and functional analysis of DNA methylation in arabidopsis. Cell 126(6):1189–1201. doi:S0092-8674(06)01018-X [pii] 10.1016/j.cell.2006.08.003
44. Zilberman D, Gehring M, Tran RK, Ballinger T, Henikoff S (2007) Genome-wide analysis of Arabidopsis thaliana DNA methylation uncovers an interdependence between methylation and transcription. Nat Genet 39(1):61–69. doi:ng1929 [pii] 10.1038/ng1929
45. Robinson MD, Stirzaker C, Statham AL, Coolen MW, Song JZ, Nair SS, Strbenac D, Speed TP, Clark SJ (2010) Evaluation of affinity-based genome-wide DNA methylation data: effects

of CpG density, amplification bias, and copy number variation. Genome Res 20(12):1719–1729. doi:gr.110601.110 [pii] 10.1101/gr.110601.110
46. Robinson MD, Statham AL, Speed TP, Clark SJ (2010) Protocol matters: which methylome are you actually studying? Epigenomics 2(4):587–598. doi:10.2217/epi.10.36
47. Down TA, Rakyan VK, Turner DJ, Flicek P, Li H, Kulesha E, Graf S, Johnson N, Herrero J, Tomazou EM, Thorne NP, Backdahl L, Herberth M, Howe KL, Jackson DK, Miretti MM, Marioni JC, Birney E, Hubbard TJ, Durbin R, Tavare S, Beck S (2008) A Bayesian deconvolution strategy for immunoprecipitation-based DNA methylome analysis. Nat Biotechnol 26(7):779–785. doi:nbt1414 [pii] 10.1038/nbt1414
48. Pomraning KR, Smith KM, Freitag M (2009) Genome-wide high throughput analysis of DNA methylation in eukaryotes. Methods 47(3):142–150. doi:S1046-2023(08)00182-5 [pii] 10.1016/j.ymeth.2008.09.022
49. Ruike Y, Imanaka Y, Sato F, Shimizu K, Tsujimoto G (2010) Genome-wide analysis of aberrant methylation in human breast cancer cells using methyl-DNA immunoprecipitation combined with high-throughput sequencing. BMC Genomics 11:137. doi:1471-2164-11-137 [pii] 10.1186/1471-2164-11-137
50. Li N, Ye M, Li Y, Yan Z, Butcher LM, Sun J, Han X, Chen Q, Zhang X, Wang J (2010) Whole genome DNA methylation analysis based on high throughput sequencing technology. Methods 52(3):203–212. doi:S1046-2023(10)00127-1 [pii] 10.1016/j.ymeth.2010.04.009
51. Chavez L, Jozefczuk J, Grimm C, Dietrich J, Timmermann B, Lehrach H, Herwig R, Adjaye J (2010) Computational analysis of genome-wide DNA methylation during the differentiation of human embryonic stem cells along the endodermal lineage. Genome Res 20(10):1441–1450. doi:gr.110114.110 [pii] 10.1101/gr.110114.110
52. Bock C, Tomazou EM, Brinkman AB, Muller F, Simmer F, Gu H, Jager N, Gnirke A, Stunnenberg HG, Meissner A (2010) Quantitative comparison of genome-wide DNA methylation mapping technologies. Nat Biotechnol 28(10):1106–1114. doi:nbt.1681 [pii] 10.1038/nbt.1681
53. Feber A, Wilson GA, Zhang L, Presneau N, Idowu B, Down TA, Rakyan VK, Noon LA, Lloyd AC, Stupka E, Schiza V, Teschendorff AE, Schroth GP, Flanagan A, Beck S (2011) Comparative methylome analysis of benign and malignant peripheral nerve sheath tumors. Genome Res 21(4):515–524. doi:gr.109678.110 [pii] 10.1101/gr.109678.110
54. Pelizzola M, Koga Y, Urban AE, Krauthammer M, Weissman S, Halaban R, Molinaro AM (2008) MEDME: an experimental and analytical methodology for the estimation of DNA methylation levels based on microarray derived MeDIP-enrichment. Genome Res 18(10):1652–1659. doi:gr.080721.108 [pii] 10.1101/gr.080721.108
55. Brinkman AB, Simmer F, Ma K, Kaan A, Zhu J, Stunnenberg HG (2010) Whole-genome DNA methylation profiling using MethylCap-seq. Methods 52(3):232–236. doi:S1046-2023(10)00166-0 [pii] 10.1016/j.ymeth.2010.06.012
56. Serre D, Lee BH, Ting AH (2010) MBD-isolated genome sequencing provides a high-throughput and comprehensive survey of DNA methylation in the human genome. Nucleic Acids Res 38(2):391–399. doi:gkp992 [pii] 10.1093/nar/gkp992
57. Lan X, Adams C, Landers M, Dudas M, Krissinger D, Marnellos G, Bonneville R, Xu M, Wang J, Huang TH, Meredith G, Jin VX (2011) High resolution detection and analysis of CpG dinucleotides methylation using MBD-Seq technology. PLoS One 6(7):e22226. doi:10.1371/journal.pone.0022226 PONE-D-11-02256 [pii]
58. Zhou X, Maricque B, Xie M, Li D, Sundaram V, Martin EA, Koebbe BC, Nielsen C, Hirst M, Farnham P, Kuhn RM, Zhu J, Smirnov I, Kent WJ, Haussler D, Madden PA, Costello JF, Wang T (2011) The human epigenome browser at washington university. Nat Methods 8(12):989–990. doi:10.1038/nmeth.1772 nmeth.1772 [pii]
59. Karpf AR, Peterson PW, Rawlins JT, Dalley BK, Yang Q, Albertsen H, Jones DA (1999) Inhibition of DNA methyltransferase stimulates the expression of signal transducer and activator of transcription 1, 2, and 3 genes in colon tumor cells. Proc Natl Acad Sci USA 96(24):14007–14012
60. Yamashita K, Upadhyay S, Osada M, Hoque MO, Xiao Y, Mori M, Sato F, Meltzer SJ, Sidransky D (2002) Pharmacologic unmasking of epigenetically silenced tumor suppressor

genes in esophageal squamous cell carcinoma. Cancer Cell 2(6):485–495. doi:S1535610802002155 [pii]
61. Foltz G, Yoon JG, Lee H, Ryken TC, Sibenaller Z, Ehrich M, Hood L, Madan A (2009) DNA methyltransferase-mediated transcriptional silencing in malignant glioma: a combined whole-genome microarray and promoter array analysis. Oncogene. doi:onc2009122 [pii] 10.1038/onc.2009.122
62. Cameron EE, Bachman KE, Myohanen S, Herman JG, Baylin SB (1999) Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. Nat Genet 21(1):103–107
63. Shames DS, Girard L, Gao B, Sato M, Lewis CM, Shivapurkar N, Jiang A, Perou CM, Kim YH, Pollack JR, Fong KM, Lam CL, Wong M, Shyr Y, Nanda R, Olopade OI, Gerald W, Euhus DM, Shay JW, Gazdar AF, Minna JD (2006) A genome-wide screen for promoter methylation in lung cancer identifies novel methylation markers for multiple malignancies. PLoS Med 3(12):e486. doi:06-PLME-RA-0315R2 [pii] 10.1371/journal.pmed.0030486
64. Gius D, Cui H, Bradbury CM, Cook J, Smart DK, Zhao S, Young L, Brandenburg SA, Hu Y, Bisht KS, Ho AS, Mattson D, Sun L, Munson PJ, Chuang EY, Mitchell JB, Feinberg AP (2004) Distinct effects on gene expression of chemical and genetic manipulation of the cancer epigenome revealed by a multimodality approach. Cancer Cell 6(4):361–371
65. Mueller W, Nutt CL, Ehrich M, Riemenschneider MJ, von Deimling A, van den Boom D, Louis DN (2007) Downregulation of RUNX3 and TES by hypermethylation in glioblastoma. Oncogene 26(4):583–593. doi:1209805 [pii] 10.1038/sj.onc.1209805
66. Meissner A, Gnirke A, Bell GW, Ramsahoye B, Lander ES, Jaenisch R (2005) Reduced representation bisulfite sequencing for comparative high-resolution DNA methylation analysis. Nucleic Acids Res 33(18):5868–5877. doi:33/18/5868 [pii] 10.1093/nar/gki901
67. Meissner A, Mikkelsen TS, Gu H, Wernig M, Hanna J, Sivachenko A, Zhang X, Bernstein BE, Nusbaum C, Jaffe DB, Gnirke A, Jaenisch R, Lander ES (2008) Genome-scale DNA methylation maps of pluripotent and differentiated cells. Nature 454(7205):766–770. doi:nature07107 [pii] 10.1038/nature07107
68. Smith ZD, Gu H, Bock C, Gnirke A, Meissner A (2009) High-throughput bisulfite sequencing in mammalian genomes. Methods 48(3):226–232. doi:S1046-2023(09)00111-X [pii] 10.1016/j.ymeth.2009.05.003
69. Cokus SJ, Feng S, Zhang X, Chen Z, Merriman B, Haudenschild CD, Pradhan S, Nelson SF, Pellegrini M, Jacobsen SE (2008) Shotgun bisulphite sequencing of the Arabidopsis genome reveals DNA methylation patterning. Nature 452(7184):215–219
70. Lister R, O'Malley RC, Tonti-Filippini J, Gregory BD, Berry CC, Millar AH, Ecker JR (2008) Highly integrated single-base resolution maps of the epigenome in Arabidopsis. Cell 133(3):523–536. doi:S0092-8674(08)00448-0 [pii] 10.1016/j.cell.2008.03.029
71. Xi Y, Li W (2009) BSMAP: whole genome bisulfite sequence MAPping program. BMC Bioinformatics 10:232. doi:1471-2105-10-232 [pii] 10.1186/1471-2105-10-232
72. Gu H, Smith ZD, Bock C, Boyle P, Gnirke A, Meissner A (2011) Preparation of reduced representation bisulfite sequencing libraries for genome-scale DNA methylation profiling. Nat Protoc 6(4):468–481. doi:nprot.2010.190 [pii] 10.1038/nprot.2010.190
73. Xiang H, Zhu J, Chen Q, Dai F, Li X, Li M, Zhang H, Zhang G, Li D, Dong Y, Zhao L, Lin Y, Cheng D, Yu J, Sun J, Zhou X, Ma K, He Y, Zhao Y, Guo S, Ye M, Guo G, Li Y, Li R, Zhang X, Ma L, Kristiansen K, Guo Q, Jiang J, Beck S, Xia Q, Wang W, Wang J (2010) Single base-resolution methylome of the silkworm reveals a sparse epigenomic map. Nat Biotechnol 28(5):516–520. doi:nbt.1626 [pii] 10.1038/nbt.1626
74. Schroeder DI, Lott P, Korf I, LaSalle JM (2011) Large-scale methylation domains mark a functional subset of neuronally expressed genes. Genome Res 21(10):1583–1591. doi:gr.119131.110 [pii] 10.1101/gr.119131.110
75. Feng S, Cokus SJ, Zhang X, Chen PY, Bostick M, Goll MG, Hetzel J, Jain J, Strauss SH, Halpern ME, Ukomadu C, Sadler KC, Pradhan S, Pellegrini M, Jacobsen SE (2010) Conservation and divergence of methylation patterning in plants and animals. Proc Natl Acad Sci USA 107(19):8689–8694. doi:1002720107 [pii] 10.1073/pnas.1002720107

76. Zemach A, McDaniel IE, Silva P, Zilberman D (2010) Genome-wide evolutionary analysis of eukaryotic DNA methylation. Science 328(5980):916–919. doi:science.1186366 [pii] 10.1126/science.1186366
77. Laurent L, Wong E, Li G, Huynh T, Tsirigos A, Ong CT, Low HM, Kin Sung KW, Rigoutsos I, Loring J, Wei CL (2010) Dynamic changes in the human methylome during differentiation. Genome Res 20(3):320–331. doi:gr.101907.109 [pii] 10.1101/gr.101907.109
78. Lister R, Pelizzola M, Kida YS, Hawkins RD, Nery JR, Hon G, Antosiewicz-Bourget J, O'Malley R, Castanon R, Klugman S, Downes M, Yu R, Stewart R, Ren B, Thomson JA, Evans RM, Ecker JR (2011) Hotspots of aberrant epigenomic reprogramming in human induced pluripotent stem cells. Nature 471(7336):68–73. doi:nature09798 [pii] 10.1038/nature09798
79. Hon GC, Hawkins RD, Caballero OL, Lo C, Lister R, Pelizzola M, Valsesia A, Ye Z, Kuan S, Edsall LE, Camargo AA, Stevenson BJ, Ecker JR, Bafna V, Strausberg RL, Simpson AJ, Ren B (2011) Global DNA hypomethylation coupled to repressive chromatin domain formation and gene silencing in breast cancer. Genome Res. doi:gr.125872.111 [pii] 10.1101/gr.125872.111
80. Berman BP, Weisenberger DJ, Aman JF, Hinoue T, Ramjan Z, Liu Y, Noushmehr H, Lange CP, van Dijk CM, Tollenaar RA, Van Den Berg D, Laird PW (2011) Regions of focal DNA hypermethylation and long-range hypomethylation in colorectal cancer coincide with nuclear lamina-associated domains. Nat Genet 44(1):40–46. doi:10.1038/ng.969 ng.969 [pii]
81. Krueger F, Andrews SR (2011) Bismark: a flexible aligner and methylation caller for Bisulfite-Seq applications. Bioinformatics 27(11):1571–1572. doi:btr167 [pii] 10.1093/bioinformatics/btr167
82. Xi Y, Bock C, Muller F, Sun D, Meissner A, Li W (2011) RRBSMAP: a fast, accurate and user-friendly alignment tool for reduced representation bisulfite sequencing. Bioinformatics. doi:btr668 [pii] 10.1093/bioinformatics/btr668
83. Ehrich M, Nelson MR, Stanssens P, Zabeau M, Liloglou T, Xinarianos G, Cantor CR, Field JK, van den Boom D (2005) Quantitative high-throughput analysis of DNA methylation patterns by base-specific cleavage and mass spectrometry. Proc Natl Acad Sci USA 102(44):15785–15790. doi:0507816102 [pii] 10.1073/pnas.0507816102
84. Raval A, Tanner SM, Byrd JC, Angerman EB, Perko JD, Chen SS, Hackanson B, Grever MR, Lucas DM, Matkovic JJ, Lin TS, Kipps TJ, Murray F, Weisenburger D, Sanger W, Lynch J, Watson P, Jansen M, Yoshinaga Y, Rosenquist R, de Jong PJ, Coggill P, Beck S, Lynch H, de la Chapelle A, Plass C (2007) Downregulation of death-associated protein kinase 1 (DAPK1) in chronic lymphocytic leukemia. Cell 129(5):879–890. doi:S0092-8674(07)00512-0 [pii] 10.1016/j.cell.2007.03.043
85. Deng J, Shoemaker R, Xie B, Gore A, LeProust EM, Antosiewicz-Bourget J, Egli D, Maherali N, Park IH, Yu J, Daley GQ, Eggan K, Hochedlinger K, Thomson J, Wang W, Gao Y, Zhang K (2009) Targeted bisulfite sequencing reveals changes in DNA methylation associated with nuclear reprogramming. Nat Biotechnol 27(4):353–360. doi:nbt.1530 [pii] 10.1038/nbt.1530
86. Ito S, D'Alessio AC, Taranova OV, Hong K, Sowers LC, Zhang Y (2010) Role of Tet proteins in 5mC to 5hmC conversion, ES-cell self-renewal and inner cell mass specification. Nature 466(7310):1129–1133. doi:nature09303 [pii] 10.1038/nature09303
87. Ono R, Taki T, Taketani T, Taniwaki M, Kobayashi H, Hayashi Y (2002) LCX, leukemia-associated protein with a CXXC domain, is fused to MLL in acute myeloid leukemia with trilineage dysplasia having t(10;11)(q22;q23). Cancer Res 62(14):4075–4080
88. Lorsbach RB, Moore J, Mathew S, Raimondi SC, Mukatira ST, Downing JR (2003) TET1, a member of a novel protein family, is fused to MLL in acute myeloid leukemia containing the t(10;11)(q22;q23). Leukemia 17(3):637–641. doi:10.1038/sj.leu.2402834 [pii]
89. Ko M, Huang Y, Jankowska AM, Pape UJ, Tahiliani M, Bandukwala HS, An J, Lamperti ED, Koh KP, Ganetzky R, Liu XS, Aravind L, Agarwal S, Maciejewski JP, Rao A (2010) Impaired hydroxylation of 5-methylcytosine in myeloid cancers with mutant TET2. Nature 468(7325):839–843. doi:nature09586 [pii] 10.1038/nature09586

90. Huang Y, Pastor WA, Shen Y, Tahiliani M, Liu DR, Rao A (2010) The behaviour of 5-hydroxymethylcytosine in bisulfite sequencing. PLoS One 5(1):e8888. doi:10.1371/journal.pone.0008888
91. Globisch D, Munzel M, Muller M, Michalakis S, Wagner M, Koch S, Bruckl T, Biel M, Carell T (2010) Tissue distribution of 5-hydroxymethylcytosine and search for active demethylation intermediates. PLoS One 5(12):e15367. doi:10.1371/journal.pone.0015367
92. Szwagierczak A, Bultmann S, Schmidt CS, Spada F, Leonhardt H (2010) Sensitive enzymatic quantification of 5-hydroxymethylcytosine in genomic DNA. Nucleic Acids Res 38(19):e181. doi:gkq684 [pii] 10.1093/nar/gkq684
93. Kinney SM, Chin HG, Vaisvila R, Bitinaite J, Zheng Y, Esteve PO, Feng S, Stroud H, Jacobsen SE, Pradhan S (2011) Tissue-specific distribution and dynamic changes of 5-hydroxymethylcytosine in mammalian genomes. J Biol Chem 286(28):24685–24693. doi:M110.217083 [pii] 10.1074/jbc.M110.217083
94. Flusberg BA, Webster DR, Lee JH, Travers KJ, Olivares EC, Clark TA, Korlach J, Turner SW (2010) Direct detection of DNA methylation during single-molecule, real-time sequencing. Nat Methods 7(6):461–465. doi:nmeth.1459 [pii] 10.1038/nmeth.1459
95. Ke X-S, Qu Y, Rostad K, Li W-C, Lin B, Halvorsen OJ, Haukaas SA, Jonassen I, Petersen K, Goldfinger N, Rotter V, Akslen LA, Oyan AM, Kalland K-H (2009) Genome-wide profiling of histone h3 lysine 4 and lysine 27 trimethylation reveals an epigenetic signature in prostate carcinogenesis. PLoS One 4:e4687
96. Fraga MF, Ballestar E, Villar-Garea A, Boix-Chornet M, Espada J, Schotta G, Bonaldi T, Haydon C, Ropero S, Petrie K, Iyer NG, Pérez-Rosado A, Calvo E, Lopez JA, Cano A, Calasanz MJ, Colomer D, Piris MA, Ahn N, Imhof A, Caldas C, Jenuwein T, Esteller M (2005) Loss of acetylation at Lys16 and trimethylation at Lys20 of histone H4 is a common hallmark of human cancer. Nat Genet 37:391–400
97. Varambally S, Dhanasekaran SM, Zhou M, Barrette TR, Kumar-Sinha C, Sanda MG, Ghosh D, Pienta KJ, Sewalt RGAB, Otte AP, Rubin MA, Chinnaiyan AM (2002) The polycomb group protein EZH2 is involved in progression of prostate cancer. Nature 419:624–629
98. Sharma SV, Lee DY, Li B, Quinlan MP, Takahashi F, Maheswaran S, McDermott U, Azizian N, Zou L, Fischbach MA (2010) A chromatin-mediated reversible drug-tolerant state in cancer cell subpopulations. Cell 141:69–80
99. Park PJ (2009) ChIP-seq: advantages and challenges of a maturing technology. Nat Rev Genet 10:669–680
100. O'Neill LP, Turner BM, Turner B (2003) ChIP with native chromatin: advantages and problems relative to methods using cross-linked material. Methods 31(1):76–82
101. Orlando V (2000) Mapping chromosomal proteins in vivo by formaldehyde-crosslinked-chromatin immunoprecipitation. Trends Biochem Sci 25:99–104
102. Barski A, Zhao K (2009) Genomic location analysis by ChIP-Seq. J Cell Biochem 107(107):11–18
103. Dingwall C, Lomonossoff GP, Laskey RA (1981) High sequence specificity of micrococcal nuclease. Nucleic Acids Res 9:2659–2673
104. Hörz W, Altenburger W (1981) Sequence specific cleavage of DNA by micrococcal nuclease. Nucleic Acids Res 9:2643–2658
105. Li H, Ruan J, Durbin R (2008) Mapping short DNA sequencing reads and calling variants using mapping quality scores. Genome Res 18:1851–1858
106. Langmead B, Trapnell C, Pop M, Salzberg SL (2009) Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol 10:R25
107. Li H, Durbin R (2010) Fast and accurate long-read alignment with Burrows-Wheeler transform. Bioinformatics 26:589–595
108. Li R, Yu C, Li Y, Lam T-W, Yiu S-M, Kristiansen K, Wang J (2009) SOAP2: an improved ultrafast tool for short read alignment. Bioinformatics 25:1966–1967
109. Li R, Li Y, Kristiansen K, Wang J (2008) SOAP: short oligonucleotide alignment program. Bioinformatics 24:713–714

110. Coarfa C, Yu F, Miller CA, Chen Z, Harris RA, Milosavljevic A (2010) Pash 3.0: a versatile software package for read mapping and integrative analysis of genomic and epigenomic variation using massively parallel DNA sequencing. BMC Bioinformatics 11:572. doi:1471-2105-11-572 [pii] 10.1186/1471-2105-11-572
111. Pepke S, Wold B, Mortazavi A (2009) Computation for ChIP-seq and RNA-seq studies. Nat Methods 6:S22–S32
112. Xu H, Handoko L, Wei X, Ye C, Sheng J, Wei C-L, Lin F, Sung W-K (2010) A signal-noise model for significance analysis of ChIP-seq with negative control. Bioinformatics 26:1199–1204
113. Xu H, Wei C-L, Lin F, Sung W-K (2008) An HMM approach to genome-wide identification of differential histone modification sites from ChIP-seq data. Bioinformatics 24:2344–2349
114. Zang C, Schones DE, Zeng C, Cui K, Zhao K, Peng W (2009) A clustering approach for identification of enriched domains from histone modification ChIP-Seq data. Bioinformatics 25:1952–1958
115. Barski A, Cuddapah S, Cui K, Roh T-Y, Schones DE, Wang Z, Wei G, Chepelev I, Zhao K (2007) High-resolution profiling of histone methylations in the human genome. Cell 129:823–837
116. Johnson DS, Mortazavi A, Myers RM, Wold B (2007) Genome-wide mapping of in vivo protein-DNA interactions. Science 316:1497–1502
117. Mikkelsen TS, Ku M, Jaffe DB, Issac B, Lieberman E, Giannoukos G, Alvarez P, Brockman W, Kim T-K, Koche RP, Lee W, Mendenhall E, O'Donovan A, Presser A, Russ C, Xie X, Meissner A, Wernig M, Jaenisch R, Nusbaum C, Lander ES, Bernstein BE (2007) Genome-wide maps of chromatin state in pluripotent and lineage-committed cells. Nature 448:553–560
118. Heintzman ND, Stuart RK, Hon G, Fu Y, Ching CW, Hawkins RD, Barrera LO, Van Calcar S, Qu C, Ching KA, Wang W, Weng Z, Green RD, Crawford GE, Ren B (2007) Distinct and predictive chromatin signatures of transcriptional promoters and enhancers in the human genome. Nat Genet 39:311–318
119. Heintzman ND, Hon GC, Hawkins RD, Kheradpour P, Stark A, Harp LF, Ye Z, Lee LK, Stuart RK, Ching CW, Ching KA, Antosiewicz-Bourget JE, Liu H, Zhang X, Green RD, Lobanenkov VV, Stewart R, Thomson JA, Crawford GE, Kellis M, Ren B (2009) Histone modifications at human enhancers reflect global cell-type-specific gene expression. Nature 459:108–112
120. Ernst J, Kheradpour P, Mikkelsen TS, Shoresh N, Ward LD, Epstein CB, Zhang X, Wang L, Issner R, Coyne M, Ku M, Durham T, Kellis M, Bernstein BE (2011) Mapping and analysis of chromatin state dynamics in nine human cell types. Nature 473:43–49
121. Wasserman NF, Aneas I, Nobrega MA (2010) An 8q24 gene desert variant associated with prostate cancer risk confers differential in vivo activity to a MYC enhancer. Genome Res 20:1191–1197
122. Adli M, Bernstein BE (2011) Whole-genome chromatin profiling from limited numbers of cells using nano-ChIP-seq. Nat Protoc 6:1656–1668
123. Goren A, Ozsolak F, Shoresh N, Ku M, Adli M, Hart C, Gymrek M, Zuk O, Regev A, Milos PM, Bernstein BE (2010) Chromatin profiling by directly sequencing small quantities of immunoprecipitated DNA. Nat Methods 7:47–49
124. Dekker J, Rippe K, Dekker M, Kleckner N (2002) Capturing chromosome conformation. Science 295:1306–1311
125. Zhao Z, Tavoosidana G, Sjölinder M, Göndör A, Mariano P, Wang S, Kanduri C, Lezcano M, Sandhu KS, Singh U, Pant V, Tiwari V, Kurukuti S, Ohlsson R (2006) Circular chromosome conformation capture (4C) uncovers extensive networks of epigenetically regulated intra- and interchromosomal interactions. Nat Genet 38:1341–1347
126. Dostie J, Richmond TA, Arnaout RA, Selzer RR, Lee WL, Honan TA, Rubio ED, Krumm A, Lamb J, Nusbaum C, Green RD, Dekker J (2006) Chromosome Conformation Capture Carbon Copy (5C): a massively parallel solution for mapping interactions between genomic elements. Genome Res 16:1299–1309

127. Lieberman-Aiden E, van Berkum NL, Williams L, Imakaev M, Ragoczy T, Telling A, Amit I, Lajoie BR, Sabo PJ, Dorschner MO, Sandstrom R, Bernstein B, Bender MA, Groudine M, Gnirke A, Stamatoyannopoulos J, Mirny LA, Lander ES, Dekker J (2009) Comprehensive mapping of long-range interactions reveals folding principles of the human genome. Science 326:289–293
128. Cai S, Lee CC, Kohwi-Shigematsu T (2006) SATB1 packages densely looped, transcriptionally active chromatin for coordinated expression of cytokine genes. Nat Genet 38:1278–1288
129. Simonis M, Kooren J, de Laat W (2007) An evaluation of 3C-based methods to capture DNA interactions. Nat Methods 4:895–901
130. Fullwood MJ, Wei C-L, Liu ET, Ruan Y (2009) Next-generation DNA sequencing of paired-end tags (PET) for transcriptome and genome analyses. Genome Res 19:521–532
131. Simonis M, Klous P, Homminga I, Galjaard R-J, Rijkers E-J, Grosveld F, Meijerink JPP, de Laat W (2009) High-resolution identification of balanced and complex chromosomal rearrangements by 4C technology. Nat Methods 6:837–842
132. Ohm JE, McGarvey KM, Yu X, Cheng L, Schuebel KE, Cope L, Mohammad HP, Chen W, Daniel VC, Yu W, Berman DM, Jenuwein T, Pruitt K, Sharkis SJ, Watkins DN, Herman JG, Baylin SB (2007) A stem cell-like chromatin pattern may predispose tumor suppressor genes to DNA hypermethylation and heritable silencing. Nat Genet 39(2):237–242. doi:ng1972 [pii] 10.1038/ng1972
133. Widschwendter M, Fiegl H, Egle D, Mueller-Holzner E, Spizzo G, Marth C, Weisenberger DJ, Campan M, Young J, Jacobs I, Laird PW (2007) Epigenetic stem cell signature in cancer. Nat Genet 39(2):157–158. doi:ng1941 [pii] 10.1038/ng1941
134. Schlesinger Y, Straussman R, Keshet I, Farkash S, Hecht M, Zimmerman J, Eden E, Yakhini Z, Ben-Shushan E, Reubinoff BE, Bergman Y, Simon I, Cedar H (2007) Polycomb-mediated methylation on Lys27 of histone H3 pre-marks genes for de novo methylation in cancer. Nat Genet 39(2):232–236. doi:ng1950 [pii] 10.1038/ng1950
135. Ley TJ, Ding L, Walter MJ, McLellan MD, Lamprecht T, Larson DE, Kandoth C, Payton JE, Baty J, Welch J, Harris CC, Lichti CF, Townsend RR, Fulton RS, Dooling DJ, Koboldt DC, Schmidt H, Zhang Q, Osborne JR, Lin L, O'Laughlin M, McMichael JF, Delehaunty KD, McGrath SD, Fulton LA, Magrini VJ, Vickery TL, Hundal J, Cook LL, Conyers JJ, Swift GW, Reed JP, Alldredge PA, Wylie T, Walker J, Kalicki J, Watson MA, Heath S, Shannon WD, Varghese N, Nagarajan R, Westervelt P, Tomasson MH, Link DC, Graubert TA, DiPersio JF, Mardis ER, Wilson RK (2010) DNMT3A mutations in acute myeloid leukemia. N Engl J Med 363(25):2424–2433. doi:10.1056/NEJMoa1005143
136. Yamashita Y, Yuan J, Suetake I, Suzuki H, Ishikawa Y, Choi YL, Ueno T, Soda M, Hamada T, Haruta H, Takada S, Miyazaki Y, Kiyoi H, Ito E, Naoe T, Tomonaga M, Toyota M, Tajima S, Iwama A, Mano H (2010) Array-based genomic resequencing of human leukemia. Oncogene 29(25):3723–3731. doi:onc2010117 [pii] 10.1038/onc.2010.117
137. Clarke J, Wu HC, Jayasinghe L, Patel A, Reid S, Bayley H (2009) Continuous base identification for single-molecule nanopore DNA sequencing. Nat Nanotechnol 4(4):265–270. doi:nnano.2009.12 [pii] 10.1038/nnano.2009.12

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Epigenetic Alterations in Oncogenesis

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Preface

Epigenetics refers to heritable changes in gene expression or genome function encoded by marks other than DNA base sequence; information literally "above" the level of genetics. Epigenetic marks include cytosine methylation and cytosine hydroxymethylation, histone tail modifications, histone variants, and nucleosome positional information, all of which are resident along the DNA duplex. Epigenetic marks frequently show interdependent relationships, for example, the close association of DNA methylation states with particular histone tail modifications and histone variants. From the standpoint of cell physiology, epigenetics provides a mechanism for cells to integrate environmental or intrinsic stimuli into heritable changes in genome function. From the standpoint of development, epigenetics provides a platform for cell differentiation and cell specialization, which in principle cannot simply be the consequence of DNA sequence. Most relevant to this book is the fact that changes in epigenetic states are now recognized to play a fundamental role in cancer development and progression. Cancer, almost uniquely among common human diseases, is characterized by natural selection for cellular variants with improved fitness, e.g., proliferative capacity and rate, evasion of cell death, invasive growth, migration to and proliferation at secondary sites, chemotherapy resistance, and a myriad of other naturally or artificially selected phenotypes. Epigenetic changes play a key role in this phenotypic selection, possibly to an equal to or even greater extent than do genetic mutations.

As a field, cancer epigenetics has now reached young adulthood. The observations that started the field were of DNA hypomethylation changes in cancer in the 1980s, followed by the discovery of DNA hypermethylation in cancer in the 1990s. In the last decade, additional alterations at other levels of epigenetic control (e.g., histone modifications) have also been discovered and characterized in cancer. Also, over the past few years rapid progress has been made in translating the findings of epigenetic alterations into new cancer biomarkers and therapeutic targets. One clear highlight in the field has been the FDA-approval of DNA methyltransferase (DNMT) inhibitors and histone deacetylase (HDAC) inhibitors to treat a select number of human malignancies.

The early work in cancer epigenetics was largely hypothesis or "candidate-gene" driven. More recent work using unbiased and global approaches (i.e., epigenomics)

have validated and greatly extended the early observations. Evidence now suggests that DNA hypomethylation is linked to oncogenic gene activation and genomic instability, and that DNA hypermethylation leads to tumor suppressor gene inactivation, including inactivation of DNA repair genes that also may promote genomic instability. Thus, epigenetic mutations (epimutations) appear to promote genetic mutations and genomic rearrangements in cancer. Intriguingly, a number of recent findings largely from cancer genome sequencing data suggest that genes involved in epigenetic control processes are commonly mutated in a variety of cancers, thus demonstrating that genetic changes can also promote epigenetic alterations in cancer. Taken together, the data now indicate that the roles of genetics and epigenetics in cancer development are highly intertwined.

Epigenetic Alterations in Oncogenesis comprises 15 chapters contributed by leading active researchers in the field. The book is divided into three sections that run the gamut from a description of the basic epigenetic mechanisms that regulate gene expression in human cancer, to how alterations in epigenetic marks contribute to cancer biology, and concluding with an account of the uses for epigenetic-targeted drugs to treat human cancer, as well as the analysis methods to decipher cancer epigenomes.

Part I, *Epigenetic Marks and Mechanisms*, provides an introduction to the major epigenetic marks and how these are altered during oncogenesis. The part begins with a discussion by Jin and Robertson in Chap. 1 on cytosine DNMTs and DNA hypermethylation in cancer, and focuses particularly on the silencing of genes involved in DNA repair, which are a frequent target of hypermethylation. In addition, the authors summarize important recent work showing that DNMTs themselves participate in DNA repair processes. In Chap. 2, Ehrlich and Lacey turn attention to the flip side of the coin, DNA hypomethylation, which was the original epigenetic alteration observed in cancer. The authors discuss the diverse genomic contexts in which DNA hypomethylation can occur and present possible mechanisms to explain DNA hypomethylation in cancer. An exciting recent development in epigenetics is the discovery of 5-hydroxymethylcytosine (5-hmC) as a novel epigenetic mark, which itself appears to be linked to DNA hypomethylation. The biological significance of 5-hmC as well as the enzymes that catalyze its formation (ten–eleven translocation or TET proteins, which can be mutated in cancer) is discussed by Kinney and Pradhan in Chap. 3. In Chap. 4, attention turns to altered histone modifications in cancer with a detailed discussion by Campbell and Turner on how posttranslational histone modifications are controlled under normal circumstances and the mechanisms driving their alteration in malignancy. A critical concept in epigenetics is that DNA methylation and histone modifications ultimately impact gene expression and genome function via their effects on nucleosomes; the important topic of altered nucleosome occupancy in cancer is covered by Andreu-Vieyra and Liang in Chap. 5.

Part III, *The Impact of Epigenetic Alterations on Cancer Biology*, discusses how epigenetic changes contribute to critically important cancer phenotypes. The section begins in Chap. 6, where Fabbri and colleagues discuss miRNA expression alterations in cancer caused by epigenetic changes, including DNA methylation, histone modifications, and Polycomb proteins. The importance of this concept is illustrated by the inherent capacity of altered miRNA expression to derange entire

transcriptional programs in cancer cells. A large family of genes known as cancer-testis or cancer-germ line genes encodes antigens that are a major target of cancer vaccines. Additionally, a number of these genes have emerging oncogenic functions. In Chap. 7, De Smet and Loriot discuss how epigenetic mechanisms, most prominently DNA hypomethylation, lead to the activation of these genes in many human malignancies. Andersen and Jones follow this with a discussion in Chap. 8 of how DNA methylation controls cell fate in the intestine and how, when the tumor suppressor gene adenomatous polyposis coli (APC) is lost, this promotes DNA hypomethylation and intestinal tumorigenesis. In Chap. 9, Futscher describes how tractable cell model systems are being used to discern the temporal epigenetic alterations that are linked to cell immortalization and transformation. It is now recognized that epigenetic regulation lies at the heart of stem cell maintenance and differentiation. In Chap. 10, Huang and colleagues discuss epigenetic regulation of mesenchymal stem cells (MSC) during tumorigenesis, and highlight recent work showing that targeted DNA methylation of tumor suppressor genes provides a model system to study MSC-driven tumorigenesis.

Part III, *Clinical Implications and Analysis Methods*, provides an overview of important topics related to the utility of epigenetic alterations as cancer biomarkers and therapeutic targets, and provides a detailed overview of the methods used to decipher cancer epigenomes. In the past few years, a major link between environmental toxicants, epigenetic changes, and cancer has become apparent. In Chap. 11, Pogribny and Rusyn discuss these developments as they pertain to chemical carcinogens such as arsenic, as well as other pharmaceutical and biological agents. While epigenetic alterations in cancer cells and tumor tissues is well established, emerging data suggest that systemic epigenetic changes (i.e., those affecting normal tissues) can also occur in cancer patients, as well as in individuals with elevated risk for cancer. Marsit and Christensen highlight the current research in this exciting and potentially high impact area in Chap. 12. Epigenetic therapies have entered the clinic and received their first widespread use in the context of myeloid malignancies, particularly myelodysplastic syndrome (MDS) and acute myelogenous leukemia (AML). In Chap. 13, Griffiths and Gore discuss the clinical work in this arena, with a focus on the FDA-approved azanucleosides 5-azacytidine (vidaza) and decitabine (dacogen), but also touching on HDAC inhibitors. In Chap. 14, Balch and Nephew discuss how epigenetic therapies may be particularly well suited for chemotherapy sensitization to overcome drug resistance, and review the extensive preclinical work and rapidly accumulating clinical knowledge in this area. Finally, in Chap. 15, Costello and colleagues review the approaches used for the analysis of cancer epigenomes. In particular, they discuss the methods appropriate for the analysis of cytosine methylation and hydroxymethylation, discuss next-generation sequencing approaches, and touch on the computational methods now being used to explore cancer epigenomes.

Omaha, Nebraska, USA Adam R. Karpf

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Part I
Epigenetic Marks and Mechanisms

Chapter 1
DNA Methyltransferases, DNA Damage Repair, and Cancer

Bilian Jin and Keith D. Robertson

Abstract The maintenance DNA methyltransferase (DNMT) 1 and the de novo methyltransferases DNMT3A and DNMT3B are all essential for mammalian development. DNA methylation, catalyzed by the DNMTs, plays an important role in maintaining genome stability. Aberrant expression of DNMTs and disruption of DNA methylation patterns are closely associated with many forms of cancer, although the exact mechanisms underlying this link remain elusive. DNA damage repair systems have evolved to act as a genome-wide surveillance mechanism to maintain chromosome integrity by recognizing and repairing both exogenous and endogenous DNA insults. Impairment of these systems gives rise to mutations and directly contributes to tumorigenesis. Evidence is mounting for a direct link between DNMTs, DNA methylation, and DNA damage repair systems, which provide new insight into the development of cancer. Like tumor suppressor genes, an array of DNA repair genes frequently sustain promoter hypermethylation in a variety of tumors. In addition, DNMT1, but not the DNMT3s, appear to function coordinately with DNA damage repair pathways to protect cells from sustaining mutagenic events, which is very likely through a DNA methylation-independent mechanism. This chapter is focused on reviewing the links between DNA methylation and the DNA damage response.

1.1 Introduction

DNA methyltransferases (DNMTs), responsible for the transfer of a methyl group from the universal methyl donor, *S*-adenosyl-L-methionine (SAM), to the 5-position of cytosine residues in DNA, are essential for mammalian development [1].

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There are four members of the DNMT family, including DNMT1, DNMT3A, DNMT3B, and DNMT3L. DNMT3L, unlike the other DNMTs, does not possess any inherent enzymatic activity [2]. The other three family members are active on DNA. *DNMT1* encodes the maintenance methyltransferase and *DNMT3A/DNMT3B* encode the de novo methyltransferases [3, 4], required to establish and maintain genomic methylation. While this maintenance vs. de novo division has been convenient, there is clear evidence for functional overlap between the maintenance and the de novo methyltransferases [5, 6]. Gene knockout analysis in mice has shown that *Dnmt1* and *Dnmt3a/Dnmt3b* genes are all essential for viability. *Dnmt1* inactivation leads to very early lethality at embryonic day (E) 9.5, shortly after gastrulation [7–9], whereas *Dnmt3b* knockout induces embryo death at E14.5–18.5, due to multiple developmental defects including growth impairment and rostral neural tube defects [3, 8, 9]. *Dnmt3a*$^{-/-}$ mice become runted and die at about 4 weeks of age, although they appear to be relatively normal at birth [3].

DNMTs play an important role in genomic integrity, disruption of which may result in chromosome instability and tumor progression. It is well established that DNMTs are required for transcriptional silencing of a number of sequence classes, including imprinted genes, genes on the inactive X chromosome and transposable elements [1, 10], and silencing of these sequences is essential for maintaining chromosome stability. Much compelling evidence has come from targeted deletion experiments showing that all three DNMTs are involved in stabilization of the genome, particularly repetitive sequences [3, 11, 12]. For example, either single knockout of *Dnmt1* or double knockout of *Dnmt3a* and *Dnmt3b* enhances telomere recombination [11]. DNMT3B is specifically required for stabilization of pericentromeric satellite repeats. DNMT3B deficiency results in expansion and rearrangements of pericentromeric repeats [3, 12]. Immunodeficiency, centromere instability, and facial anomalies (ICF) syndrome is the only human genetic disorder known to involve biallelic mutations in *DNMT3B*. It is characterized by chromosomal instability arising due to destabilization of pericentromeric repeats, particularly those at juxtacentromeric regions of chromosomes 1, 9, and 16 [3, 12]. Of note, cells null for *DNMT1* or with hypomorphic mutations in *DNMT1* that partially reduce its levels to 30% of WT DNMT1 display significantly greater microsatellite instability (MSI) [13–17], a greater frequency of chromosomal translocations [18] and much higher sensitivity to genotoxic agents [17], which may promote the development of cancer.

The DNA damage response (DDR) is a genome-wide surveillance system that protects cells from potentially mutagenic DNA insults derived from either endogenous or exogenous sources. The DDR usually functions through the coordinated actions of DNA repair and checkpoint systems to promote DNA damage repair before replication or to activate cell death pathways if excessive damage exists [19]. Like the cellular DNA methylation machinery, an intact DDR is crucial for preventing cancer. Evidence is mounting to support a link between the DNA methylation and DNA damage repair systems, as first suggested by promoter hypermethylation and silencing of DNA repair genes in multiple types of cancer [20]. More importantly, DNMT1 may be directly involved in DNA damage repair in a DNA methylation-independent manner [14, 17, 21–23]. Strong support for this latter notion

comes from recent observations that DNMT1 is rapidly and transiently recruited to regions of DNA double-strand breaks (DSBs) via its interaction with proliferating cell nuclear antigen (PCNA) [21, 24], as well the PCNA-like DNA damage sliding clamp component RAD9 (of the 9-1-1 complex) [21]. In this chapter, we examine and outline the links between DNMTs and DNA repair systems and discuss the possible mechanisms of how they are orchestrated, with a focus on cancer.

1.2 Epigenetic Silencing of DNA Repair Pathways Through Aberrant Promoter Hypermethylation

DNA repair systems have evolved to maintain genomic integrity by countering threats posed by DNA lesions [19]. Deficiency in the DNA repair pathways may leave these lesions unrepaired or cause them to be repaired incorrectly, eventually leading to genome instability or mutations that contribute directly to a large array of human diseases including cancer. Carcinogenesis is believed to originate from and be driven by the acquisition of abnormal genetic and/or epigenetic changes. Aberrant DNA hypermethylation, when it occurs at promoter CpG islands (CGIs), leads to potent and heritable transcriptional silencing that inactivates key cellular pathways much like genetic changes (e.g., mutation/deletion) do. In addition to genetic mutations, promoter hypermethylation in DNA repair genes is closely linked to a variety of human tumor types including colorectal, breast, lung cancers, and glioma [20] (Table 1.1), suggesting that epigenetic silencing of DNA repair pathways is an important contributor to the development of cancer.

1.2.1 Epigenetic Inactivation of the DNA Mismatch Repair Pathway

Mismatch repair (MMR) is a genome surveillance system to maintain genomic integrity through recognizing and correcting mismatched nucleotides arising during DNA replication, homologous recombination (HR), or other forms of DNA damage. Impairment of this system gives rise to MSI [25, 26], which has now been recognized as a hallmark of MMR gene-deficient cancers. Microsatellite loci, widely dispersed in the genome, are repetitive sequences consisting of short runs of nucleotides, typically one to four bases in length. Repetitive regions may give rise to the formation of secondary structures, which are subject to expansion or contraction. The secondary structures, if incorrectly resolved, lead to slippage of DNA polymerases along repetitive sequences during replication. Microsatellites are particularly susceptible to length change mutations during replication and transcription, resulting in frameshift mutations if they are located within a gene [25, 26]. MMR deals with these changes to maintain microsatellite stability. MMR comprises

Table 1.1 Genes in DNA damage repair pathways that are hypermethylated in cancer

Repair pathway	Methylated gene	Cancer type	Samples studied	Samples methylated	Methylation frequency (%)	References
MMR	MLH1	Sporadic CRC (MSI+)	110	67	61	[41–44]
		Sporadic CRC (MSI–)	128	38	30	[42, 43]
		Sporadic early-onset CRC	110	55	50	[45]
		NSCLC	77	43	56	[32]
		Acute myeloid leukemia	177	11	6	[34–36]
		Ovarian cancer	672	72	11	[33]
		Oral squamous cell carcinoma	99	8	8	[29]
		HNPCC	179	2	1	[39, 40]
		Gastric cancer	306	58	19	[30, 31]
		HNSCC	49	14	29	[37]
	MSH2	NSCLC	14	4	29	[32]
		Gastric cancer	200	27	14	[30]
		Ovarian cancer	56	29	52	[46]
		Sporadic CRC	36	1	3	[47]
		HNPCC	46	11	24	[48]
	MSH3	Gastric cancer	200	25	13	[30]
	MSH6	Breast cancer	33		92–95[a]/20©	[50]
BER	TDG	Multiple myeloma	KAS-6/1 cell line			[52]
	MBD4	CRC	39		24[a]/14©	[53]
	OGG1	Thyroid cancer	38	2	5	[54]
NER	XPC	Bladder cancer	37	12	32	[56]
	ERCC1	Glioma	32	Unknown		[57]
	XRCC1	Gastric cancer	25	Unknown		[60]
	RAD23B	Multiple myeloma	KAS-6/1 cell line			[61]

HR	BRCA1	NSCLC	98	29	30	[69]
		Sporadic ovarian cancer	81	12	15	[66]
		Sporadic breast cancer	190	24	13	[64, 70]
		Hereditary breast cancer	162	18	11	[70]
		Early onset gastric cancer	104	0.6	1	[67]
		Bladder cancer	96	0.71	1	[68]
	BRCA2	Breast cancer	33		59–64[a]/10©	[50]
		NSCLC	98	41	42	[69]
	FANCC	Acute lymphoblastic leukemia	97	3	3	[74]
		Acute myeloid leukemia	143	1	1	[74]
	FANCF	HNSCC	89	13	15	[75]
		NSCLC	158	22	14	[75]
		Cervical cancer	91	27	30	[76]
		Ovarian cancer	53	7	13	[77]
	FANCL	Acute lymphoblastic leukemia	97	1	1	[74]
NHEJ	XRCC5	NSCLC	98	19	19	[69]
ATM/ATR	ATM	HNSCC	100	25	25	[101]
		Breast cancer	23	18	78	[100]
		CRC	HCT116 cell line			[99]
	CHK2	NSCLC	139	39	28	[106]
		Glioma	5	5	100	[107]

(continued)

Table 1.1 (continued)

Repair pathway	Methylated gene	Cancer type	Samples studied	Samples methylated	Methylation frequency (%)	References
Others	MGMT	Oral squamous cell carcinoma	99	40	40	[29]
		Gastric cancer	200	50	25	[30]
		CRC	36	14	39	[80]
		HNSCC	21	6	29	[80]
		NSCLC	34	10	29	[80]
		Lymphomas	61	15	25	[80]
		Glioma	140	54	39	[80]
	WRN	Gastric cancer	38	10	26	[91]
		CRC	182	69	38	[91]
		NSCLC	56	21	38	[91]
		Prostate cancer	20	4	20	[91]
		Breast cancer	58	10	17	[91]
		Thyroid cancer	32	4	13	[91]
		Non-Hodgkin lymphoma	118	28	24	[91]
		Acute lymphoblastic leukemia	21	2	10	[91]
		Acute myeloblastic leukemia	36	3	8	[91]
		Chondrosarcomas	15	5	33	[91]
		Osteosarcomas	27	3	11	[91]

CRC colorectal cancer; *NSCLC* non-small cell lung cancer; *HNPCC* hereditary non-polyposis colorectal cancer; *HNSCC* head and neck squamous cell carcinoma

[a]Mean methylation level (%) in cancer vs. © mean methylation level (%) in control

©Indicates references from which methylation data derived from similar samples was pooled for this summary

the MutS complex and the MutL complex. MutS recognizes the mismatched base, while MutL recruits repair enzymes to damage sites via its binding with MutS [27]. There are two main MutS complexes in humans, MutSα and MutSβ. MutSα, consisting of the MutS homologue 2 (MSH2) protein bound to MSH6, recognizes single-base mismatches or small insertion/deletion loops (indels), whereas MutSβ, consisting of MSH2 and MSH3, repairs only indels [28]. The main complex for MutL in humans is MutLα, consisting of a heterodimer of MLH1 and PMS2 [26]. Mutations in or epigenetic silencing of MMR genes like *MLH1* and *MSH2* is closely associated with a variety of human cancers such as hereditary non-polyposis colon cancer (HNPCC), sporadic colon cancer, and ovarian cancer [29].

MLH1 plays a central role in coordinating various steps in MMR via interacting with other MMR proteins and modulating their activities. Hypermethylation of the *MLH1* promoter is observed in a variety of cancers including oral squamous cell carcinoma [30], gastric cancer [31, 32], non-small cell lung cancer (NSCLC) [33], ovarian cancer [34], acute myeloid leukemia [35–37], head and neck squamous cell carcinoma (HNSCC) [38], HNPCC [39–41], and particularly in colorectal cancer (CRC) [42–45] (Table 1.1). The reduced MLH1 protein expression is correlated with high-level methylation detected in human CRC samples, whereas samples with low-level methylation display expression levels similar to those observed in methylation-negative samples [46], strongly suggesting that the *MLH1* gene is inactivated via promoter hypermethylation in a dose-dependent manner. Nonetheless, it is not clear whether a moderate degree of methylation affects MLH1 gene expression or not. On the basis of observations made in germ line cells, it has long been believed that *MLH1* promoter methylation involves only one allele of maternal origin. However, more recent findings demonstrate that there is biallelic involvement of *MLH1* promoter hypermethylation in many cancers [46]. The causal link between MSI and epigenetic inactivation of *MLH1* is further highlighted by the observation that 90% of MSI+ HNPCC have *MLH1* hypermethylation, while 95% of MSI samples do not [20].

MSH2 is also hypermethylated in multiple tumor types, including gastric cancer [31], NSCLC [33], ovarian cancer [47], sporadic CRC [48], and HNPCC [49] (Table 1.1). Interestingly, promoter methylation of *MSH2* in HNPCC occurs primarily in patients with germ line mutations in *MSH2* rather than in germ line mutation-negative cases [49]. Seventy percent of patients with *MSH2* methylation also present germ line mutations in this gene, clearly indicating that methylation is the second inactivating hit in these tumors [49]. DNA hypermethylation can be caused by transcription across a CGI within a promoter region. Recent studies have revealed that deletions of the last exons of the *EpCAM* gene, located immediately upstream of *MSH2*, give rise to somatic hypermethylation of the *MSH2* promoter [50]. Deletions at the most 3′-end of the *EpCAM* gene result in loss of its polyadenylation signal, which abolishes transcription termination. Transcription of *EpCAM* then continues downstream into the *MSH2* promoter and induces promoter hypermethylation of *MSH2*. DNA methylation triggered by transcriptional read-through of a neighboring gene, in either sense or antisense, direction may represent a general mutational mechanism that promotes aberrant epigenetic changes. Like *MLH2*, other MutS

homologues, including *MSH3* and *MSH6*, are also inactivated by hypermethylation in tumors such as breast [51] and gastric cancers [31] (Table 1.1).

1.2.2 Epigenetic Inactivation of the Base Excision Repair and Nucleotide Excision Repair Pathways

The specific pairing of DNA bases in the genome is constantly challenged by endogenous metabolic by-products and environmental insults. Base excision repair (BER) is responsible for the removal of damaged DNA bases and their backbones to prevent mutations that could give rise to cancer [19, 52]. In BER, abnormal DNA bases are recognized and removed by specific glycosylases, followed by recruitment of other enzymes including nuclease, polymerase, and ligase proteins, to complete the repair process via excising the remaining sugar fragments and reinstalling an intact correctly based-paired nucleotide [19].

Either thymine DNA glycosylase (TDG) or methyl-CpG-binding domain 4 (MBD4) mediate a specific BER pathway for the correction of G/T mismatches arising due to 5-methylcytosine deamination leading to C to T transitions. DNA hypermethylation-mediated silencing of *TDG* and *MBD4* may contribute to the frequent genomic instability that occurs in cancer cells [53] (Table 1.1). *TDG* promoter hypermethylation negatively correlates with its expression. TDG down-regulation leads to less efficient DNA repair activity in response to hydrogen peroxide-induced DNA damage. Ectopic expression of TDG, however, functionally compensates for lower repair activities of damaged DNA in the KAS-6/1 myeloma cell line with extensive endogenous *TDG* gene hypermethylation [53]. *MBD4*, like *TDG*, is also subject to promoter hypermethylation and gene silencing in tumors like sporadic CRC and ovarian cancer [54]. Another DNA glycosylase, OGG1, which mediates removal of 8-oxoguanine induced by oxidative damage, is also subject to inactivation via promoter methylation in cancer cells [55] (Table 1.1).

Of all the repair systems, nucleotide excision repair (NER) recognizes the most varied types of DNA lesions, contending with the diverse class of helix-distorting damage that interferes with base pairing and obstructs replication and transcription. In NER, there exist two sub-pathways that differ in the mechanism of lesion recognition: global genome-NER (GG-NER) that surveys the entire genome for distortions, and transcription-coupled repair (TCR), which targets damage that blocks elongating RNA polymerases [19, 56]. NER, therefore, plays a particularly important role in preventing mutations. Thus far, three syndromes, xeroderma pigmentosum, Cockayne syndrome, and trichothiodystrophy (TTD), are closely associated with NER defects [56]. Of these, patients with xeroderma pigmentosum, attributable to mutations in one of the seven xeroderma pigmentosum (XP) group genes (*XPA*–*XPG*), show a dramatically increased incidence of UV light-induced skin cancer [19, 56].

It was reported recently that the *XPC* promoter is epigenetically inactivated in bladder cancer [57] (Table 1.1). *XPC* promoter methylation is significantly elevated

in cancerous bladder compared to normal tissue, leading to reduced mRNA levels in the tumor [57]. Epigenetic defects in the *XPC* gene may also influence malignant behavior and prognosis. ERCC1 is a crucial protein in the NER pathway primarily involved in the repair of platinum-DNA adducts. Aberrant CGI methylation in the *ERCC1* promoter region has been observed in human glioma cell lines and primary tumors, which is associated with cisplatin chemosensitivity [58]. In a rat lung cancer model, however, *ERCC1* methylation is detected in only a very small proportion of samples [59]. Deficiency in XRCC1, a scaffolding protein for BER and single-strand break repair (SSBR), is associated with enhanced risk of lung cancer [60]. *XRCC1* is subject to aberrant promoter methylation in human gastric cancer tissues [61]. In lung cancer, infiltrating carcinomas exhibit statistically higher levels of methylation at the *XRCC1* promoter compared to normal, hyperplastic, and squamous metaplastic tissues [59]. RAD23B, a key component for damage recognition in NER, is also hypermethylated in multiple myeloma [62].

1.2.3 Epigenetic Inactivation of HR and Nonhomologous End-Joining DNA Repair Pathway Components

HR not only provides an important mechanism to repair several types of DNA lesions that pose a threat to genome integrity, including DNA DSBs, DNA damage encountered during DNA replication, and DNA interstrand cross-links (ICLs), but is also required to restart stalled replication forks during the late S and G2 phases of the cell cycle [63, 64]. HR promotes precise repair of DNA damage using the intact sister chromatid as a template. Deficiency of HR leads to more error-prone repair, which is associated with mutagenesis and predisposition to cancer [63].

The *BRCA1* and *BRCA2* genes are both essential for HR-mediated DNA repair. BRCA1 appears to act as a signal integrator that links DNA damage sensors with response mechanisms. BRCA2, however, is more directly involved in homology-directed DSB repair, as it mediates formation of a RAD51-DNA nucleoprotein filament that catalyzes strand invasion during HR. *BRCA1* and *BRCA2* are frequently mutated in hereditary breast and ovarian cancers, but seldom in sporadic cases of these tumor types. Epigenetic inactivation of *BRCA1* via promoter hypermethylation, however, plays an important role in tumorigenesis in a wide array of cancers including breast [65, 66], ovarian [67], gastric [68], bladder [69], and NSCLCs [70], both hereditary [71] and sporadic forms [20, 39] (Table 1.1). It is believed that epigenetic silencing of BRCA1 creates a new mutator pathway that generates mutations and gross chromosomal rearrangements via p53 signaling. This idea is supported by several observations including one demonstrating that p53 inactivation rescues the impact of BRCA1 deficiency on cell survival [20, 72]. Although much less frequently than *BRCA1*, *BRCA2* also acquires promoter region hypermethylation that is closely associated with its reduced expression in breast cancer [51] and NSCLC [70] (Table 1.1).

The primary function of the Fanconi anemia (FA) pathway is to repair interstrand DNA cross-links, which promotes HR via coordinating other DNA damage-responsive events to stabilize stalled replication forks, to convey signals to DNA checkpoint pathways, and to facilitate recovery of replication forks [73]. FA is a genomic instability syndrome characterized by bone marrow failure, developmental abnormalities, and increased cancer incidence, which is caused by mutations in one of thirteen distinct genes (*FANCA*, *FANCB*, *FANCC*, *FANCD1*, *FANCD2*, *FANCE*, *FANCF*, *FANCG*, *FANCI*, *FANCJ*, *FANCL*, *FANCM*, and *FANCN*) [73]. Eight of them (FANCA, B, C, E, F, G, L, and M) form the FA core complex. This group of genes contains a high GC content and CGIs at their promoter regions, making them potential targets for aberrant hypermethylation-mediated silencing [74]. This idea has received support from observations that *FANCC*, *FANCF,* and *FANCL* acquire promoter methylation during human carcinogenesis [39, 75]. Of these, *FANCF* displays hypermethylation the most frequently, occurring in 14–28% of different cancers including NSCLC [76], HNSCC [76], cervical [77], and ovarian [39, 78] (Table 1.1).

Unlike HR, which performs error-free repair, nonhomologous end-joining (NHEJ) simply restores DNA integrity by joining the two DNA ends. This type of repair is error-prone and frequently results in the loss or addition of several nucleotides at the break site. Despite its mutagenic consequences, NHEJ is the major DSB repair pathway in mammalian cells. Defects in NHEJ lead to chromosomal translocations and genomic instability. In NHEJ, DSBs are detected by the KU70/KU80 heterodimer; the KU complex then activates the protein kinase DNA-PKcs (DNA-dependent protein kinase catalytic subunit), leading to recruitment and activation of end-processing enzymes, polymerases, and finally ligation of the breaks by the XRCC4/DNA ligase IV complex. In the NHEJ pathway, only the *XRCC5* gene, encoding the KU80 protein, has been reported to be inactivated via epigenetic mechanisms [70] (Table 1.1). Low expression of XRCC5 in squamous cell carcinoma and NSCLC is significantly associated with promoter region hypermethylation. Treatment of NSCLC cells with the DNA demethylating agent 5-aza-2′-deoxycytidine (5-aza-CdR), however, does not result in increased KU80 expression [70]. Thus, the underlying mechanisms promoting and maintaining XRCC5 silencing await further investigation, particularly in more samples and more types of cancer.

1.2.4 Epigenetic Silencing of O⁶-Methylguanine-DNA Methyltransferase

O^6-methylguanine, which arises due to alkylation reactions, pairs with thymine rather than cytosine, resulting in G:C to A:T mutations during DNA replication. O^6-methylguanine-DNA methyltransferase (MGMT), also known as O^6-alkylguanine-DNA alkyltransferase (AGT), repairs DNA damage by transferring the methyl groups on the O^6 position of guanine to an active site cysteine residue to protect cells from sustaining mutagenic events, which has been demonstrated by gain- or loss-of-function experiments in vitro and in vivo [79]. The MGMT protein is unique among DNA-repair components because it acts alone to remove DNA adducts.

Although MGMT is ubiquitously expressed in normal human tissues, mean enzymatic activity in malignant tissues is usually higher than in their normal counterparts. However, there is a variety of tumors such as glioma, CRC, NSCLC, and HNSCC that lack MGMT expression [20, 39] (Table 1.1). It has been well documented that MGMT deficiency often arises due to abnormal promoter methylation [20, 39, 80]. For example, 29% of NSCLCs and 38% of CRCs display aberrant *MGMT* methylation, in which the presence of hypermethylation is highly associated with loss of MGMT protein [81]. *MGMT* is the most frequently methylated gene in central nervous system tumors. Epigenetic silencing of *MGMT* via promoter hypermethylation occurs in approximately 40% of primary glioblastomas and over 70% of secondary glioblastomas. It is also detected in 50% of the diffuse and anaplastic astrocytomas and approximately two-thirds of oligodendroglial and mixed tumors [82]. These results, together with a causal relationship between DNA methylation of the *MGMT* CGI and decreased transcription of the gene in cell culture-based studies, demonstrate that DNA methylation is an important mechanism for silencing the *MGMT* gene in human cancers.

Epigenetic silencing of *MGMT* may initiate an important mutator signaling cascade in human cancers since MGMT loss causes G:C to A:T transitions, which lead to downstream gene mutations. This proposal is strongly supported by an analysis of point mutations in *KRAS* and *p53*. KRAS, the most commonly altered oncogene in cancer, is an early key player in multiple signal pathways. Loss of MGMT is associated with increased *KRAS* mutations possessing G:C to A:T transitions in colon [83] and gastric cancer [84]. *p53* is the most frequently mutated tumor suppressor gene (TSG) in human cancer, and the majority of known *p53* mutations are G:C to A:T transitions [66, 85]. Epigenetic inactivation of *MGMT* may lead to G:C to A:T transition mutations in *p53*, which has been observed in several types of cancer including colorectal [66], liver [86], lung [87], esophageal squamous cell carcinomas [88], and glioma [89]. Interestingly, *MGMT* promoter methylation is associated with improved disease chemosensitivity and prolonged survival time in patients treated with alkylating agent-based therapies [90]. However, it is unclear whether the improved survival is specifically due to loss of MGMT expression or accompanying drug sensitivity.

1.2.5 Epigenetic Silencing of WRN

Werner syndrome (WS) is a rare autosomal recessive disease, characterized by premature onset of aging, genomic instability, and increased cancer incidence. WS is caused by null mutations at the *WRN* locus at 8p11.2–p12, which codes for a DNA helicase belonging to the RecQ family. Deficiency in WRN function causes defects in DNA replication and recombination, as well as DNA repair.

WRN is a 180-kd nuclear protein that has a unique interaction with its DNA substrates through its C terminal RQC domain during base separation [91]. In addition to two C-terminal ATPase domains encoding for helicase activity, the WRN protein contains an N-terminal domain coding for exonuclease activity. Its helicase

and exonuclease activities function in a coordinated manner, suggestive of roles in DNA repair, recombination, and replication. Recently, the WRN protein was also shown to be involved in telomere maintenance based on the discovery that its deficiency leads to accelerated telomere shortening in WS cells [92]. These multiple roles of the WRN protein highlight its importance in aging and cancer.

The evidence suggesting that WRN acts as a TSG is derived primarily from WS, which is characterized by the early onset development of a variety of cancers due to germ line *WRN* mutation; somatic mutations in the *WRN* gene have not been reported. Epigenetic inactivation of *WRN* provides additional support for its TSG role in sporadic cancer. The *WRN* promoter undergoes hypermethylation in a wide array of tumors including colorectal, gastric, prostate, non-small cell lung, and breast cancers [93, 94] (Table 1.1). Epigenetic silencing of *WRN* via methylation not only leads to the loss of protein and enzyme activity, but also to chromosomal instability. Furthermore, the above phenotype is reversed by DNA-demethylating agents. Most importantly, restoration of WRN expression induces its tumor-suppressor effects, such as inhibition of colony formation and tumor growth [93]. Taken together, aberrant epigenetic silencing of *WRN*, a candidate TSG, may play an important role in human cancers. Interestingly, WRN was recently shown to be associated with promoter methylation of the *OCT4* gene [95], which encodes a crucial transcription factor for the maintenance of cell pluripotency. During differentiation of human pluripotent NCCIT embryonic carcinoma cells, WRN localizes to the *OCT4* promoter region with *de novo* DNA methyltransferase DNMT3B and promotes differentiation-dependent *OCT4* silencing and promoter methylation [95]. Deficiency in WRN blocks DNMT3B recruitment to the promoter and leads to decreased promoter methylation of *OCT4* [95]. Therefore, WRN may also contribute to the control of stem cell differentiation via epigenetic silencing of the key pluripotency transcription factor OCT4.

1.2.6 Epigenetic Inactivation of ATM/ATR Signaling

DNA damage signaling requires the coordinated action of a large array of molecules that can be categorized as DNA damage sensors, transducers, mediators, and effectors according to their functions. Upon damage of DNA, the MRE11–RAD50–NBS1 (MRN) sensor complex recognizes DSBs and the replication protein A (RPA) complex processes accumulated single-stranded DNA (ssDNA). The transducer ataxia-telangiectasia mutated (ATM) and ATR kinases are recruited to and activated by DSBs and RPA-coated ssDNA, respectively. With the help of mediators (including 53BP1, MDC1, BRCA1, MCPH1, and PTIP in ATM signaling, and TopBP1 and Claspin in ATR signaling), ATM and ATR activate the effector kinases CHK2 and CHK1, respectively, which then spread the signal throughout the nucleus [96–98]. CHK1 and CHK2 decrease cyclin-dependent kinase (CDK) activity, which slows down or arrests cell cycle progression. Meanwhile, ATM/ATR

signaling promotes DNA repair through various mechanisms. Through ATM/ATR signaling, DNA repair and cell cycle progression are closely coordinated. The coordinated action of DNA repair and cell cycle controls either promotes the resumption of normal cell functioning before replication or triggers apoptosis/cell death when normal cell functioning cannot be restored; both mechanisms act as barriers to tumorigenesis [19].

Ataxia-telangiectasia (AT) is a rare autosomal recessive disorder, characterized by progressive cerebellar ataxia, oculocutaneous telangiectasia, susceptibility to bronchopulmonary disease, and lymphoid tumors. AT is caused by deficiency in the *ATM* gene, localized on chromosome 11q22–23. ATM is an Ser/Thr protein kinase of the phosphoinositide 3-kinase (PI3K)-related protein kinase (PIKK) family, which also includes ATR, DNA-PKcs, and SMG1. ATM may have as many as 700 substrates [99, 100], highlighting its multiple functions in various biological processes including cancer. Loss of heterozygosity in *ATM* results in reduced protein expression; however, this mechanism explains only a small proportion of cancers where ATM down-regulation is observed. In sporadic cancer, which accounts for 90–95% of tumors, the probability of *ATM* gene mutations is low, whereas altered expression of ATM is frequently observed. It is therefore likely that epigenetic modifications have an impact on ATM expression in these cases (Table 1.1). Initial proof for this idea came from studies using the human colon cancer cell line HCT116 [101]. In this cell line, *ATM* displays aberrant promoter methylation, which inversely correlates with its low expression and low radiosensitivity. The significance of this finding is underscored by further observations that treatment of HCT116 cells with 5-azacytidine (a DNA demethylating agent) restores expression of ATM and radiosensitivity [101]. *ATM* is also epigenetically silenced in primary cancers. For example, 78% of surgically removed breast tumors [102] and 25% of HNSCC [103] display aberrant methylation in the *ATM* promoter region accompanied by reduced ATM.

CHK2, the mammalian homologue of the yeast Rad53 and Cds1, is located at chromosome 22q12.1, spans approximately 50 kb, and consists of 14 exons [104]. CHK2, activated by ATM, responds primarily to DSBs. Its fundamental role is to coordinate cell cycle progression with DNA repair and cell survival or death. Germ line mutations in the *CHK2* gene predispose to Li–Fraumeni syndrome (LFS), characterized by multiple tumors at early age with a predominance of breast cancer and sarcomas [105]. Somatic mutations in *CHK2* exist also, although they occur in only a small subset of sporadic human malignancies, including carcinomas of the breast, lung, colon, and ovary, osteosarcomas, and lymphomas [106]. The finding of both germ line and somatic mutations suggests that *CHK2* acts as a TSG. This is further supported by the observation that down-regulation of CHK2 is associated with promoter methylation in sporadic cancers including lung cancer, glioma, and Hodgkin's lymphoma [107–109]. For example, DNA hypermethylation of the distal *CHK2* CGIs occurs in 28.1% of NSCLCs and 40.0% of squamous cell carcinomas, which inversely correlates with *CHK2* mRNA levels. It should be noted, however, that observations in breast, colon, and

ovarian cancers do not support a causative link between DNA methylation and gene expression of CHK2 [110, 111].

1.3 DNA Methyltransferase 1 and Mismatch Repair

The function of the MMR pathway is to correct base substitution mismatches and insertion–deletion mismatches generated in newly replicated DNA [112]. Deficiencies in or inactivation of this pathway has profound biological consequences. Loss of MMR activity is attributed to the initiation and promotion of multistage carcinogenesis [113]. A growing number of reports have demonstrated that loss of DNMT1 function has a significant impact on MSI—a hallmark of MMR efficiency, suggesting it has a role in the MMR pathway (Fig. 1.1). Using genetic screens in *Blm*-deficient embryonic stem (ES) cells, *Dnmt1* was identified as an MMR modifier gene. *Dnmt1* deficiency in murine ES cells results in a fourfold increase in the MSI rate [13]. Further support for this finding comes from several other laboratories [14–17, 114]. DNMT1 deficiency enhances microsatellite mutations for both integrated reporter genes [13, 14, 16, 17] and endogenous repeats [15]. This finding holds true for both ES cells and somatic cells. In a murine ES cell line with homologous deletion of *Dnmt1*, the stability of five endogenous microsatellite repeats (two mononucleotides and three dinucleotides), exhibiting instabilities in MMR-deficient cells was analyzed. A significantly higher frequency of instability was detected at three of the five markers in *Dnmt1*$^{-/-}$ ES cells compared to the wild-type ES cells [15]. The slippage rate of a stable reporter gene was also monitored. Dnmt1 deficiency led to a sevenfold higher rate of microsatellite slippage in *Dnmt1*$^{-/-}$ ES cells compared to wild-type cells [14]. Notably, no DNA methylation in the region flanking the reporter gene was discovered, regardless of Dnmt1 status, suggesting that the effect of Dnmt1 on MMR was not at the level of DNA methylation [14]. Enhanced MSI is associated with higher levels of histone H3 acetylation and lower MeCP2 binding at regions near the assayed microsatellite, suggesting that Dnmt1 loss decreases MMR efficiency by modifying chromatin structure. CAG repeat expansions are closely associated with human age-related diseases including 12 neurodegenerative disorders. Repeat instability induced by CAG repeat expansion requires the MMR components [16, 115]. DNMT1 deficiency induces destabilization and intergenerational expansion of CAG triplet repeats [16]. Double knockdown of MLH1 and DNMT1, however, additively increases the frequency of CAG contraction [114]. Specific targeting of DNMT1 in hTERT-immortalized normal human fibroblasts by siRNA induces both resistance to MSI and the drug 6-thioguanine (which induces cytotoxic DNA damage due to its misincorporation opposite thymine [116]) at a CA17 reporter gene; two hallmarks of MMR deficiency. Mutation rates correspond well with DNMT1 levels, ranging from 4.1-fold in cells with 31% of the normal DNMT1 protein level to tenfold in cells with 12% of the normal DNMT1 protein level [17]. This suggests that DNMT1 regulates microsatellite stability in a dose-dependent manner. The exact underlying mechanism of how

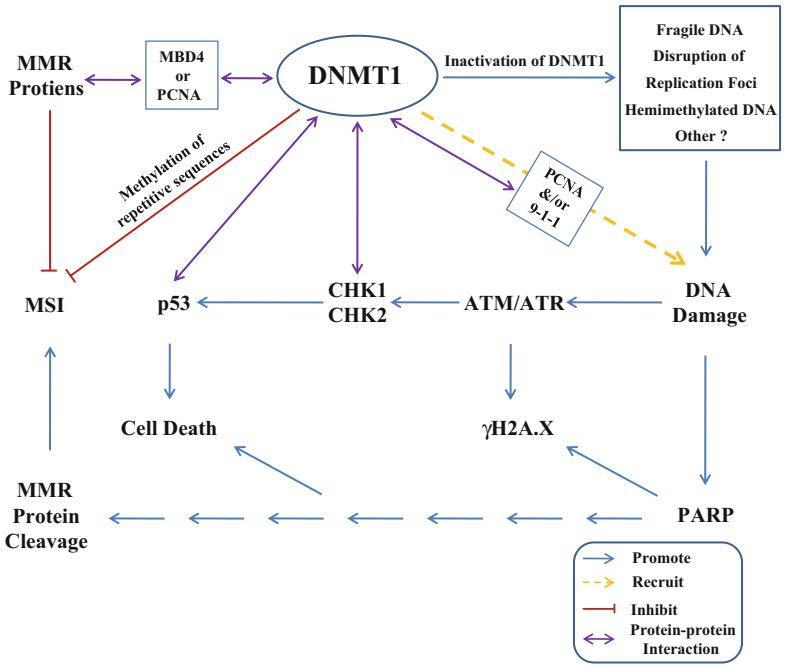

Fig. 1.1 Impact of DNMT1 on MMR and DDR. DNMT1 may promote stabilization of microsatellites via methylation of CpG repeats and it also interacts with DNA repair proteins via third-party mediators (e.g., MBD4 and PCNA). Moreover, deficiency in DNMT1 leads to activation of PARP signaling, eventually resulting in MMR protein cleavage. DNMT1 is also closely associated with DDR. Inactivation of DNMT1 may induce several changes to DNA and/or chromatin including increased DNA fragility, disruption of replication foci, and accumulation of hemimethylated DNA, which may be recognized as "damage" and activate the DDR. Strong support for a direct link between DNMT1 and DDR comes from the identification of several protein-protein interactions involving DNMT1 and DDR proteins. DNMT1 is recruited to sites of DNA damage via its interaction with PCNA and 9-1-1. DNMT1 is also capable of binding CHK1 and p53, which promote cell cycle arrest and apoptosis, respectively

DNMT1 is involved in MSI appears complex and remains elusive. Microsatellite methylation probably provides a mechanism for length stabilization by subsequent transcriptional repression of genes containing or proximal to microsatellites with methylated CpG repeats. However, increased mutations usually occur at microsatellite repeats that do not contain any CpG sites in the repeat itself [13, 15, 16, 114] or nearby [14], indicating that DNA methylation changes around microsatellite repeats, at least in some cases, are not the primary cause of the instability. Alternatively, DNMT1 might influence transcriptional repression and MSI through chromatin remodeling [14].

The impact of DNMT1 on the MMR pathway is further highlighted by the observation that DNMT1 and the MMR proteins probably interact with each other through a third-party mediator (Fig. 1.1). The methyl CpG-binding protein MBD4/MED1 may provide a functional link between MMR and DNMT1 through protein–protein

interaction. MBD4, which possesses glycosylase repair activity for G:T mismatches, is involved in NER as well as MMR. MBD4 binds MLH1 via its C-terminal glycosylase domain [117, 118]. Deletion of *Mbd4* in MEFs induced destabilization of MMR proteins and conferred resistance to antitumor drugs including 5-FU and platinum [119]. MBD4 and TDG have functional overlap and they interact with the de novo methyltransferases DNMT3A and DNMT3B [120, 121]. MBD4 also interacts with maintenance methyltransferase DNMT1 via its N-terminal MBD domain [118]. Based on a combination of immunoprecipitation and GST-pull down experiments in mouse, rat, and *Xenopus*, a minimal domain of approximately 70 amino acids in the N-terminal targeting sequence region of DNMT1 was shown to be required for MBD4 to bind to DNMT1 [118], which overlaps with a region in rat DNMT1 that interacts with MECP2 [122]. Through interacting directly with both DNMT1 and MLH1, MBD4 recruits MLH1 to heterochromatic sites that are coincident with DNMT1 localization [118]. Similarly, MBD4/MLH1 accumulates at DNA damage sites where DNMT1 is recruited after laser microirradiation [118]. Loss of DNMT1 induces p53-dependent apoptosis, which can be rescued by inactivation of p53 [123]. The MBD4/MLH1 complex also mediates the apoptotic response to DNMT1 depletion [118]. Colocalization of these proteins at damaged regions implies that they function coordinately in the cellular decision to repair the lesion or activate apoptosis. Like MBD4, PCNA may act as a mediator between MMR and DNMT1 because of its direct interaction with both systems. PCNA interacts with multiple components of the MMR pathway including MSH6, MSH3, and MLH1. Disruption of this interaction confers an MMR defect in vivo and in vitro [124–126]. Both MSH6 and MSH3 colocalize with PCNA at replication foci during S-phase [127]. MLH1 is recruited to damage sites where PCNA and DNMT1 also accumulate, although with slower kinetics than DNMT1 [118, 128]. The recruitment of DNMT1 to both the replication fork and DNA damage sites is through a direct interaction with PCNA and possibly CHK1 and the 9-1-1 complex as well [21, 24]. However, there is no report showing that PCNA, MLH1, and DNMT1 colocalize together, implying that PCNA might interact with each protein at a different time. Nonetheless, the functional mechanisms of whether and how these factors are orchestrated in response to DNA damage requires further investigation.

Most recently, DNMT1 deficiency has been shown to induce the depletion of multiple repair factors at the protein level (Fig. 1.1) [17], highlighting its importance not only in MMR efficiency, but also in DDR signaling. In normal human fibroblasts and CRC cell lines, DNMT1 knockdown leads to a matching decrease in MLH1 at the protein, but not the mRNA level [17]. Loss of MLH1, however, does not lead to expression changes in DNMT1 [17]. Promoter hypermethylation of *MLH1*, although frequently observed in sporadic colon cancers [39], does not appear to be the cause leading to gene inactivation in the context of DNMT1 deficiency. *MLH1* hypermethylation in DNMT1-deficient cells was further ruled out using a bisulfite pyrosequencing assay [17]. Further observations suggest that DNMT1 deficiency affects the steady-state levels of a number of repair proteins, including MSH2, MSH6, and PMS2, as well as MBD4 [17]. Loss of multiple MMR components in DNMT1 hypomorphic cells indicates that DNMT1 might play an

indirect role in the stabilization or proteolytic cleavage of these proteins, rather than directly interacting with each of them. It is documented that DNMT1 deficiency activates the DDR, which leads to cell cycle arrest [21, 123] and the triggering of cell death pathways [123] that may result in cleavage of proteins including MLH1 [129], which might account for MMR protein depletion after DNMT1 knockdown. Loss of DNMT1 activates ATM/ATR, which normally phosphorylate H2A.X leading to focal accumulation of γH2A.X, a hallmark of DDR [21]. If excessive damage exists, p53-dependent [123] and other cell death pathways are activated to maintain genomic integrity. Elevated γH2A.X levels in DNMT1 hypomorphic cells can be partially reduced through inhibition of ATM/ATR signaling [17]. However, the PAR polymerase (PARP) inhibitor DPQ also reduces the level of γH2A.X, to an extent exceeding that observed with the ATM/ATR inhibitor caffeine. In keeping with these observations, the viability of DNMT1-depleted cells treated with DPQ is enhanced to a greater extent than treatment of cells with agents that inhibit caspases or p53 [17]. These findings, together with the observation that PARylation increases after DNMT1 loss, clearly demonstrate that PARP is involved in the DDR and cell death process in cells deficient in DNMT1 (Fig. 1.1). PARP catalyzes the polymerization of ADP-ribose (PAR) units on target proteins using nicotinamide adenine dinucleotide (NAD^+) molecules as a donor [130]. NAD^+ depletion, induced by severe DNA damage, gives rise to mitochondrial membrane depolarization and apoptosis initiation factor (AIF) translocation. It eventually results in an activation of caspases that lead to protein cleavage and cell death. DNA repair protein MLH1 [129], along with BLM1 [131] and ATM [132], are preferred targets of caspases. Treatment with the PARP inhibitor DPQ, as expected, leads to an increase in full-length MLH1 protein levels in DNMT1-depleted cells [17]. Taken together, DDR signaling, particularly the cell death pathway mediated by PARP, may play a substantial role in regulating cleavage of MMR repair proteins in cells deficient for DNMT1 (Fig. 1.1).

1.4 DNMT1 and the DNA Damage Response

Reduction of DNMT1 levels activates a DDR usually initiated by the most lethal form of DNA damage-DSBs (Fig. 1.1). DNMT1 deficiency also inhibits DNA replication [22, 23, 133]. It was reported that DNMT1 knockdown triggers an intra-S-phase arrest of DNA replication, independent of DNA demethylation [22]. Similar to the observations for DNA damage checkpoints [134], the intra-S-phase arrest is transient, disappearing after 10 days of treatment with *DNMT1* siRNA. The S-phase cells induced by DNMT1 knockdown exist in two distinct populations: 70% incorporate BrdUr, while 30% do not, consistent with the presence of an intra-S-phase checkpoint triggering cell cycle arrest [134]. Cells are arrested at different positions throughout S-phase, suggesting that this response is not specific to distinct classes of origins of DNA replication. 5-aza-CdR, a nucleoside analogue, is a well-characterized and widely used inhibitor of DNA methylation, which inhibits

DNA methylation by trapping DNMT1 at the replication fork after being incorporated into DNA. 5-aza-CdR does not inhibit the de novo synthesis of DNMT1 protein or its presence in the nucleus. S-phase cells treated with 5-aza-CdR, which causes genome-wide demethylation, do not exhibit two distinct population distributions as observed in cells deficient in DNMT1. These results suggest that the intra-S-phase arrest is not correlated with the degree of DNA methylation, consistent with observations that DNA replication arrest following DNMT1 inhibition is probably due to a reduction in the physical presence of DNMT1 at the replication fork, rather than DNA demethylation [133]. As discussed above, the cell cycle distribution in DNMT1 knockdown cells resembles the transient intra-S-phase arrest in DNA replication that is evoked by genotoxic insults [135–137]. In addition, DNMT1 inhibition also leads to the induction of a set of genes that are implicated in the genotoxic stress response including *p21* [133], *p53* [123], and the growth arrest DNA damage inducible 45β gene (GADD45*β*) [22]. These results imply that DNMT1 is linked to DNA damage repair machineries to maintain chromosome integrity via blocking DNA replication, a notion further strengthened by observations that DNMT1 knockdown activates the checkpoint pathways in an ATR-dependent manner [23]. Upon DNMT1 depletion, CHK1 and CHK2, key proteins in ATM/ATR signaling, are phosphorylated, which in turn induce phosphorylation and degradation of cell division control protein 25 A (CDC25A) as well as CDC25B [23]. As a consequence, the capacity for loading CDC45, an essential factor for DNA replication [138], onto replication forks is decreased, resulting in replication arrest. DNMT1 knockdown also induces the formation of histone γH2A.X foci, a hallmark of the DNA DSB response. The response elicited by DNMT1 knockdown is blocked by siRNA-mediated depletion of ATR, suggestive of its ATR dependency. Further support for the importance of ATR came from the finding that the cellular response to DNMT1 depletion is markedly attenuated in cells derived from a patient with Seckel syndrome, a disorder due to ATR deficiency [23]. However, it is not clear whether ATM, another key transducer like ATR in the checkpoint pathway, is involved in the process or not. DNA demethylating agents do not trigger the stress response like genetic DNMT1 depletion does [23]. Moreover, this response is abolished by ectopic expression of either wild-type DNMT1 or a mutant form of DNMT1 lacking the catalytic domain [23], suggesting that loss of catalytic activity of DNMT1 is not driving this response. Also of importance, DNMT1 knockdown leads to very limited genomic demethylation [22, 23], consistent with observations made in cells containing hypomorphic mutations in *DNMT1* [139, 140]. One explanation for this limited demethylation is that de novo DNMTs compensate for the reduction of DNMT1 activity [139]. Another possibility is that DNMT1 loss triggers a checkpoint pathway (Fig. 1.1) to block DNA replication, preventing loss of DNA methylation in an attempt to maintain genome stability. Double knockdown of DNMT1 and ATR does indeed induce global DNA demethylation, whereas single knockdowns of either DNMT1 or ATR do not, implying that the arrest of DNA replication activated by ATR signaling following DNMT1 depletion prevents loss of DNA methylation and that blocking this

response results in global loss of DNA methylation [23]. Taken together, it appears that reduction of DNMT1 levels activates ATR signaling to block DNA replication in a DNA methylation-independent manner (Fig. 1.1). How this response to DNMT1 reduction is initiated, however, is still uncertain. It is possible that removal of DNMT1 from replication forks disrupts fork progression and eventually results in DSBs that elicit checkpoint signaling (Fig. 1.1). Alternatively, the presence of low levels of hemimethylated DNA due to the absence of DNMT1 may trigger this response (Fig. 1.1).

Complete inactivation of DNMT1 via genetic mechanisms also activates the DDR and causes genomic demethylation. The degree of demethylation, however, varies greatly depending on cellular context, ranging from 20% loss in human cancer cells [141] to 90% loss of genomic methylation in murine ES cells [7, 8]. As the principal enzyme responsible for maintaining DNA methylation, DNMT1 is essential for embryonic development and cell survival. Disruption of *Dnmt1* in mice results in loss of 90% of genomic methylation and embryonic lethality [7, 8]. Murine ES cells deficient for *Dnmt1* die when introduced to differentiate [7], mouse fibroblasts die within 2–4 cell divisions after conditional deletion in *Dnmt1* [123], and the human colon cancer cell line HCT116 undergoes marked apoptosis and cell death within one cell division if *DNMT1* is completely inactivated by cre-mediated conditional knockout [141, 142]. Notably, complete inactivation of DNMT1 triggers the DDR before cells die [141]. Deletion of *DNMT1* activates p53 [123, 141], a target of ATM whose phosphorylation correlates with accumulation of p53 in response to DNA damage [143]. Disruption of both alleles of *DNMT1* leads to activation of the G2/M checkpoint and G2 arrest, as verified by the presence of phosphorylated ATM and γH2A.X at discrete nuclear DNA damage foci [141]. Further support for checkpoint activation comes from the finding that treatment of cells with an ATM/ATR inhibitor, caffeine, facilitates mitotic entry and cell death in *DNMT1* null cells [141]. Most of these cells, however, eventually escape G2 arrest and reenter interphase with their unrepaired DNA, resulting in severe chromosomal and mitotic abnormalities (mitotic catastrophe) [141]. Thus far, the mechanisms by which DNMT1 inactivation leads to activation of DNA damage repair remains elusive. In the complete absence of DNMT1, DNA may become more fragile owing to reduced methylation and/or defective chromatin structure in critical regions of the genome, leading to activation of DNA damage signaling (Fig. 1.1) [142]. Alternatively, the accumulation of hemimethylated DNA in *DNMT1* mutant cells may be recognized as damage and trigger the damage response (Fig. 1.1). Both of these possibilities are consistent with the observation that agents that affect overall chromatin structure without damaging DNA also activate ATM [144]. Nonetheless, it cannot be excluded that oncogene activation or gene mutations initiate the DDR, as Dnmt1-deficient ES cells exhibit significantly increased mutation rates, particularly in the form of deletions and mutations [145].

Recruitment of DNMT1 to sites of DNA damage has been observed by our laboratory [21, 146] and others [24], providing compelling evidence to support the notion that DNMT1 is directly involved in DNA damage repair (Fig. 1.1).

Immediately after laser microirradiation-induced DSBs, an accumulation of DNMT1 and PCNA occurs at the damage sites in S and non-S phase cells, colocalizing with γH2A.X—a marker of DSBs. Recruitment of DNMT1 to damage sites is dependent on its interaction with PCNA through its PCNA-binding domain (PBD) [21, 24], but is independent of its catalytic activity [21]. In addition to PCNA, DNMT1 also interacts with other components of the DNA damage machinery including CHK1 [21, 146] and the 9-1-1 complex [21]. PCNA, along with CHK1 and 9-1-1, is essential for DNMT1's recruitment to DNA damage sites. After recruitment to damaged regions, DNMT1 modulates the rate of ATR signaling and is essential for suppressing abnormal activation of the DDR in the absence of exogenous damage [21]. Taken together, these data have revealed a direct link between DNMT1 and the DNA damage repair process.

PCNA mediates recruitment of DNMT1, not only to DNA replication sites, but also to DNA damage sites. The DNMT1–PCNA interaction implies that the role of DNMT might be to restore epigenetic information after damage repair. However, recent studies demonstrate that this interaction is not essential for maintaining DNA methylation [5, 147]. Furthermore, the observation [21] that DNMT1 is very rapidly recruited and retained only transiently, likely before resynthesis is completed, suggest that genomic methylation is not the main function of DNMT1 at these sites, at least in the early part of the DDR. The recruitment kinetics of WT *DNMT1* and *DNMT1* with a point mutation in the catalytic domain are almost identical [21]. CHK1/CHK2 activation and γH2A.X foci formation induced by DNMT1 deficiency are rescued by expression of a catalytically inactive form of DNMT1 [23]. Therefore, although the possibility that DNMT1 participates in the restoration of DNA methylation patterns during damage repair cannot be excluded, it seems more likely that DNMT1 functions in sensing and/or mobilizing the response to certain forms of DNA damage (Fig. 1.1).

In summary, both DNMTs and DNA damage repair systems have evolved to maintain genomic integrity and disruption of these pathways contributes to the development of cancer [19]. Therefore, we have examined and outlined the interaction of DNMTs and DNA methylation with DNA damage repair systems and have discussed possible mechanisms for how the two systems may function coordinately to deal with DNA damage. Promoter methylation, catalyzed by DNMTs, plays an established role in silencing key genes in multiple DNA damage repair pathways; inactivation of these pathways may predispose to a large array of tumors [20]. These findings are consistent with observations that TSGs are frequently silenced via epigenetic mechanisms in cancer cells. Unexpectedly perhaps, more recent observations strongly suggest that DNMTs, particular DNMT1, are directly involved in DNA damage repair systems via what is likely to be a DNA-methylation-independent mechanism [17, 21–23, 141]. The exact nature of the links between the DNMTs, DNA methylation, and DNA damage repair systems is complex and remains to be further investigated. A more thorough understanding of these links will not only help dissect the mechanisms of tumor development, but also identify new antitumor targets and therapeutic strategies.

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References

1. Robertson KD (2005) DNA methylation and human disease. Nat Rev Genet 6(8):597–610
2. Kareta MS, Botello ZM, Ennis JJ, Chou C, Chedin F (2006) Reconstitution and mechanism of the stimulation of de novo methylation by human DNMT3L. J Biol Chem 281(36): 25893–25902
3. Okano M, Bell DW, Haber DA, Li E (1999) DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell 99(3):247–257
4. Okano M, Xie S, Li E (1998) Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases. Nat Genet 19(3):219–220
5. Egger G, Jeong S, Escobar SG et al (2006) Identification of DNMT1 (DNA methyltransferase 1) hypomorphs in somatic knockouts suggests an essential role for DNMT1 in cell survival. Proc Natl Acad Sci USA 103(38):14080–14085
6. Riggs AD, Xiong Z (2004) Methylation and epigenetic fidelity. Proc Natl Acad Sci USA 101(1):4–5
7. Li E, Bestor TH, Jaenisch R (1992) Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. Cell 69(6):915–926
8. Lei H, Oh SP, Okano M et al (1996) De novo DNA cytosine methyltransferase activities in mouse embryonic stem cells. Development 122(10):3195–3205
9. Li E (2002) Chromatin modification and epigenetic reprogramming in mammalian development. Nat Rev Genet 3(9):662–673
10. Yoder JA, Walsh CP, Bestor TH (1997) Cytosine methylation and the ecology of intragenomic parasites. Trends Genet 13(8):335–340
11. Gonzalo S, Jaco I, Fraga MF et al (2006) DNA methyltransferases control telomere length and telomere recombination in mammalian cells. Nat Cell Biol 8(4):416–424
12. Xu GL, Bestor TH, Bourc'his D et al (1999) Chromosome instability and immunodeficiency syndrome caused by mutations in a DNA methyltransferase gene. Nature 402(6758): 187–191
13. Guo G, Wang W, Bradley A (2004) Mismatch repair genes identified using genetic screens in Blm-deficient embryonic stem cells. Nature 429(6994):891–895
14. Kim M, Trinh BN, Long TI, Oghamian S, Laird PW (2004) Dnmt1 deficiency leads to enhanced microsatellite instability in mouse embryonic stem cells. Nucleic Acids Res 32(19): 5742–5749
15. Wang KY, James Shen CK (2004) DNA methyltransferase Dnmt1 and mismatch repair. Oncogene 23(47):7898–7902
16. Dion V, Lin Y, Hubert L Jr, Waterland RA, Wilson JH (2008) Dnmt1 deficiency promotes CAG repeat expansion in the mouse germline. Hum Mol Genet 17(9):1306–1317
17. Loughery JE, Dunne PD, O'Neill KM, Meehan RR, McDaid JR, Walsh CP (2011) DNMT1 deficiency triggers mismatch repair defects in human cells through depletion of repair protein levels in a process involving the DNA damage response. Hum Mol Genet 20(16): 3241–3255
18. Karpf AR, Matsui S (2005) Genetic disruption of cytosine DNA methyltransferase enzymes induces chromosomal instability in human cancer cells. Cancer Res 65(19):8635–8639
19. Jackson SP, Bartek J (2009) The DNA-damage response in human biology and disease. Nature 461(7267):1071–1078

20. Jacinto FV, Esteller M (2007) Mutator pathways unleashed by epigenetic silencing in human cancer. Mutagenesis 22(4):247–253
21. Ha K, Lee GE, Palii SS et al (2011) Rapid and transient recruitment of DNMT1 to DNA double-strand breaks is mediated by its interaction with multiple components of the DNA damage response machinery. Hum Mol Genet 20(1):126–140
22. Milutinovic S, Zhuang Q, Niveleau A, Szyf M (2003) Epigenomic stress response. Knockdown of DNA methyltransferase 1 triggers an intra-S-phase arrest of DNA replication and induction of stress response genes. J Biol Chem 278(17):14985–14995
23. Unterberger A, Andrews SD, Weaver IC, Szyf M (2006) DNA methyltransferase 1 knockdown activates a replication stress checkpoint. Mol Cell Biol 26(20):7575–7586
24. Mortusewicz O, Schermelleh L, Walter J, Cardoso MC, Leonhardt H (2005) Recruitment of DNA methyltransferase I to DNA repair sites. Proc Natl Acad Sci USA 102(25):8905–8909
25. Laghi L, Bianchi P, Malesci A (2008) Differences and evolution of the methods for the assessment of microsatellite instability. Oncogene 27(49):6313–6321
26. Kunkel TA, Erie DA (2005) DNA mismatch repair. Annu Rev Biochem 74:681–710
27. Raschle M, Dufner P, Marra G, Jiricny J (2002) Mutations within the hMLH1 and hPMS2 subunits of the human MutLalpha mismatch repair factor affect its ATPase activity, but not its ability to interact with hMutSalpha. J Biol Chem 277(24):21810–21820
28. Kantelinen J, Kansikas M, Korhonen MK et al (2010) MutSbeta exceeds MutSalpha in dinucleotide loop repair. Br J Cancer 102(6):1068–1073
29. Thibodeau SN, French AJ, Cunningham JM et al (1998) Microsatellite instability in colorectal cancer: different mutator phenotypes and the principal involvement of hMLH1. Cancer Res 58(8):1713–1718
30. Viswanathan M, Tsuchida N, Shanmugam G (2003) Promoter hypermethylation profile of tumor-associated genes p16, p15, hMLH1, MGMT and E-cadherin in oral squamous cell carcinoma. Int J Cancer 105(1):41–46
31. Kim HG, Lee S, Kim DY et al (2010) Aberrant methylation of DNA mismatch repair genes in elderly patients with sporadic gastric carcinoma: A comparison with younger patients. J Surg Oncol 101(1):28–35
32. Brucher BL, Geddert H, Langner C et al (2006) Hypermethylation of hMLH1, HPP1, p14(ARF), p16(INK4A) and APC in primary adenocarcinomas of the small bowel. Int J Cancer 119(6):1298–1302
33. Wang YC, Lu YP, Tseng RC et al (2003) Inactivation of hMLH1 and hMSH2 by promoter methylation in primary non-small cell lung tumors and matched sputum samples. J Clin Invest 111(6):887–895
34. Murphy MA, Wentzensen N (2011) Frequency of mismatch repair deficiency in ovarian cancer: a systematic review. Int J Cancer 129:1914–1922
35. Seedhouse CH, Das-Gupta EP, Russell NH (2003) Methylation of the hMLH1 promoter and its association with microsatellite instability in acute myeloid leukemia. Leukemia 17(1):83–88
36. Lenz G, Hutter G, Hiddemann W, Dreyling M (2004) Promoter methylation and expression of DNA repair genes hMLH1 and MGMT in acute myeloid leukemia. Ann Hematol 83(10):628–633
37. Nomdedeu JF, Perea G, Estivill C et al (2005) Microsatellite instability is not an uncommon finding in adult de novo acute myeloid leukemia. Ann Hematol 84(6):368–375
38. Tawfik HM, El-Maqsoud NM, Hak BH, El-Sherbiny YM (2011) Head and neck squamous cell carcinoma: mismatch repair immunohistochemistry and promoter hypermethylation of hMLH1 gene. Am J Otolaryngol 32(6):528–36
39. Lahtz C, Pfeifer GP (2011) Epigenetic changes of DNA repair genes in cancer. J Mol Cell Biol 3(1):51–58
40. Valle L, Carbonell P, Fernandez V et al (2007) MLH1 germline epimutations in selected patients with early-onset non-polyposis colorectal cancer. Clin Genet 71(3):232–237
41. Gazzoli I, Loda M, Garber J, Syngal S, Kolodner RD (2002) A hereditary nonpolyposis colorectal carcinoma case associated with hypermethylation of the MLH1 gene in normal tissue

and loss of heterozygosity of the unmethylated allele in the resulting microsatellite instability-high tumor. Cancer Res 62(14):3925–3928
42. Herman JG, Umar A, Polyak K et al (1998) Incidence and functional consequences of hMLH1 promoter hypermethylation in colorectal carcinoma. Proc Natl Acad Sci USA 95(12):6870–6875
43. Nakagawa H, Nuovo GJ, Zervos EE et al (2001) Age-related hypermethylation of the 5′ region of MLH1 in normal colonic mucosa is associated with microsatellite-unstable colorectal cancer development. Cancer Res 61(19):6991–6995
44. Kuismanen SA, Holmberg MT, Salovaara R et al (1999) Epigenetic phenotypes distinguish microsatellite-stable and -unstable colorectal cancers. Proc Natl Acad Sci USA 96(22): 12661–12666
45. Wheeler JM, Beck NE, Kim HC, Tomlinson IP, Mortensen NJ, Bodmer WF (1999) Mechanisms of inactivation of mismatch repair genes in human colorectal cancer cell lines: the predominant role of hMLH1. Proc Natl Acad Sci USA 96(18):10296–10301
46. Auclair J, Vaissiere T, Desseigne F et al (2011) Intensity-dependent constitutional MLH1 promoter methylation leads to early onset of colorectal cancer by affecting both alleles. Genes Chromosomes Cancer 50(3):178–185
47. Zhang H, Zhang S, Cui J, Zhang A, Shen L, Yu H (2008) Expression and promoter methylation status of mismatch repair gene hMLH1 and hMSH2 in epithelial ovarian cancer. Aust N Z J Obstet Gynaecol 48(5):505–509
48. Vlaykova T, Mitkova A, Stancheva G et al (2011) Microsatellite instability and promoter hypermethylation of MLH1 and MSH2 in patients with sporadic colorectal cancer. J BUON 16(2):265–273
49. Nagasaka T, Rhees J, Kloor M et al (2010) Somatic hypermethylation of MSH2 is a frequent event in Lynch Syndrome colorectal cancers. Cancer Res 70(8):3098–3108
50. Ligtenberg MJ, Kuiper RP, Chan TL et al (2009) Heritable somatic methylation and inactivation of MSH2 in families with Lynch syndrome due to deletion of the 3′ exons of TACSTD1. Nat Genet 41(1):112–117
51. Moelans CB, Verschuur-Maes AH, van Diest PJ (2011) Frequent promoter hypermethylation of BRCA2, CDH13, MSH6, PAX5, PAX6 and WT1 in ductal carcinoma in situ and invasive breast cancer. J Pathol 225(2):222–231
52. David SS, O'Shea VL, Kundu S (2007) Base-excision repair of oxidative DNA damage. Nature 447(7147):941–950
53. Peng B, Hurt EM, Hodge DR, Thomas SB, Farrar WL (2006) DNA hypermethylation and partial gene silencing of human thymine- DNA glycosylase in multiple myeloma cell lines. Epigenetics 1(3):138–145
54. Howard JH, Frolov A, Tzeng CW et al (2009) Epigenetic downregulation of the DNA repair gene MED1/MBD4 in colorectal and ovarian cancer. Cancer Biol Ther 8(1):94–100
55. Guan H, Ji M, Hou P et al (2008) Hypermethylation of the DNA mismatch repair gene hMLH1 and its association with lymph node metastasis and T1799A BRAF mutation in patients with papillary thyroid cancer. Cancer 113(2):247–255
56. Hoeijmakers JH (2001) Genome maintenance mechanisms for preventing cancer. Nature 411(6835):366–374
57. Yang J, Xu Z, Li J et al (2010) XPC epigenetic silence coupled with p53 alteration has a significant impact on bladder cancer outcome. J Urol 184(1):336–343
58. Chen HY, Shao CJ, Chen FR, Kwan AL, Chen ZP (2010) Role of ERCC1 promoter hypermethylation in drug resistance to cisplatin in human gliomas. Int J Cancer 126(8):1944–1954
59. Liu WB, Ao L, Cui ZH et al (2011) Molecular analysis of DNA repair gene methylation and protein expression during chemical-induced rat lung carcinogenesis. Biochem Biophys Res Commun 408(4):595–601
60. Jiang J, Liang X, Zhou X et al (2010) DNA repair gene X-ray repair cross complementing group 1 Arg194Trp polymorphism on the risk of lung cancer: a meta-analysis on 22 studies. J Thorac Oncol 5(11):1741–1747

61. Wang P, Tang JT, Peng YS, Chen XY, Zhang YJ, Fang JY (2010) XRCC1 downregulated through promoter hypermethylation is involved in human gastric carcinogenesis. J Dig Dis 11(6):343–351
62. Peng B, Hodge DR, Thomas SB et al (2005) Epigenetic silencing of the human nucleotide excision repair gene, hHR23B, in interleukin-6-responsive multiple myeloma KAS-6/1 cells. J Biol Chem 280(6):4182–4187
63. Moynahan ME, Jasin M (2010) Mitotic homologous recombination maintains genomic stability and suppresses tumorigenesis. Nat Rev Mol Cell Biol 11(3):196–207
64. Mazon G, Mimitou EP, Symington LS. SnapShot: homologous recombination in DNA double-strand break repair. Cell. 2010;142(4):646, 646.e1.
65. Esteller M, Silva JM, Dominguez G et al (2000) Promoter hypermethylation and BRCA1 inactivation in sporadic breast and ovarian tumors. J Natl Cancer Inst 92(7):564–569
66. Esteller M, Risques RA, Toyota M et al (2001) Promoter hypermethylation of the DNA repair gene O(6)-methylguanine-DNA methyltransferase is associated with the presence of G:C to A:T transition mutations in p53 in human colorectal tumorigenesis. Cancer Res 61(12): 4689–4692
67. Baldwin RL, Nemeth E, Tran H et al (2000) BRCA1 promoter region hypermethylation in ovarian carcinoma: a population-based study. Cancer Res 60(19):5329–5333
68. Bernal C, Vargas M, Ossandon F et al (2008) DNA methylation profile in diffuse type gastric cancer: evidence for hypermethylation of the BRCA1 promoter region in early-onset gastric carcinogenesis. Biol Res 41(3):303–315
69. Cabello MJ, Grau L, Franco N et al (2011) Multiplexed methylation profiles of tumor suppressor genes in bladder cancer. J Mol Diagn 13(1):29–40
70. Lee MN, Tseng RC, Hsu HS et al (2007) Epigenetic inactivation of the chromosomal stability control genes BRCA1, BRCA2, and XRCC5 in non-small cell lung cancer. Clin Cancer Res 13(3):832–838
71. Esteller M, Fraga MF, Guo M et al (2001) DNA methylation patterns in hereditary human cancers mimic sporadic tumorigenesis. Hum Mol Genet 10(26):3001–3007
72. Ludwig T, Chapman DL, Papaioannou VE, Efstratiadis A (1997) Targeted mutations of breast cancer susceptibility gene homologs in mice: lethal phenotypes of Brca1, Brca2, Brca1/Brca2, Brca1/p53, and Brca2/p53 nullizygous embryos. Genes Dev 11(10): 1226–1241
73. Kee Y, D'Andrea AD (2010) Expanded roles of the Fanconi anemia pathway in preserving genomic stability. Genes Dev 24(16):1680–1694
74. Meier D, Schindler D (2011) Fanconi anemia core complex gene promoters harbor conserved transcription regulatory elements. PLoS One 6(8):e22911
75. Hess CJ, Ameziane N, Schuurhuis GJ et al (2008) Hypermethylation of the FANCC and FANCL promoter regions in sporadic acute leukaemia. Cell Oncol 30(4):299–306
76. Marsit CJ, Liu M, Nelson HH, Posner M, Suzuki M, Kelsey KT (2004) Inactivation of the Fanconi anemia/BRCA pathway in lung and oral cancers: implications for treatment and survival. Oncogene 23(4):1000–1004
77. Narayan G, Arias-Pulido H, Nandula SV et al (2004) Promoter hypermethylation of FANCF: disruption of Fanconi anemia-BRCA pathway in cervical cancer. Cancer Res 64(9): 2994–2997
78. Lim SL, Smith P, Syed N et al (2008) Promoter hypermethylation of FANCF and outcome in advanced ovarian cancer. Br J Cancer 98(8):1452–1456
79. Pegg AE, Dolan ME, Moschel RC (1995) Structure, function, and inhibition of O6-alkylguanine-DNA alkyltransferase. Prog Nucleic Acid Res Mol Biol 51:167–223
80. Gerson SL (2004) MGMT: its role in cancer aetiology and cancer therapeutics. Nat Rev Cancer 4(4):296–307
81. Esteller M, Hamilton SR, Burger PC, Baylin SB, Herman JG (1999) Inactivation of the DNA repair gene O6-methylguanine-DNA methyltransferase by promoter hypermethylation is a common event in primary human neoplasia. Cancer Res 59(4):793–797

82. Weller M, Stupp R, Reifenberger G et al (2010) MGMT promoter methylation in malignant gliomas: ready for personalized medicine? Nat Rev Neurol 6(1):39–51
83. Whitehall VL, Walsh MD, Young J, Leggett BA, Jass JR (2001) Methylation of O-6-methylguanine DNA methyltransferase characterizes a subset of colorectal cancer with low-level DNA microsatellite instability. Cancer Res 61(3):827–830
84. Park TJ, Han SU, Cho YK, Paik WK, Kim YB, Lim IK (2001) Methylation of O(6)-methylguanine-DNA methyltransferase gene is associated significantly with K-ras mutation, lymph node invasion, tumor staging, and disease free survival in patients with gastric carcinoma. Cancer 92(11):2760–2768
85. Greenblatt MS, Bennett WP, Hollstein M, Harris CC (1994) Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. Cancer Res 54(18): 4855–4878
86. Zhang YJ, Chen Y, Ahsan H et al (2003) Inactivation of the DNA repair gene O6-methylguanine-DNA methyltransferase by promoter hypermethylation and its relationship to aflatoxin B1-DNA adducts and p53 mutation in hepatocellular carcinoma. Int J Cancer 103(4): 440–444
87. Wolf P, Hu YC, Doffek K, Sidransky D, Ahrendt SA (2001) O(6)-Methylguanine-DNA methyltransferase promoter hypermethylation shifts the p53 mutational spectrum in non-small cell lung cancer. Cancer Res 61(22):8113–8117
88. Zhang L, Lu W, Miao X, Xing D, Tan W, Lin D (2003) Inactivation of DNA repair gene O6-methylguanine-DNA methyltransferase by promoter hypermethylation and its relation to p53 mutations in esophageal squamous cell carcinoma. Carcinogenesis 24(6):1039–1044
89. Nakamura M, Watanabe T, Yonekawa Y, Kleihues P, Ohgaki H (2001) Promoter methylation of the DNA repair gene MGMT in astrocytomas is frequently associated with G:C –> A:T mutations of the TP53 tumor suppressor gene. Carcinogenesis 22(10):1715–1719
90. Sabharwal A, Middleton MR (2006) Exploiting the role of O6-methylguanine-DNA-methyltransferase (MGMT) in cancer therapy. Curr Opin Pharmacol 6(4):355–363
91. Kitano K, Kim SY, Hakoshima T (2010) Structural basis for DNA strand separation by the unconventional winged-helix domain of RecQ helicase WRN. Structure 18(2):177–187
92. Opresko PL (2008) Telomere ResQue and preservation—roles for the Werner syndrome protein and other RecQ helicases. Mech Ageing Dev 129(1–2):79–90
93. Agrelo R, Cheng WH, Setien F et al (2006) Epigenetic inactivation of the premature aging Werner syndrome gene in human cancer. Proc Natl Acad Sci USA 103(23):8822–8827
94. Kawasaki T, Ohnishi M, Suemoto Y et al (2008) WRN promoter methylation possibly connects mucinous differentiation, microsatellite instability and CpG island methylator phenotype in colorectal cancer. Mod Pathol 21(2):150–158
95. Smith JA, Ndoye AM, Geary K, Lisanti MP, Igoucheva O, Daniel R (2010) A role for the Werner syndrome protein in epigenetic inactivation of the pluripotency factor Oct4. Aging Cell 9(4):580–591
96. Polo SE, Jackson SP (2011) Dynamics of DNA damage response proteins at DNA breaks: a focus on protein modifications. Genes Dev 25(5):409–433
97. Harrison JC, Haber JE (2006) Surviving the breakup: the DNA damage checkpoint. Annu Rev Genet 40:209–235
98. Lazzaro F, Giannattasio M, Puddu F et al (2009) Checkpoint mechanisms at the intersection between DNA damage and repair. DNA Repair (Amst) 8(9):1055–1067
99. Linding R, Jensen LJ, Ostheimer GJ et al (2007) Systematic discovery of in vivo phosphorylation networks. Cell 129(7):1415–1426
100. Matsuoka S, Ballif BA, Smogorzewska A et al (2007) ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage. Science 316(5828): 1160–1166
101. Kim WJ, Vo QN, Shrivastav M, Lataxes TA, Brown KD (2002) Aberrant methylation of the ATM promoter correlates with increased radiosensitivity in a human colorectal tumor cell line. Oncogene 21(24):3864–3871

102. Vo QN, Kim WJ, Cvitanovic L, Boudreau DA, Ginzinger DG, Brown KD (2004) The ATM gene is a target for epigenetic silencing in locally advanced breast cancer. Oncogene 23(58): 9432–9437
103. Ai L, Vo QN, Zuo C et al (2004) Ataxia-telangiectasia-mutated (ATM) gene in head and neck squamous cell carcinoma: promoter hypermethylation with clinical correlation in 100 cases. Cancer Epidemiol Biomarkers Prev 13(1):150–156
104. Bartek J, Falck J, Lukas J (2001) CHK2 kinase—a busy messenger. Nat Rev Mol Cell Biol 2(12):877–886
105. Bell DW, Varley JM, Szydlo TE et al (1999) Heterozygous germ line hCHK2 mutations in Li-Fraumeni syndrome. Science 286(5449):2528–2531
106. Bartek J, Lukas J (2003) Chk1 and Chk2 kinases in checkpoint control and cancer. Cancer Cell 3(5):421–429
107. Kato N, Fujimoto H, Yoda A et al (2004) Regulation of Chk2 gene expression in lymphoid malignancies: involvement of epigenetic mechanisms in Hodgkin's lymphoma cell lines. Cell Death Differ 11(Suppl 2):S153–161
108. Kim DS, Kim MJ, Lee JY et al (2009) Epigenetic inactivation of checkpoint kinase 2 gene in non-small cell lung cancer and its relationship with clinicopathological features. Lung Cancer 65(2):247–250
109. Wang H, Wang S, Shen L et al (2010) Chk2 down-regulation by promoter hypermethylation in human bulk gliomas. Life Sci 86(5–6):185–191
110. Sullivan A, Yuille M, Repellin C et al (2002) Concomitant inactivation of p53 and Chk2 in breast cancer. Oncogene 21(9):1316–1324
111. Williams LH, Choong D, Johnson SA, Campbell IG (2006) Genetic and epigenetic analysis of CHEK2 in sporadic breast, colon, and ovarian cancers. Clin Cancer Res 12(23):6967–6972
112. Jascur T, Boland CR (2006) Structure and function of the components of the human DNA mismatch repair system. Int J Cancer 119(9):2030–2035
113. Loeb LA, Loeb KR, Anderson JP (2003) Multiple mutations and cancer. Proc Natl Acad Sci USA 100(3):776–781
114. Lin Y, Wilson JH (2009) Diverse effects of individual mismatch repair components on transcription-induced CAG repeat instability in human cells. DNA Repair (Amst) 8(8):878–885
115. Lin Y, Dion V, Wilson JH (2006) Transcription promotes contraction of CAG repeat tracts in human cells. Nat Struct Mol Biol 13(2):179–180
116. Karran P (2006) Thiopurines, DNA damage, DNA repair and therapy-related cancer. Br Med Bull 79–80:153–170
117. Bellacosa A, Cicchillitti L, Schepis F et al (1999) MED1, a novel human methyl-CpG-binding endonuclease, interacts with DNA mismatch repair protein MLH1. Proc Natl Acad Sci USA 96(7):3969–3974
118. Ruzov A, Shorning B, Mortusewicz O, Dunican DS, Leonhardt H, Meehan RR (2009) MBD4 and MLH1 are required for apoptotic induction in xDNMT1-depleted embryos. Development 136(13):2277–2286
119. Cortellino S, Turner D, Masciullo V et al (2003) The base excision repair enzyme MED1 mediates DNA damage response to antitumor drugs and is associated with mismatch repair system integrity. Proc Natl Acad Sci USA 100(25):15071–15076
120. Boland MJ, Christman JK (2008) Characterization of Dnmt3b:thymine-DNA glycosylase interaction and stimulation of thymine glycosylase-mediated repair by DNA methyltransferase(s) and RNA. J Mol Biol 379(3):492–504
121. Li YQ, Zhou PZ, Zheng XD, Walsh CP, Xu GL (2007) Association of Dnmt3a and thymine DNA glycosylase links DNA methylation with base-excision repair. Nucleic Acids Res 35(2): 390–400
122. Kimura H, Shiota K (2003) Methyl-CpG-binding protein, MeCP2, is a target molecule for maintenance DNA methyltransferase, Dnmt1. J Biol Chem 278(7):4806–4812
123. Jackson-Grusby L, Beard C, Possemato R et al (2001) Loss of genomic methylation causes p53-dependent apoptosis and epigenetic deregulation. Nat Genet 27(1):31–39

124. Flores-Rozas H, Clark D, Kolodner RD (2000) Proliferating cell nuclear antigen and Msh2p-Msh6p interact to form an active mispair recognition complex. Nat Genet 26(3):375–378
125. Iyer RR, Pohlhaus TJ, Chen S et al (2008) The MutSalpha-proliferating cell nuclear antigen interaction in human DNA mismatch repair. J Biol Chem 283(19):13310–13319
126. Plotz G, Welsch C, Giron-Monzon L et al (2006) Mutations in the MutSalpha interaction interface of MLH1 can abolish DNA mismatch repair. Nucleic Acids Res 34(22):6574–6586
127. Kleczkowska HE, Marra G, Lettieri T, Jiricny J (2001) hMSH3 and hMSH6 interact with PCNA and colocalize with it to replication foci. Genes Dev 15(6):724–736
128. Umar A, Buermeyer AB, Simon JA et al (1996) Requirement for PCNA in DNA mismatch repair at a step preceding DNA resynthesis. Cell 87(1):65–73
129. Chen F, Arseven OK, Cryns VL (2004) Proteolysis of the mismatch repair protein MLH1 by caspase-3 promotes DNA damage-induced apoptosis. J Biol Chem 279(26):27542–27548
130. Kim MY, Zhang T, Kraus WL (2005) Poly(ADP-ribosyl)ation by PARP-1: 'PAR-laying' NAD+ into a nuclear signal. Genes Dev 19(17):1951–1967
131. Bischof O, Galande S, Farzaneh F, Kohwi-Shigematsu T, Campisi J (2001) Selective cleavage of BLM, the bloom syndrome protein, during apoptotic cell death. J Biol Chem 276(15): 12068–12075
132. Wang J, Pabla N, Wang CY, Wang W, Schoenlein PV, Dong Z (2006) Caspase-mediated cleavage of ATM during cisplatin-induced tubular cell apoptosis: inactivation of its kinase activity toward p53. Am J Physiol Renal Physiol 291(6):F1300–1307
133. Knox JD, Araujo FD, Bigey P et al (2000) Inhibition of DNA methyltransferase inhibits DNA replication. J Biol Chem 275(24):17986–17990
134. Bartek J, Lukas J (2001) Mammalian G1- and S-phase checkpoints in response to DNA damage. Curr Opin Cell Biol 13(6):738–747
135. Kastan MB, Lim DS (2000) The many substrates and functions of ATM. Nat Rev Mol Cell Biol 1(3):179–186
136. Falck J, Mailand N, Syljuasen RG, Bartek J, Lukas J (2001) The ATM-Chk2-Cdc25A checkpoint pathway guards against radioresistant DNA synthesis. Nature 410(6830):842–847
137. Maser RS, Mirzoeva OK, Wells J et al (2001) Mre11 complex and DNA replication: linkage to E2F and sites of DNA synthesis. Mol Cell Biol 21(17):6006–6016
138. Hardy CF (1997) Identification of Cdc45p, an essential factor required for DNA replication. Gene 187(2):239–246
139. Rhee I, Bachman KE, Park BH et al (2002) DNMT1 and DNMT3b cooperate to silence genes in human cancer cells. Nature 416(6880):552–556
140. Ting AH, Jair KW, Suzuki H, Yen RW, Baylin SB, Schuebel KE (2004) CpG island hypermethylation is maintained in human colorectal cancer cells after RNAi-mediated depletion of DNMT1. Nat Genet 36(6):582–584
141. Chen T, Hevi S, Gay F et al (2007) Complete inactivation of DNMT1 leads to mitotic catastrophe in human cancer cells. Nat Genet 39(3):391–396
142. Brown KD, Robertson KD (2007) DNMT1 knockout delivers a strong blow to genome stability and cell viability. Nat Genet 39(3):289–290
143. Canman CE, Lim DS, Cimprich KA et al (1998) Activation of the ATM kinase by ionizing radiation and phosphorylation of p53. Science 281(5383):1677–1679
144. Bakkenist CJ, Kastan MB (2003) DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. Nature 421(6922):499–506
145. Chen RZ, Pettersson U, Beard C, Jackson-Grusby L, Jaenisch R (1998) DNA hypomethylation leads to elevated mutation rates. Nature 395(6697):89–93
146. Palii SS, Van Emburgh BO, Sankpal UT, Brown KD, Robertson KD (2008) DNA methylation inhibitor 5-Aza-2′-deoxycytidine induces reversible genome-wide DNA damage that is distinctly influenced by DNA methyltransferases 1 and 3B. Mol Cell Biol 28(2):752–771
147. Spada F, Haemmer A, Kuch D et al (2007) DNMT1 but not its interaction with the replication machinery is required for maintenance of DNA methylation in human cells. J Cell Biol 176(5):565–571

Chapter 2
DNA Hypomethylation and Hemimethylation in Cancer

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Abstract In contrast to earlier views that there was much compartmentalization of the types of sequences subject to cancer-linked changes in DNA epigenetics, it is now clear that both cancer-associated DNA hypomethylation and hypermethylation are found throughout the genome. The hypermethylation includes promoters of tumor suppressor genes whose expression becomes repressed, thereby facilitating cancer formation. How hypomethylation contributes to carcinogenesis has been less clear. Recent insights into tissue-specific intra- and intergenic methylation and into cancer methylomes suggest that some of the DNA hypomethylation associated with cancers is likely to aid in tumor formation and progression by many different pathways, including effects on transcription in *cis*. Cancer-associated loss of DNA methylation from intergenic enhancers, promoter regions, silencers, and chromatin boundary elements may alter transcription rates. In addition, cancer-associated intragenic DNA hypomethylation might modulate alternative promoter usage,

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production of intragenic noncoding RNA transcripts, cotranscriptional splicing, and transcription initiation or elongation. Initial studies of hemimethylation of DNA in cancer and many new studies of DNA demethylation in normal tissues suggest that active demethylation with spreading of hypomethylation can explain much of the cancer-associated DNA hypomethylation. The new discoveries that genomic 5-hydroxymethylcytosine is an intermediate in DNA demethylation, a base with its own functionality, and a modified base that, like 5-methylcytosine, exhibits cancer-associated losses, suggest that both decreased hydroxymethylation and decreased methylation of DNA play important roles in carcinogenesis.

2.1 Introduction

Altered methylation of DNA in human cancers was first described as overall genomic hypomethylation in various cancers vs. a wide variety of normal tissues [1] and as hypomethylation of a few gene regions in colon adenocarcinomas vs. normal colonic epithelium [2]. Almost all types of cancers exhibit both hypermethylation of some DNA sequences and hypomethylation of others relative to appropriate controls that account for the tissue specificity of DNA methylation [3]. The cancer-associated hypermethylation and hypomethylation of the genome are generally independent of each other [4, 5]. Until recently, it appeared that cancer-specific changes in DNA methylation were usually hypermethylation of unique gene regions and hypomethylation of DNA repeats, albeit with many notable exceptions [6–11]. Deep sequencing of the genome has revealed far greater size and complexity to the transcriptome than previously appreciated [12]. Similarly, recent whole-genome analysis of the cancer methylome demonstrates that there is much more cancer-linked hypomethylation of unique gene sequences and hypermethylation of repeated sequences than previously found, although there are differences in the frequency with which subsets of sequences undergo hypo- or hypermethylation [13–18].

This chapter reviews new insights into genome-wide DNA and chromatin epigenetics in normal cell populations as well as in cancers [19–29]. Recent studies are drawing attention to previously unsuspected roles of epigenetic marks in the body of genes as well as at promoters and intergenic transcription control regions. These findings are likely to be relevant to the biological impact of cancer-associated DNA hypomethylation. In addition to effects on normal gene expression, cancer-associated DNA hypomethylation probably favors oncogenesis by enhancing recombination [30–33]; occasionally activating a small number of endogenous retroviral elements [34, 35]; altering the intranuclear positioning of chromatin; and modulating the sequestration transcription factors at tandem DNA repeats, as reviewed previously [3, 6]. In addition, the little-studied area of DNA hemimethylation in cancer is discussed in this chapter in the context of our growing understanding of pathways for the conversion of genomic 5-methylcytosine (5mC) residues to C residues.

2.2 Genomic Hypomethylation Profiles in Cancer and Their Relevance at Promoters and Enhancers

Until recent high-resolution genome-wide analyses of DNA methylation, cancer-specific portions of methylomes were considered to consist predominantly of hypomethylated DNA repeats and hypermethylated gene regions [3, 7, 36]. DNA repeats are often used as a surrogate for average genomic methylation changes (usually losses of 5mC), with DNA epigenetic changes in some classes of repeats more closely associated with certain tumor types [6, 18, 35, 37–39]. In our 1983 analysis of global DNA hypomethylation in human cancers by high performance liquid chromatography analysis of enzymatic DNA digests [1], we fractionated one adenocarcinoma DNA into highly repetitive, moderately repetitive, and unique sequence classes. Because we found that each of these cancer DNA fractions had similar ratios of mol% 5mC to those from normal human tissues, we concluded that cancer-linked hypomethylation was not confined to repeated DNA. Indeed, cancer-linked DNA hypomethylation often occurs in unique sequences in and around genes, including metastasis-associated genes, as originally revealed in studies using CpG methylation-sensitive restriction endonucleases or sodium sulfite-based methods to study individual gene regions [2, 6, 40].

Recent genome-wide studies of DNA methylation in various normal and cancer cell populations indicate much tissue specificity throughout the genome in normal samples and pervasive cancer-linked DNA hypomethylation and hypermethylation [13, 15, 16, 41–45]. Regions of cancer-associated changes in DNA methylation are found in short interspersed or clustered regions as well as in long blocks [7, 42, 44, 46, 47]. There is increasing evidence for cause-and-effect relationships between normal tissue-specific DNA hypomethylation and increased transcription as well as many associations between cancer-linked hypomethylation and cancer-linked increases in gene expression [16, 17, 19, 21, 24, 48–55]. The inverse relationships between expression and DNA methylation include imprinted genes implicated in carcinogenesis [56].

A small percentage of annotated gene promoters overlap tissue-specific (T-DMR) or cancer-specific (C-DMR) differentially methylated DNA regions [49, 57]. However, most of the non-imprinted, autosomal T-DMR promoters are not the main type of vertebrate DNA promoters, which are part of CpG islands (CGIs, a class of CpG-rich regions surrounded by CpG-poor DNA). Among the genes with T-DMR promoters are some that become activated upon experimentally induced demethylation with a low dose of 5-deoxyazacytidine but not upon treatment with a histone deacetylase inhibitor, trichostatin A [49].

Enhancers too sometimes show a correlation between upregulation of expression of the associated gene and DNA demethylation in normal cells. For example, the binding of FoxA1/FOXA1 to enhancers is inhibited by site-specific DNA methylation at the corresponding binding site [58]. This differentiation-associated transcription regulatory factor can open up DNA compacted in chromatin of inactive enhancers (as a “pioneer” factor) and then recruit effector transcription factors to

make the enhancer active [59–61]. A window of DNA demethylation provided by previous binding of FoxD3, another pioneer factor, allows recruitment of FoxA1 and conversion of the enhancer to a state that is poised for activity. Moreover, in embryonal stem cells, local DNA demethylation per se, rather than any changes in histone H3K27 or H3K9 methylation, is associated with the binding of pioneer factors to certain tissue-specific non-CGI promoters [58]. Pioneer factors, including FOXA1, are implicated in various types of carcinogenesis [62]. Given the extensive hypomethylation of DNA in cancers, many known and yet more unknown enhancer regions are likely to become demethylated specifically in tumors. However, specific losses of DNA methylation from transcription regulatory regions might facilitate, but not independently cause, changes in expression [63].

Broad DNA regions enriched in hypomethylation are sometimes also associated with increases in copy number of DNA regions and can, thereby, synergistically increase expression of some of the affected genes [13, 33, 42]. Such broad regional hypomethylation (which can encompass occasional sites of persistent methylation) might reflect higher order chromatin structure. The latter is influenced, in turn, by the type, frequency, and spacing of DNA repeats; the G+C and CpG contents of subregions; the gene density; the nucleosome density; broad regions of distinct histone composition modification; and the presence of clusters of co-regulated genes. Nonetheless, a long region of cancer-linked DNA hypermethylation can be adjacent to a region of cancer-linked DNA hypomethylation with a sharp border between them, as demonstrated for a tandem repeat array (D4Z4) and its border sequences [9]. Despite evidence for functionality, DNA demethylation in cancer probably involves frequent overshooting of targeted sequences. These are referred to as *passenger* DNA methylation changes [64]. The hypomethylation in cancers of many more sites than are biologically relevant is probably due to a relaxed specificity of the demethylation apparatus during carcinogenesis and tumor progression and to the spreading of DNA demethylation patterns.

2.3 Genomic Hypomethylation in Cancer Within Gene Bodies

Recent findings implicate intragenic epigenetic marks in the regulation of normal gene expression. T-DMRs have been found inside many genes, and increased methylation in the central gene body or downstream promoter-flanking region of certain subsets of genes is associated with increased transcription [23, 65–68]. Moreover, there are nonrandom associations between positions of CpG methylation within genes and exon–intron boundaries, distance from the transcription start site, and distance from the 3′ end of the gene [66, 69]. Besides first exons, T-DMRs are present in various exonic and intronic sequences, including internal CGIs, sequences adjacent to internal CGIs (“CGI shores”), insulators, intragenic ncRNA genes, and 3′ terminal regions [17, 19, 28, 59, 70, 71]. They are present in both repeated and unique sequences. These findings are consistent with the many interrelationships between DNA and chromatin epigenetics and tissue-specific chromatin epigenetic marks inside genes [65, 68, 72, 73].

Differentiation-related DNA and/or chromatin epigenetic marks within genes may help determine alternative promoter usage, modulate the rate of transcription initiation or elongation, and possibly help direct the choice of alternative splice sites [19, 21, 24, 26, 27, 29, 34, 52, 74, 75]. The average DNA methylation level in the central portion of moderately expressed genes is associated with higher average transcription levels, possibly by being related to nucleosome positioning [76]. For example, immediately downstream of proximal CpG-poor promoters, it was unexpectedly found that methylation of sequences antagonizes binding of Polycomb repressor complexes [68]. Methylation of gene-body CGIs appears to be associated with repression of intragenic promoters [28]. However, for some sets of genes under certain conditions, lower expression was correlated with increases in gene-body methylation [69].

With respect to alternative splicing, evidence implicates certain histone modifications in helping to regulate the choice of splice junctions by altering rates of transcription, nucleosome positioning, or direct interactions with proteins that mark exon–intron junctions of pre-mRNA [77, 78]. Changes in physiological conditions can alter the chromatin modifications at these junctions and concomitantly modulate exon skipping [78]. DNA methylation may also be involved in regulating alternative splicing because of the many DNA methylation/chromatin epigenetic interrelationships and the finding that intron–exon junctions are enriched in sharp transitions in DNA methylation levels [66]. A recent report that malignant prostate cancer cells have enrichment of DNA hypermethylation at exon–intron junctions [45] is consistent with the cancer-linked involvement of DNA methylation levels in determining alternative splicing.

Programmed changes in DNA methylation in intra- and intergenic regions are not restricted to differentiation-related events. For example, electroconvulsive stimulation of mouse neuronal cells in vivo was recently demonstrated to cause rapid decreases and increases in DNA methylation in a substantial minority of CpG sites, especially at CpG-poor regions [69]. The physiologically linked DNA demethylation included rapid demethylation of exons and introns in various positions of the genes. Importantly, there was enrichment in these DNA epigenetic changes in the vicinity of brain-related genes. Thus, there is ample precedent from studies of normal cell functioning to suggest that cancer-associated DNA hypomethylation in intronic and exonic sequences can modulate the amount and type of gene products and thereby contribute to tumor formation or progression.

Cancer-linked DNA hypomethylation in the gene body is illustrated in Fig. 2.1 for three genes whose expression has been reported to be altered in certain cancers [79–81]. *TGFB2* has an intronic Alu repeat that was hypomethylated in some cancer cell lines relative to a wide variety of normal tissues (Fig. 2.1a) and untransformed cell cultures. The only exceptions to this intronic region being highly methylated in normal tissues and cell strains were found in skeletal muscle (Fig. 2.1a), myoblasts, and myotubes (data not shown). Their hypomethylation at this site might be related to the significant upregulation of *TGFB2* in myoblasts and myotubes vs. 19 types of non-muscle cell cultures [82] and is an example of the frequent relationship between targets for cancer-associated hypo- or hypermethylation and targets for differentiation-associated epigenetic changes [17, 83]. Like *TGFB2, PRDM16* (Fig. 2.1b) exhibited gene-body hypomethylation in

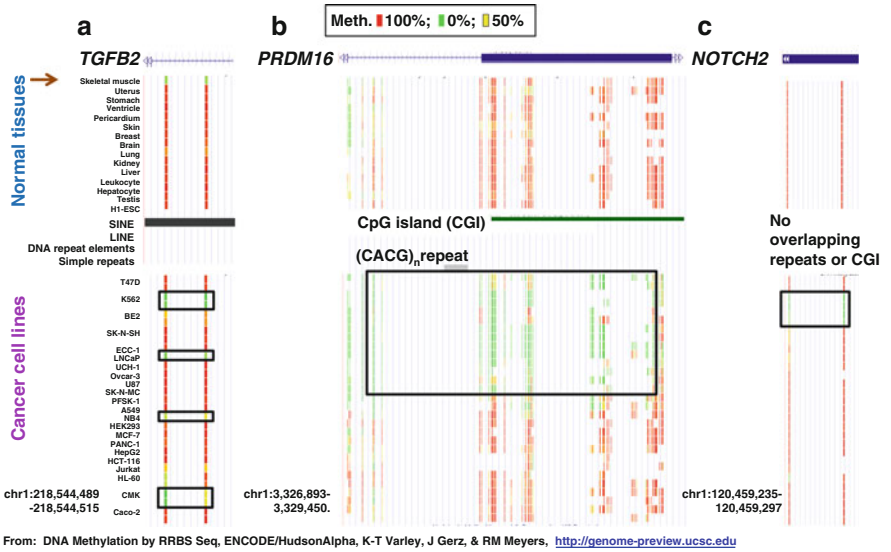

Fig. 2.1 Examples of cancer cell-associated hypomethylation (*boxed*) within gene bodies and overlapping a DNA repeat (**a**), a CGI (**b**), or neither (**c**) as determined by whole-genome analysis using reduced representation bisulfite sequencing (RRBS). (**a**), *TGFB2* , intron 1; the cancer hypomethylation overlaps an Alu repeat that is also hypomethylated in skeletal muscle (see *arrow*). (**b**), *PRDM16,* exon 9 and intron 8; the cancer hypomethylation overlaps a CGI and CGI shore. (**c**), *NOTCH2*, exon 34; no overlapping repeats or CGI. In contrast to the cancer-derived cell lines, non-immortalized cell strains (not shown) showed the same hypermethylation seen in normal tissues with the exception of myoblasts and myotubes for *TGFB2.* Myoblasts and myotubes overexpress *TGFB2* relative to 19 other types of cultured cell popula*tions*. All analyses were done in duplicate, and representative duplicates are shown

some of the cancer cell lines; however, this hypomethylation was in a region largely overlapping a CGI in an exon. *NOTCH2* (Fig. 2.1c) also showed gene-body hypomethylation in several cancer cell lines, but this hypomethylation was neither in a subregion with a CGI nor a DNA repeat. We note that some of the cancer cell lines with *TGFB2* or *PRDM16* gene hypomethylation also displayed cancer cell-linked promoter hypermethylation (data not shown).

Recently, the presence of 5-hydroxymethylcytosine (5hmC) as the sixth naturally programmed base in vertebrate DNA has been established [84]. It is generated from 5mC by hydroxylation via the enzymes TET1, TET2, or TET3 and is even more highly tissue specific in its relative levels in DNA than is 5mC [84–86]. It is implicated in stem cell renewal and distinct types of differentiation [87–89], as described further in an accompanying chapter by Pradhan and Kinney. Like 5mC, 5hmC is enriched in certain intragenic regions and exhibits major decreases in its genomic levels in cancer [84–86]. However, unlike 5mC, exons, intragenic CGIs, and enhancers have significantly elevated 5hmC levels relative to other portions of the genome [87, 90, 91]. These findings further highlight the need for studies of the functional significance of decreases in intragenic DNA epigenetic marks in cancer. In addition, they introduce a complication into almost all studies to date of 5mC that use either bisulfite or conventional CpG methylation-sensitive

restriction analysis to distinguish 5mC from unmethylated C, as these methods cannot resolve 5hmC and 5mC [69, 92, 93]. Therefore, a caveat to conclusions about 5mC distribution is that 5hmC might have been monitored instead, especially in exonic or enhancer regions in more 5hmC-rich tissues like brain [84, 85]. However, in some other cell types, like breast, heart, cell lines, and cancers, 5hmC is very much lower [84–86, 93], and 5hmC levels are also low in intronic and intergenic regions [90, 94].

2.4 Hypomethylation of DNA Repeats in Cancer

Global losses of DNA methylation with less numerous increases in methylation in other portions of the genome are typical of cancer [5, 6] although there are exceptions [18]. A major contributor toward the overall DNA hypomethylation is hypomethylation of tandem and interspersed DNA repeats, which is observed in most examined cancers [6, 95–97]. Most hypomethylation of DNA repeats in cancers is apparently the result of demethylation and not preexisting hypomethylation in a cancer stem cell [3], with the exception of seminomas as discussed below. Besides the effects on transcription and possible effects on alternative splicing described in the previous section, hypomethylation of a minor portion of interspersed DNA repeats may occasionally cause induction of retroviral element transcription [35]. In addition, hypomethylation of certain promoter-containing interspersed DNA repeats may affect chromatin boundaries resulting in effects on transcription of nearby genes [98, 99].

In a study of mononuclear cells from a few patients with chronic lymphocytic leukemia vs. the analogous cells from controls, Dante et al. described hypomethylation of LINE-1, a highly repeated interspersed repeat [100]. Hypomethylation of LINE-1 and Alu repeats was subsequently observed in many other types of cancers [38, 101–104]. Similarly, we found that tandem repeats in centromeric and juxtacentromeric satellite DNA are frequently hypomethylated in breast adenocarcinomas, ovarian epithelial cancers, and Wilms tumors [30, 105, 106], as confirmed for many other types of cancers [3, 107]. Additional classes of tandem repeats (including macrosatellite DNAs) and segmental duplications are also susceptible to DNA hypomethylation in malignancies [9, 18, 39, 43, 83, 108–110], although different subclasses of DNA repeat families can vary in their susceptibility to loss of DNA methylation in cancer [38, 39, 102, 111–113]. In some cancers, satellite DNA repeats showed the strongest DNA hypomethylation of all types of sequences analyzed [18, 33].

The frequency of cancer-associated hypomethylation of DNA repeats depends on the grade, the stage, and the individual tumor specimen [46, 114]. This hypomethylation is seen sometimes in non-tumor tissue adjacent to the cancer and in benign neoplasms and tissue lesions such as breast fibroadenomas and ovarian cystadenomas, although often to a lesser extent than in cancers [13, 51, 95, 105, 106, 112, 115]. In a mouse model of prostate tumor progression, repeat DNA hypomethylation was observed at the stage of prostatic intraepithelial neoplasia and prior to promoter hypermethylation [116]. However, depending on the tumor type

or specimen, repeat DNA hypomethylation may increase with tumor progression, a relationship inferred since the 1980s [1, 117]. In many types of cancer, repeat DNA hypomethylation is a highly informative prognostic marker and/or predictor of survival [46, 107, 118–122].

2.5 DNA Hypomethylation and Germ Cells: Comparison to Cancer Hypomethylation

Differential methylation of testes-specific genes has some similarities to cancer-associated DNA hypomethylation. Most genes that are specifically expressed in testis (like the cancer-testis genes) have little or no methylation in their promoter regions in testis and sperm although they are highly methylated, and transcriptionally repressed, in somatic tissues [123]. In sperm, as well as in many cancers, tandem DNA repeats and certain subclasses of interspersed DNA repeats display low methylation levels compared with normal postnatal somatic tissues [38, 112, 124–126]. Reminiscent of the tendency (with many exceptions, as described above) towards DNA repeats and unique sequences having opposite methylation changes in cancer, single-copy genes become demethylated but tandem and interspersed repeats retain their methylation in murine primordial germ cells at 12.5–13.5 dpc [123].

Another interface between the germ line epigenome and cancer is seen in the exceptionally strong global DNA hypomethylation in seminomatous testicular germ cell tumors. In our 1982 study of 62 tumors representing 23 different types, we found that a testicular seminoma had only 1.4% of its genomic C present as 5mC, while the next lowest 5mC level for a cancer was 2.4% [1]. The range of genomic 5mC levels among the normal tissues that we studied was 3.5–4.1% of C residues methylated. Smiraglia et al. confirmed the extraordinary depletion of 5mC in the genomes of many seminomas [127]. This finding has been ascribed to the origin of seminomas from primordial germ cells that had undergone massive demethylation before oncogenic transformation without subsequent de novo methylation thereafter [127, 128]. Importantly, seminomas generally show none of the CGI hypermethylation so prevalent in other types of cancer, but rather display extreme overall DNA hypomethylation [127]. Therefore, cancers can develop without gene region hypermethylation but with extreme overall genomic hypomethylation.

2.6 Opposite Cancer-Linked Changes in DNA Methylation in DNA Repeats: Hypo- and Hypermethylation

Opposite types of cancer-linked DNA methylation changes can occur in the same DNA sequence, as we found in a Southern blot study of methylation of NBL2, a 1.4-kb sequence repeated in tandem mostly near the centromeres of acrocentric chromosomes [39]. NBL2 was hypomethylated at HhaI sites (5′-CGCG-3′ sites) in

17% of ovarian carcinomas and hypermethylated in >70% of ovarian carcinomas and Wilms tumors at the same sites [39]. Various normal postnatal somatic tissues exhibited partial methylation at HhaI sites in NBL2 and were similar to each other in their methylation patterns at this tandem repeat. Using NotI (5′-GCGGCCGC-3′) for Southern blotting, only the cancer-linked hypomethylation of NBL2 was previously observed [108, 110] because NotI cleaves control somatic DNA too infrequently to reveal hypermethylation in cancers. This is an example of the importance of considering the technique used in evaluating results on DNA methylation [92] as well as the appropriate control DNA for comparison to the cancer. A few cancer DNAs digested with HhaI displayed two distinct fractions of NBL2 sequences, one with overall hypermethylation and the other with overall hypomethylation relative to all the somatic controls, which suggests that the repeats at one chromosomal location underwent de novo methylation and at another underwent demethylation during carcinogenesis. Hairpin genomic sequencing [129] (see below) at two ~0.3-kb subregions of the 1.4-kb *NBL2* ([8] and Nishiyama and Ehrlich, unpublished data) confirmed that hypomethylation at NBL2 predominated in some cancers and hypermethylation in others in comparison to normal somatic tissues, which displayed much site specificity in the methylation status of individual CpG sites. Therefore, a small region of DNA can be made unstable epigenetically during carcinogenesis so that CpG sites that are very near to each other undergo opposite changes in DNA methylation. The plasticity of the directionality of methylation changes at DNA repeats in cancers has also been seen in recent genome-wide studies [15, 18].

D4Z4, a heterologous tandem array (macrosatellite) located at subtelomeric 4q and 10q, also exhibited strong hypomethylation in the bulk of the array in some cancers and hypermethylation in others of the same type [9]. Several of the cancers had extremely high levels of methylation in more than three consecutive 3.3-kb repeat units of D4Z4, indicative of the spreading of de novo methylation. This methylation spreading seems to have limits to its processivity and to be prone to stop at certain subregions of the repeat unit.

2.7 Tagging Classes of DNA Sequences for Demethylation

Because NBL2 and D4Z4 tandem repeats displayed overall hypomethylation in some cancers and hypermethylation in others, it was highly informative to compare their methylation changes in a given cancer. Among 17 ovarian carcinomas and 44 Wilms tumors, there was a significant correlation ($p<0.001$) between the direction (either hypo- or hypermethylation) and degree of methylation change (strong, moderate, or weak) at D4Z4 and the dissimilar NBL2 [9]. This suggests that diverse sequences on different chromosomes may be similarly tagged for demethylation or de novo methylation (methylation of symmetrically unmethylated CpG dyads) during carcinogenesis. However, many cancers with extensive hypermethylation of D4Z4 and NBL2 repeats displayed hypomethylation of another, heterologous tandem repeat, juxtacentromeric satellite 2 on chromosome 1 (Sat2) [39].

NBL2 (mostly in the short arm of the acrocentric chromosomes) and D4Z4 (in the subtelomeric region of chromosomes 4 and 10) are both rich in G+C and look like very long CGIs. However, they differ appreciably in their G+C composition (61% and 73%, respectively) and their CpG content (5.7% and 9.9%, respectively). Analysis of histone modification and DNaseI sensitivity has been done for D4Z4 and indicates that its chromatin has properties midway between constitutive heterochromatin and unexpressed euchromatin [130, 131]. In contrast, Sat2, which is in the pericentromeric region, is constitutively heterochromatic and highly condensed in interphase. It has only 38% G+C but, nonetheless, it has 5.1% CpG. Therefore, the CpG suppression seen in the overall genome is not evident in Sat2. Sometimes even Sat2, with its rather CpG-rich character, becomes hypermethylated in cancers at a CpG dyad that exhibits a low methylation level in normal somatic tissues [132].

That the G+C content and chromatin structure is important for recruiting machinery for either demethylation or de novo methylation is consistent with our findings on the HpaII site immediately proximal to the D4Z4 array. It is located in a 0.2-kb D4Z4-proximal subregion that has 43% G+C, while D4Z4 has 73% G+C in all of its essentially identical, tandem 3.3-kb repeats. This 0.2-kb sequence immediately adjacent to the array is prone to tumor-linked hypomethylation even in cancers displaying strong hypermethylation within the array [9]. Surprisingly, even the adjacent D4Z4 repeat unit at the proximal end of the array became hypomethylated in cancers with hypermethylation of the bulk of the array. Probably, the array-adjacent sequence with its much lower G+C content helps confer a different chromatin structure on the neighboring D4Z4 repeat unit, which, in turn, affects the directionality of cancer-linked methylation change. Interestingly, a study of tandem transgenic repeats in mice revealed that, in some animals, all of the (G+C)-rich transgene units became methylated except for one copy adjacent to cellular DNA [133]. Despite the regional properties of DNA and chromatin that may recruit cancer-associated DNA methylation or demethylation apparati, there are, as mentioned above, very local sequence-specific effects which allow individual CpG dyads to circumvent regional demethylation or de novo methylation [8, 9].

DNA demethylation both influences and is strongly influenced by histone modifications. For example, histone H3 trimethylation at lysine 4 (H3K4me3) correlates best with the lack of DNA methylation around the transcription start site [66]. This was found for both CGI promoters [134] and promoters that do not contain a CGI, and for CpG methylation as well as the appreciable amount of CpA methylation in embryonal stem cells [66]. A histone H3 unmethylated at lysine 4 has been implicated as necessary for de novo methylation by DNMT3A in conjunction with its interacting partner DNMT3L [135]. Increased activity of the histone lysine demethylase LSD1 (KDM1A), which, depending on its interacting partners, demethylates K4- or K9-methylated histone H3, has been found to correlate with an adverse outcome and a less differentiated phenotype in neuroblastomas [136]. Conversely, mutation of the *Lsd1* gene blocks murine gastrulation [137] and results in global DNA hypomethylation. This may be partially due to the need for Lsd1/LSD1 to demethylate the DNMT1 enzyme itself and thereby increase its stability

[137] but also could reflect the role of this enzyme in the demethylation of H3K9me3. There are many other players that could influence DNA methylation during carcinogenesis by their effects on chromatin structure, e.g., poly(ADP-ribosyl)ation, other types of histone modifications, histone variants, nonhistone chromatin proteins, specific interactions with DNMT proteins, and modulation of the set of DNA methyltransferase isoforms produced at the RNA or protein levels [138–143]. Nonetheless, multi-functionality of LSD1 in its ability to demethylate proteins and both activating and repressive histone methylation marks may serve as a paradigm for how, paradoxically, there can be both increases and decreases in DNA methylation in a given cancer cell.

2.8 Active Versus Passive DNA Demethylation

There are two broad classes of mechanisms by which 5mC residues can be replaced by C residues (DNA demethylation). During replicative or repair DNA synthesis there may be a failure to methylate the newly synthesized DNA strand at a symmetrically methylated CpG dyad (passive demethylation), which will initially result in a hemimethylated dyad (Fig. 2.2). If this failure occurs again at the same CpG dyad in the next round of replication, then a symmetrically unmethylated CpG dyad will be the result. Active demethylation involves 5mC residues being physically replaced with C residues (at the base or mononucleotide level) or, less likely, the methyl group being removed enzymatically. Accumulating evidence favors mainly active demethylation contributing to the naturally occurring DNA demethylation by the replacement of C residues [144, 145]. Active demethylation is consistent with

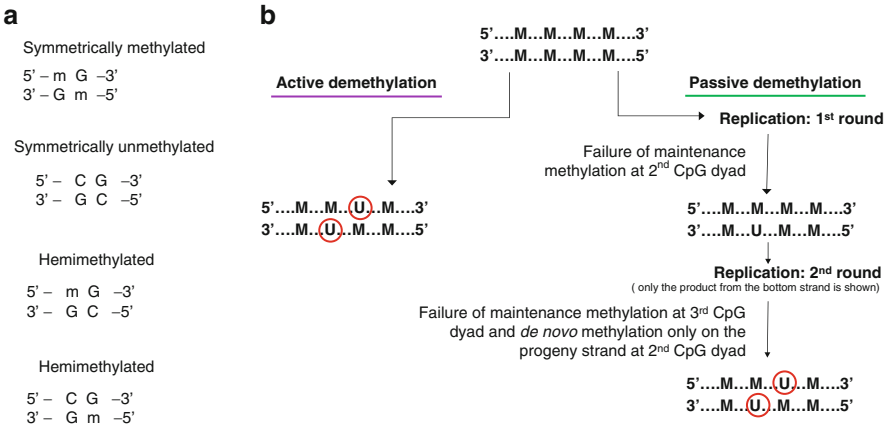

Fig. 2.2 Findings of consecutive hemimethylated dyads of opposite orientation in normal and cancer cells are best explained by active demethylation. (**a**) m, 5mC; C, unmethylated cytosine. (**b**) M, 5′-5mCpG-3′; U, 5′-CpG-3′. The generation of hemimethylated dyads of opposite orientation by passive demethylation would involve improbable changes in the second round of replication

the rapid and distributive loss of 5mC and the replication independence that has been demonstrated for many examples of naturally programmed demethylation of mammalian genomes [146, 147]. However, passive demethylation or a combination of active and passive demethylation due to inadequate maintenance methylation [148] is likely to also play a role in normal and pathological decreases in DNA methylation. Hemimethylated dyads (Fig. 2.2) can be intermediates in both active and passive demethylation of DNA as well as being intermediates in maintenance methylation.

2.9 Maintenance of DNA Methylation Patterns Through Hemimethylated Intermediates

The processes by which DNA methylation patterns are maintained are highly relevant to understanding how DNA demethylation occurs. Over 30 years ago, mechanisms for the inheritance of DNA methylation were initially proposed [149, 150]. In the traditional view, methylation at each site is assumed to be governed by the processes of de novo methylation and maintenance methylation, and these processes are independent of one another. The maintenance of methylation patterns has been attributed to the methyltransferase Dnmt1. As summarized in a 2009 review by Jones and Liang, "The basis of this model is that DNA methylation patterns are established in germ cells and in developing embryos by the activity of the de novo DNA methyltransferases Dnmt3a and Dnmt3B. Subsequently, methylation patterns are inherited after DNA replication primarily owing to the activity of Dnmt1, which has a preference for hemimethylated sites that are generated through DNA synthesis" [151]. The premise of independently acting mechanisms for de novo and maintenance methylation has led to the construction of stochastic models for methylation inheritance [152–157].

2.10 Alternative Mechanisms for Maintenance Methylation

The accepted dogma of de novo methylation catalyzed by DNMT3A/Dnmt3a, DNMT3B/Dnmt3b, and maintenance methylation through obligatory hemimethylated intermediates via DNMT1/Dnmt1 has recently been called into question. According to the original model for maintenance methylation, hemimethylated CpG dyads (Fig. 2.2) should be short-lived and difficult to detect. However, as early as 1986, demethylation with long-lived hemimethylated CpG dyads was observed at individual CpG sites in the avian vitellogenin II gene following treatment with estradiol, which suggested an active pathway through excision repair and/or enzymatic demethylation [158]. A later study of the rat alpha-actin gene promoter provided evidence for hemimethylated intermediates persisting more than 48 hours prior to becoming fully demethylated and suggested active demethylation involving

cis-acting DNA elements [159]. Subsequently, Liang et al. [160] developed an assay that allowed determination of hemimethylation at *Hpa*II sites (CCGG). In mouse embryonic stem cells, levels of hemimethylation in some repetitive sequence regions were significantly higher than the traditional model of maintenance methylation by Dnmt1 would predict. By looking at gene knockouts for *Dnmt1* and *Dnmt3a* and *Dnmt3b*, they deduced that ongoing de novo methylation by Dnmt3a or Dnmt3b in a highly cooperative manner with Dnmt1 in embryonal stem cells compensated for inefficient maintenance methylation by Dnmt1 in these regions. These results suggest a constant, rather than sporadic or only differentiation-associated, role for de novo methylation in vivo. They concluded that sequences would gradually become demethylated without this constant role for de novo methylation to compensate for inefficient replication-coupled maintenance methylation. Furthermore, in a study by Chen et al. [161], loss of Dnmt1 gave only a 10% decrease in methylation overall following one cell cycle of replication in human colorectal carcinoma cells. This conditional knockout resulted in hemimethylation of 18% of sites analyzed by hairpin genomic sequencing in the CGI of an L1 transposable element. The overall level of methylation at CpG dyads in these sequences in cells with normal Dnmt1 was around 85% with no detectable hemimethylation.

In the alternative model for maintaining DNA methylation patterns that was proposed by Jones and Liang [151], DNMT1, the most abundant DNA methyltransferase is still considered to be primarily a maintenance methylase and is responsible for most of the replication-associated DNA methylation. However, they propose that DNMT3A and DNMT3B enzymes remain bound to nucleosomes that contain high levels of DNA methylation. Following replication, CpG dyads whose methylation fails to be correctly maintained by DNMT1 would then be "corrected" by DNMT3A and DNMT3B, so that these enzymes would preserve highly methylated regions without strictly "reading" the patterns on the parental strand. In this way, the methylation state of a region is maintained rather than a site-specific methylation pattern. In addition, DNMT1 might participate in some of this correction of lingering hemimethylated sites that have left the vicinity of the replication fork, perhaps recruited by proteins such as UHRF1 which recognizes hemimethylated sites (see below). This concept of repair methylation is consistent with findings that methylation patterns in highly methylated regions tend to vary among molecules and higher rates of de novo methylation are observed in highly methylated sequences [129]. Moreover, non-CpG methylation at asymmetrical sites, which is found mostly in embryonal stem cells [70], should rely on de novo methyltransferase activity for perpetuating the DNA methylation patterns, as described below.

In cancers, the frequent presence of long blocks of hypomethylated DNA [7, 16, 42, 47, 105] and the usual predominance of overall decreases rather than increases in 5mC content of the genome suggest that passive demethylation contributes to cancer-associated genomic hypomethylation. Passive demethylation might involve either a lack of methylation of hemimethylated sites by DNMT1 or a failure of DNMT3A or DNMT3B to retain dense methylation of a normally highly methylated region. However, the current, more layered view of the maintenance of DNA methylation patterns suggests that while some of the demethyla-

tion of DNA in cancer occurs by a failure of maintenance methylation, most is due to an active mechanism. Recent studies of normal differentiation- or physiology-associated DNA demethylation support an active type of DNA methylation involving enzymatically catalyzed modification of 5mC residues to 5hmC residues (and subsequent oxidation products) or thymine residues followed by DNA repair [162–164]. Three-step processes for active DNA demethylation have been proposed in which 5mC is first enzymatically modified; then demethylated on one strand, most likely by excision repair; and later fully demethylated by a mechanism that avoids inducing double-strand breaks during removal of both 5mCs of a 5mCpG dyad [165]. The last step could involve a repair mechanism that preferentially acts on hemimethylated substrates [165] or passive demethylation of a hemimethylated or hemihydroxymethylated dyad. The latter could be due to the 5hmC residues on one strand of a hemihydroxymethylated dyad not being recognized for maintenance methylation [148].

UHRF1 (also known as NP95) is a cofactor that interacts specifically with hemimethylated DNA and may participate in demethylation as well as de novo methylation of cancer epigenomes. UHRF1 also interacts with DNMT1, and even more strongly with DNMT3A and DNMT3B [166], and thereby, may be involved in the recruitment of DNMT3A/3B to unmethylated regions during tumorigenesis leading to de novo methylation [167]. However, recent work on gliomas has identified the disruption of DNMT1, PCNA, and UHRF1 interactions as a crucial oncogenic event promoting DNA hypomethylation-induced tumorigenesis in the absence of DNMT1 deficiencies [168]. Thus, while upregulation of UHRF1 may contribute to the silencing of tumor suppressors through de novo methylation, the disruption of DNMT1/PCNA/UHRF1 interactions might result in cancer-associated DNA hypomethylation affecting transcription.

2.11 Insights into Cancer-Associated DNA Demethylation from Studies of DNA Hemimethylation

The introduction of hairpin-bisulfite PCR (hairpin genomic sequencing) by Laird et al. in 2004 [129] has enabled the observation of the methylation status on both strands of individual DNA molecules on a site-by-site basis. In bisulfite-based genomic sequencing, bisulfite causes deamination of unmethylated C residues, but not methylated C residues [169]. Hairpin genomic sequencing allows analysis of methylation at every CG dinucleotide pair in a given region on covalently linked DNA strands of a restriction fragment. A caveat about these studies of DNA hemimethylation is that bisulfite-based DNA methylation analysis cannot distinguish between 5hmC and 5mC, as described above, and 5hmC on one strand at a CpG dyad is not recognized for maintenance methylation [170]. Therefore, it is possible that the detected hemimethylation is actually a CpG dyad with one unmethylated C residue and one 5hmC residue. However, in the studies of tandem DNA repeats in cancers described below, this is unlikely because 5hmC is predominantly in gene

regions and all studied cancers and cancer cell lines have extremely low levels of 5hmC [84–86].

By sodium bisulfite-based whole-methylome analysis using next-generation sequencing, Lister et al. analyzed more than 90% of the cytosines in human H1 embryonic stem cells (H1 ES) and IMR90 fetal lung fibroblasts [70]. While nearly all of the methylcytosines detected in the IMR90 fibroblasts were in the CG dinucleotide context, considerable methylation in non-CG contexts (mCHG and mCHH, where H = A, C or T) was observed in the H1 stem cells, comprising almost 25% of the total methylation, in agreement with a recent study by Laurent et al. [66]. Methylation at mCHG sites in H1 ES was also highly asymmetrical, with 98% of such sites observed to be methylated on only one strand. Non-CpG methylation was also found to be significantly higher on the antisense strand of gene bodies, suggesting a nonrandom bias in the observed asymmetry. Non-CpG methylation disappeared upon differentiation of the H1 stem cells, but was restored in differentiated cells induced to form pluripotent stem cells. These findings suggest that asymmetrical methylation at non-CG dinucleotide sites may contribute to maintenance of the pluripotent state. They are reminiscent of the less frequent, hemimethylated CG dinucleotide sites that we and Laird et al. have seen in various DNA repeats [8, 132, 171] or single-copy sequences [129] in normal or cancer tissues.

2.12 Hemimethylated CpG Dyads in Cancer

Although reports of DNA hemimethylation in cancer are few, our studies of hemimethylated DNA in cancers support the involvement of active demethylation in generating cancer-linked genomic hypomethylation. We analyzed DNA methylation changes in depth at the above-mentioned tandem repeats NBL2 and at Sat2 in ovarian epithelial tumors and Wilms tumors by hairpin genomic sequencing [8, 132]. In a study of 13 CpGs in a 0.2-kb subregion of Sat2 in ovarian carcinomas and somatic control tissues, hairpin genomic sequencing not only revealed significantly greater clonal variability in methylation patterns in the cancers than in diverse control tissues but also provided statistically significant evidence of clustering among both hemimethylated and fully demethylated sites [132]. Runs of hemimethylated sites with identical orientation were seen at higher than expected rates in the cancers. Similarly, an analysis of 14 CpGs in the NBL2 repeat unit identified both hypomethylation and hypermethylation in ovarian carcinomas and Wilms tumors, again with a high degree of clonal variation in methylation patterns within each sample [8].

Diverse control and cancer samples contained some DNA clones derived from unusual, consecutive hemimethylated CpG dyads of opposite polarity. Figure 2.2b illustrates how an M/U (5′-5mCpG-3′/3′-GpC-5′) dyad near a U/M dyad (5′-CpG-3′/3′-Gp5mC-5′) could be generated by active vs. passive demethylation. Passive demethylation would require inhibition of maintenance methylation (by DNMT1 alone or in conjunction with DNMT3A and DNMT3B, as discussed above) at a single CpG dyad in one round of replicative DNA synthesis. The next round of replica-

tion would then have to involve both asymmetrical de novo methylation of only the opposite strand of this dyad and inhibition, once again, of maintenance methylation at a neighboring CpG dyad. In contrast to this highly unlikely sequence of events, active demethylation can easily explain the generation of various patterns of hemimethylation in contiguous CpG dyads with either identical or opposite orientation.

In a simulation study jointly analyzing the Sat2 and NBL2 regions, we found that the observed methylation patterns in the carcinomas were best explained by a mechanism that accounted for site-to-site correlation [157]. Prior studies have produced evidence of spreading of methylation in cancer [172–176]. Our analysis suggests that demethylation may progress by spreading as well.

We propose that during carcinogenesis a highly methylated DNA sequence becomes partially demethylated by active demethylation. The sequence may then attain a density of 5mC residues in an atypical intermediate range. This intermediate level of methylation might confer less stability during successive cell divisions for maintenance of the methylation pattern or methylation density. The stability of a given partially methylated sequence could be determined, in part, by the efficiency with which DNMT3A and DNMT3B recognize unmethylated CpG sites in the sequence for repair methylation. Abnormally low methylation levels may favor the generation of yet lower levels, with some site-specific effects superimposed on the regional ones. Thus, active demethylation might start cancer-associated demethylation and a failure of maintenance methylation (including repair methylation) might continue it. The result could explain the observation that tumor progression is frequently linked to a progressive decrease in methylation.

2.13 Conclusions

Recently, there has been a burst of studies increasing our understanding of the importance of changes in DNA methylation in intragenic, promoter, and intergenic regions during differentiation and in response to some types of physiological change. These findings suggest that much more of the cancer-associated DNA hypomethylation contributes to tumor formation and progression than previously recognized. Similarly, high-resolution analysis of cancer methylomes in comparison to appropriate controls indicates that the extent of cancer-linked hypomethylation is larger than previously appreciated and affects a greater variety of DNA sequences. We propose that the pathways for normal DNA demethylation that operate during differentiation or induction of certain physiological changes become hijacked during carcinogenesis and tumor progression, leading to the initiation of cancer-associated DNA demethylation. This demethylation then may spread in *cis* by both additional rounds of active demethylation and by passive demethylation involving failures in classical maintenance methylation and replication-associated repair methylation. The net result of some of this cancer-associated DNA demethylation could be abnormal modulation of transcription and even some aberrant posttranscriptional processing of transcripts as well as increases in DNA recombination, thereby contributing to tumor formation and progression.

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References

1. Gama-Sosa MA, Slagel VA, Trewyn RW, Oxenhandler R, Kuo KC, Gehrke CW, Ehrlich M (1983) The 5-methylcytosine content of DNA from human tumors. Nucleic Acids Res 11:6883–6894
2. Feinberg AP, Vogelstein B (1983) Hypomethylation distinguishes genes of some human cancers from their normal counterparts. Nature 301(5895):89–92
3. Ehrlich M (2009) DNA hypomethylation in cancer cells. Epigenomics 1(2):239–259
4. Ehrlich M, Jiang G, Fiala ES, Dome JS, Yu MS, Long TI, Youn B, Sohn O-S, Widschwendter M, Tomlinson GE, Chintagumpala M, Champagne M, Parham DM, Liang G, Malik K, Laird PW (2002) Hypomethylation and hypermethylation of DNA in Wilms tumors. Oncogene 21(43):6694–6702
5. Ehrlich M (2006) Cancer-linked DNA hypomethylation and its relationship to hypermethylation. Curr Top Microbiol Immunol 310:251–274
6. Ehrlich M (2002) DNA methylation in cancer: too much, but also too little. Oncogene 21(35):5400–5413
7. Pfeifer GP, Rauch TA (2009) DNA methylation patterns in lung carcinomas. Semin Cancer Biol 19(3):181–187
8. Nishiyama R, Qi L, Lacey M, Ehrlich M (2005) Both hypomethylation and hypermethylation in a 0.2-kb region of a DNA repeat in cancer. Molec Cancer Res 3:617–626
9. Tsumagari K, Qi L, Jackson K, Shao C, Lacey M, Sowden J, Tawil R, Vedanarayanan V, Ehrlich M (2008) Epigenetics of a tandem DNA repeat: chromatin DNaseI sensitivity and opposite methylation changes in cancers. Nucleic Acids Res 36:2196–2207
10. Lindsey JC, Lusher ME, Anderton JA, Gilbertson RJ, Ellison DW, Clifford SC (2007) Epigenetic deregulation of multiple S100 gene family members by differential hypomethylation and hypermethylation events in medulloblastoma. Br J Cancer 97(2):267–274
11. Grunau C, Brun ME, Rivals I, Selves J, Hindermann W, Favre-Mercuret M, Granier G, De Sario A (2008) BAGE hypomethylation, a new epigenetic biomarker for colon cancer detection. Cancer Epidemiol Biomarkers Prev 17(6):1374–1379
12. Sultan M, Schulz MH, Richard H, Magen A, Klingenhoff A, Scherf M, Seifert M, Borodina T, Soldatov A, Parkhomchuk D, Schmidt D, O'Keeffe S, Haas S, Vingron M, Lehrach H, Yaspo ML (2008) A global view of gene activity and alternative splicing by deep sequencing of the human transcriptome. Science 321(5891):956–960
13. Alvarez H, Opalinska J, Zhou L, Sohal D, Fazzari MJ, Yu Y, Montagna C, Montgomery EA, Canto M, Dunbar KB, Wang J, Roa JC, Mo Y, Bhagat T, Ramesh KH, Cannizzaro L, Mollenhauer J, Thompson RF, Suzuki M, Meltzer SJ, Melnick A, Greally JM, Maitra A, Verma A (2011) Widespread hypomethylation occurs early and synergizes with gene amplification during esophageal carcinogenesis. PLoS Genet 7(3):e1001356
14. Sandoval J, Heyn HA, Moran S, Serra-Musach J, Pujana MA, Bibikova M, Esteller M (2011) Validation of a DNA methylation microarray for 450,000 CpG sites in the human genome. Epigenetics 6(6):692–702
15. Ruike Y, Imanaka Y, Sato F, Shimizu K, Tsujimoto G (2010) Genome-wide analysis of aberrant methylation in human breast cancer cells using methyl-DNA immunoprecipitation combined with high-throughput sequencing. BMC Genomics 11:137
16. Hansen KD, Timp W, Bravo HC, Sabunciyan S, Langmead B, McDonald OG, Wen B, Wu H, Liu Y, Diep D, Briem E, Zhang K, Irizarry RA, Feinberg AP (2011) Increased methylation variation in epigenetic domains across cancer types. Nat Genet 43(8):768–775

17. Irizarry RA, Ladd-Acosta C, Wen B, Wu Z, Montano C, Onyango P, Cui H, Gabo K, Rongione M, Webster M, Ji H, Potash JB, Sabunciyan S, Feinberg AP (2009) The human colon cancer methylome shows similar hypo- and hypermethylation at conserved tissue-specific CpG island shores. Nat Genet 41(2):178–186
18. Feber A, Wilson GA, Zhang L, Presneau N, Idowu B, Down TA, Rakyan VK, Noon LA, Lloyd AC, Stupka E, Schiza V, Teschendorff AE, Schroth GP, Flanagan A, Beck S (2011) Comparative methylome analysis of benign and malignant peripheral nerve sheath tumors. Genome Res 21(4):515–524
19. Brenet F, Moh M, Funk P, Feierstein E, Viale AJ, Socci ND, Scandura JM (2011) DNA methylation of the first exon is tightly linked to transcriptional silencing. PLoS One 6(1):e14524
20. Song L, Zhang Z, Grasfeder LL, Boyle AP, Giresi PG, Lee BK, Sheffield NC, Graf S, Huss M, Keefe D, Liu Z, London D, McDaniell RM, Shibata Y, Showers KA, Simon JM, Vales T, Wang T, Winter D, Zhang Z, Clarke ND, Birney E, Iyer VR, Crawford GE, Lieb JD, Furey TS (2011) Open chromatin defined by DNaseI and FAIRE identifies regulatory elements that shape cell-type identity. Genome Res 21(10):1757–67
21. Tao Y, Xi S, Briones V, Muegge K (2010) Lsh mediated RNA polymerase II stalling at HoxC6 and HoxC8 involves DNA methylation. PLoS One 5(2):e9163
22. Bauer AP, Leikam D, Krinner S, Notka F, Ludwig C, Langst G, Wagner R (2010) The impact of intragenic CpG content on gene expression. Nucleic Acids Res 38(12):3891–3908
23. Schwartz S, Ast G (2010) Chromatin density and splicing destiny: on the cross-talk between chromatin structure and splicing. Embo J 29(10):1629–1636
24. Okitsu CY, Hsieh CL (2007) DNA methylation dictates histone H3K4 methylation. Mol Cell Biol 27(7):2746–2757
25. Okitsu CY, Hsieh JC, Hsieh CL (2010) Transcriptional activity affects the H3K4me3 level and distribution in the coding region. Mol Cell Biol 30(12):2933–2946
26. Lorincz MC, Dickerson DR, Schmitt M, Groudine M (2004) Intragenic DNA methylation alters chromatin structure and elongation efficiency in mammalian cells. Nat Struct Mol Biol 11(11):1068–1075
27. Deaton AM, Webb S, Kerr AR, Illingworth RS, Guy J, Andrews R, Bird A (2011) Cell type-specific DNA methylation at intragenic CpG islands in the immune system. Genome Res 21(7):1074–1086
28. Maunakea AK, Nagarajan RP, Bilenky M, Ballinger TJ, D'Souza C, Fouse SD, Johnson BE, Hong C, Nielsen C, Zhao Y, Turecki G, Delaney A, Varhol R, Thiessen N, Shchors K, Heine VM, Rowitch DH, Xing X, Fiore C, Schillebeeckx M, Jones SJ, Haussler D, Marra MA, Hirst M, Wang T, Costello JF (2010) Conserved role of intragenic DNA methylation in regulating alternative promoters. Nature 466(7303):253–257
29. Aporntewan C, Phokaew C, Piriyapongsa J, Ngamphiw C, Ittiwut C, Tongsima S, Mutirangura A (2011) Hypomethylation of intragenic LINE-1 represses transcription in cancer cells through AGO2. PLoS One 6(3):e17934
30. Qu G, Grundy PE, Narayan A, Ehrlich M (1999) Frequent hypomethylation in Wilms tumors of pericentromeric DNA in chromosomes 1 and 16. Cancer Genet Cytogenet 109:34–39
31. Eden A, Gaudet F, Waghmare A, Jaenisch R (2003) Chromosomal instability and tumors promoted by DNA hypomethylation. Science 300(5618):455
32. Yamada Y, Jackson-Grusby L, Linhart H, Meissner A, Eden A, Lin H, Jaenisch R (2005) Opposing effects of DNA hypomethylation on intestinal and liver carcinogenesis. Proc Natl Acad Sci USA 102(38):13580–13585
33. Cadieux B, Ching TT, Vandenberg SR, Costello JF (2006) Genome-wide hypomethylation in human glioblastomas associated with specific copy number alteration, methylenetetrahydrofolate reductase allele status, and increased proliferation. Cancer Res 66(17):8469–8476
34. Trejbalova K, Blazkova J, Matouskova M, Kucerova D, Pecnova L, Vernerova Z, Heracek J, Hirsch I, Hejnar J (2011) Epigenetic regulation of transcription and splicing of syncytins, fusogenic glycoproteins of retroviral origin. Nucleic Acids Res 39(20):8728–39

35. Goering W, Ribarska T, Schulz WA (2011) Selective changes of retroelement expression in human prostate cancer. Carcinogenesis 32(10):1484–92
36. Park SY, Yoo EJ, Cho NY, Kim N, Kang GH (2009) Comparison of CpG island hypermethylation and repetitive DNA hypomethylation in premalignant stages of gastric cancer, stratified for Helicobacter pylori infection. J Pathol 219(4):410–6
37. Yang AS, Estecio MR, Doshi K, Kondo Y, Tajara EH, Issa JP (2004) A simple method for estimating global DNA methylation using bisulfite PCR of repetitive DNA elements. Nucleic Acids Res 32(3):e38
38. Weisenberger DJ, Campan M, Long TI, Kim M, Woods C, Fiala E, Ehrlich M, Laird PW (2005) Analysis of repetitive element DNA methylation by MethyLight. Nucleic Acids Res 33(21):6823–6836
39. Nishiyama R, Qi L, Tsumagari K, Dubeau L, Weissbecker K, Champagne M, Sikka S, Nagai H, Ehrlich M (2005) A DNA repeat, NBL2, is hypermethylated in some cancers but hypomethylated in others. Cancer Biol Ther 4(4):440–448
40. Pulukuri SM, Estes N, Patel J, Rao JS (2007) Demethylation-linked activation of urokinase plasminogen activator is involved in progression of prostate cancer. Cancer Res 67(3): 930–939
41. Clark SJ (2007) Action at a distance: epigenetic silencing of large chromosomal regions in carcinogenesis. Hum Mol Genet 16 Spec No 1:R88–95
42. Andrews J, Kennette W, Pilon J, Hodgson A, Tuck AB, Chambers AF, Rodenhiser DI (2010) Multi-platform whole-genome microarray analyses refine the epigenetic signature of breast cancer metastasis with gene expression and copy number. PLoS One 5(1):e8665
43. Novak P, Jensen T, Oshiro MM, Watts GS, Kim CJ, Futscher BW (2008) Agglomerative epigenetic aberrations are a common event in human breast cancer. Cancer Res 68(20): 8616–8625
44. Coolen MW, Stirzaker C, Song JZ, Statham AL, Kassir Z, Moreno CS, Young AN, Varma V, Speed TP, Cowley M, Lacaze P, Kaplan W, Robinson MD, Clark SJ (2010) Consolidation of the cancer genome into domains of repressive chromatin by long-range epigenetic silencing (LRES) reduces transcriptional plasticity. Nat Cell Biol 12(3):235–246
45. Yegnasubramanian S, Wu Z, Haffner MC, Esopi D, Aryee MJ, Badrinath R, He TL, Morgan JD, Carvalho B, Zheng Q, De Marzo AM, Irizarry RA, Nelson WG (2011) Chromosome-wide mapping of DNA methylation patterns in normal and malignant prostate cells reveals pervasive methylation of gene-associated and conserved intergenic sequences. BMC Genomics 12:313
46. Widschwendter M, Jiang G, Woods C, Muller HM, Fiegl H, Goebel G, Marth C, Holzner EM, Zeimet AG, Laird PW, Ehrlich M (2004) DNA hypomethylation and ovarian cancer biology. Cancer Res 64(13):4472–4480
47. Weber M, Davies JJ, Wittig D, Oakeley EJ, Haase M, Lam WL, Schubeler D (2005) Chromosome-wide and promoter-specific analyses identify sites of differential DNA methylation in normal and transformed human cells. Nat Genet 37(8):853–862
48. Ehrlich M (2003) Expression of various genes is controlled by DNA methylation during mammalian development. J Cell Biochem 88:899–910
49. Shen L, Kondo Y, Guo Y, Zhang J, Zhang L, Ahmed S, Shu J, Chen X, Waterland RA, Issa JP (2007) Genome-wide profiling of DNA methylation reveals a class of normally methylated CpG island promoters. PLoS Genet 3(10):2023–2036
50. Ortmann CA, Eisele L, Nuckel H, Klein-Hitpass L, Fuhrer A, Duhrsen U, Zeschnigk M (2008) Aberrant hypomethylation of the cancer-testis antigen PRAME correlates with PRAME expression in acute myeloid leukemia. Ann Hematol 87(10):809–818
51. Milicic A, Harrison LA, Goodlad RA, Hardy RG, Nicholson AM, Presz M, Sieber O, Santander S, Pringle JH, Mandir N, East P, Obszynska J, Sanders S, Piazuelo E, Shaw J, Harrison R, Tomlinson IP, McDonald SA, Wright NA, Jankowski JA (2008) Ectopic expression of P-cadherin correlates with promoter hypomethylation early in colorectal carcinogenesis and enhanced intestinal crypt fission in vivo. Cancer Res 68(19):7760–7768

52. Cheung HH, Davis AJ, Lee TL, Pang AL, Nagrani S, Rennert OM, Chan WY (2011) Methylation of an intronic region regulates miR-199a in testicular tumor malignancy. Oncogene 30(31):3404–3415
53. Colaneri A, Staffa N, Fargo DC, Gao Y, Wang T, Peddada SD, Birnbaumer L (2011) Expanded methyl-sensitive cut counting reveals hypomethylation as an epigenetic state that highlights functional sequences of the genome. Proc Natl Acad Sci USA 108(23):9715–9720
54. Kwon MJ, Shin YK (2011) Epigenetic regulation of cancer-associated genes in ovarian cancer. Int J Mol Sci 12(2):983–1008
55. Laursen KB, Wong PM, Gudas LJ (2011) Epigenetic regulation by RAR{alpha} maintains ligand-independent transcriptional activity. Nucleic Acids Res 40(1):102–15
56. Baba Y, Nosho K, Shima K, Huttenhower C, Tanaka N, Hazra A, Giovannucci EL, Fuchs CS, Ogino S (2010) Hypomethylation of the IGF2 DMR in colorectal tumors, detected by bisulfite pyrosequencing, is associated with poor prognosis. Gastroenterology 139(6):1855–1864
57. Eckhardt F, Lewin J, Cortese R, Rakyan VK, Attwood J, Burger M, Burton J, Cox TV, Davies R, Down TA, Haefliger C, Horton R, Howe K, Jackson DK, Kunde J, Koenig C, Liddle J, Niblett D, Otto T, Pettett R, Seemann S, Thompson C, West T, Rogers J, Olek A, Berlin K, Beck S (2006) DNA methylation profiling of human chromosomes 6, 20 and 22. Nat Genet 38(12):1378–1385
58. Smale ST (2010) Pioneer factors in embryonic stem cells and differentiation. Curr Opin Genet Dev 20(5):519–526
59. Serandour AA, Avner S, Percevault F, Demay F, Bizot M, Lucchetti-Miganeh C, Barloy-Hubler F, Brown M, Lupien M, Metivier R, Salbert G, Eeckhoute J (2011) Epigenetic switch involved in activation of pioneer factor FOXA1-dependent enhancers. Genome Res 21(4): 555–565
60. Xu J, Pope SD, Jazirehi AR, Attema JL, Papathanasiou P, Watts JA, Zaret KS, Weissman IL, Smale ST (2007) Pioneer factor interactions and unmethylated CpG dinucleotides mark silent tissue-specific enhancers in embryonic stem cells. Proc Natl Acad Sci USA 104(30): 12377–12382
61. Taube JH, Allton K, Duncan SA, Shen L, Barton MC (2010) Foxa1 functions as a pioneer transcription factor at transposable elements to activate Afp during differentiation of embryonic stem cells. J Biol Chem 285(21):16135–16144
62. Magnani L, Eeckhoute J, Lupien M (2011) Pioneer factors: directing transcriptional regulators within the chromatin environment. Trends Genet 27(11):465–74
63. Hatada I, Namihira M, Morita S, Kimura M, Horii T, Nakashima K (2008) Astrocyte-specific genes are generally demethylated in neural precursor cells prior to astrocytic differentiation. PLoS One 3(9):e3189
64. Kalari S, Pfeifer GP (2010) Identification of driver and passenger DNA methylation in cancer by epigenomic analysis. Adv Genet 70:277–308
65. Meissner A, Mikkelsen TS, Gu H, Wernig M, Hanna J, Sivachenko A, Zhang X, Bernstein BE, Nusbaum C, Jaffe DB, Gnirke A, Jaenisch R, Lander ES (2008) Genome-scale DNA methylation maps of pluripotent and differentiated cells. Nature 454(7205):766–770
66. Laurent L, Wong E, Li G, Huynh T, Tsirigos A, Ong CT, Low HM, Kin Sung KW, Rigoutsos I, Loring J, Wei CL (2010) Dynamic changes in the human methylome during differentiation. Genome Res 20(3):320–331
67. Ball MP, Li JB, Gao Y, Lee JH, LeProust EM, Park IH, Xie B, Daley GQ, Church GM (2009) Targeted and genome-scale strategies reveal gene-body methylation signatures in human cells. Nat Biotechnol 27(4):361–368
68. Wu H, Coskun V, Tao J, Xie W, Ge W, Yoshikawa K, Li E, Zhang Y, Sun YE (2010) Dnmt3a-dependent nonpromoter DNA methylation facilitates transcription of neurogenic genes. Science 329(5990):444–448
69. Guo JU, Ma DK, Mo H, Ball MP, Jang MH, Bonaguidi MA, Balazer JA, Eaves HL, Xie B, Ford E, Zhang K, Ming GL, Gao Y, Song H (2011) Neuronal activity modifies the DNA methylation landscape in the adult brain. Nat Neurosci 14(10):1345–1351
70. Lister R, Pelizzola M, Dowen RH, Hawkins RD, Hon G, Tonti-Filippini J, Nery JR, Lee L, Ye Z, Ngo QM, Edsall L, Antosiewicz-Bourget J, Stewart R, Ruotti V, Millar AH, Thomson

JA, Ren B, Ecker JR (2009) Human DNA methylomes at base resolution show widespread epigenomic differences. Nature 462(7271):315–322
71. De Bustos C, Ramos E, Young JM, Tran RK, Menzel U, Langford CF, Eichler EE, Hsu L, Henikoff S, Dumanski JP, Trask BJ (2009) Tissue-specific variation in DNA methylation levels along human chromosome 1. Epigenetics Chromatin 2(1):7
72. Ke XS, Qu Y, Cheng Y, Li WC, Rotter V, Oyan AM, Kalland KH (2010) Global profiling of histone and DNA methylation reveals epigenetic-based regulation of gene expression during epithelial to mesenchymal transition in prostate cells. BMC Genomics 11:669
73. Cheng X, Blumenthal RM (2010) Coordinated chromatin control: structural and functional linkage of DNA and histone methylation. Biochemistry 49(14):2999–3008
74. Stengel S, Fiebig U, Kurth R, Denner J (2010) Regulation of human endogenous retrovirus-K expression in melanomas by CpG methylation. Genes Chromosomes Cancer 49(5):401–411
75. Appanah R, Dickerson DR, Goyal P, Groudine M, Lorincz MC (2007) An unmethylated 3' promoter-proximal region is required for efficient transcription initiation. PLoS Genet 3(2):e27
76. Chodavarapu RK, Feng S, Bernatavichute YV, Chen PY, Stroud H, Yu Y, Hetzel JA, Kuo F, Kim J, Cokus SJ, Casero D, Bernal M, Huijser P, Clark AT, Kramer U, Merchant SS, Zhang X, Jacobsen SE, Pellegrini M (2010) Relationship between nucleosome positioning and DNA methylation. Nature 466(7304):388–392
77. Hodges E, Smith AD, Kendall J, Xuan Z, Ravi K, Rooks M, Zhang MQ, Ye K, Bhattacharjee A, Brizuela L, McCombie WR, Wigler M, Hannon GJ, Hicks JB (2009) High definition profiling of mammalian DNA methylation by array capture and single molecule bisulfite sequencing. Genome Res 19(9):1593–1605
78. Luco RF, Allo M, Schor IE, Kornblihtt AR, Misteli T (2011) Epigenetics in alternative pre-mRNA splicing. Cell 144(1):16–26
79. Shing DC, Trubia M, Marchesi F, Radaelli E, Belloni E, Tapinassi C, Scanziani E, Mecucci C, Crescenzi B, Lahortiga I, Odero MD, Zardo G, Gruszka A, Minucci S, Di Fiore PP, Pelicci PG (2007) Overexpression of sPRDM16 coupled with loss of p53 induces myeloid leukemias in mice. J Clin Invest 117(12):3696–3707
80. Chu D, Zhang Z, Zhou Y, Wang W, Li Y, Zhang H, Dong G, Zhao Q, Ji G (2011) Notch1 and Notch2 have opposite prognostic effects on patients with colorectal cancer. Ann Oncol 22(11):2440–7
81. Figueroa JD, Flanders KC, Garcia-Closas M, Anderson WF, Yang XR, Matsuno RK, Duggan MA, Pfeiffer RM, Ooshima A, Cornelison R, Gierach GL, Brinton LA, Lissowska J, Peplonska B, Wakefield LM, Sherman ME (2010) Expression of TGF-beta signaling factors in invasive breast cancers: relationships with age at diagnosis and tumor characteristics. Breast Cancer Res Treat 121(3):727–735
82. Tsumagari K, Chang S-C, Lacey M, Baribault C, Chittur SV, Sowden J, Tawil R, Crawford GE, Ehrlich M (2011) Gene expression during normal and FSHD myogenesis. BMC Medical Genomics 4:67
83. Nagai H, Kim YS, Yasuda T, Ohmachi Y, Yokouchi H, Monden M, Emi M, Konishi N, Nogami M, Okumura K, Matsubara K (1999) A novel sperm-specific hypomethylation sequence is a demethylation hotspot in human hepatocellular carcinomas. Gene 237(1): 15–20
84. Kriaucionis S, Heintz N (2009) The nuclear DNA base 5-hydroxymethylcytosine is present in Purkinje neurons and the brain. Science 324(5929):929–930
85. Li W, Liu M (2011) Distribution of 5-hydroxymethylcytosine in different human tissues. J Nucleic Acids 2011:870726
86. Haffner MC, Chaux A, Meeker AK, Esopi DM, Gerber J, Pellakuru LG, Toubaji A, Argani P, Iacobuzio-Donahue C, Nelson WG, Netto GJ, De Marzo AM, Yegnasubramanian S (2011) Global 5-hydroxymethylcytosine content is significantly reduced in tissue stem/progenitor cell compartments and in human cancers. Oncotarget 2(8):627–37
87. Stroud H, Feng S, Morey Kinney S, Pradhan S, Jacobsen SE (2011) 5-Hydroxymethylcytosine is associated with enhancers and gene bodies in human embryonic stem cells. Genome Biol 12(6):R54

88. Ficz G, Branco MR, Seisenberger S, Santos F, Krueger F, Hore TA, Marques CJ, Andrews S, Reik W (2011) Dynamic regulation of 5-hydroxymethylcytosine in mouse ES cells and during differentiation. Nature 473(7347):398–402
89. Pastor WA, Pape UJ, Huang Y, Henderson HR, Lister R, Ko M, McLoughlin EM, Brudno Y, Mahapatra S, Kapranov P, Tahiliani M, Daley GQ, Liu XS, Ecker JR, Milos PM, Agarwal S, Rao A (2011) Genome-wide mapping of 5-hydroxymethylcytosine in embryonic stem cells. Nature 473(7347):394–397
90. Szulwach KE, Li X, Li Y, Song CX, Han JW, Kim S, Namburi S, Hermetz K, Kim JJ, Rudd MK, Yoon YS, Ren B, He C, Jin P (2011) Integrating 5-hydroxymethylcytosine into the epigenomic landscape of human embryonic stem cells. PLoS Genet 7(6):e1002154
91. Song CX, Szulwach KE, Fu Y, Dai Q, Yi C, Li X, Li Y, Chen CH, Zhang W, Jian X, Wang J, Zhang L, Looney TJ, Zhang B, Godley LA, Hicks LM, Lahn BT, Jin P, He C (2011) Selective chemical labeling reveals the genome-wide distribution of 5-hydroxymethylcytosine. Nat Biotechnol 29(1):68–72
92. Robinson MD, Statham AL, Speed TP, Clark SJ (2010) Protocol matters: which methylome are you actually studying? Epigenomics 2(4):587–598
93. Kinney SM, Chin HG, Vaisvila R, Bitinaite J, Zheng Y, Esteve PO, Feng S, Stroud H, Jacobsen SE, Pradhan S (2011) Tissue-specific distribution and dynamic changes of 5-hydroxymethylcytosine in mammalian genomes. J Biol Chem 286(28):24685–24693
94. Jin SG, Wu X, Li AX, Pfeifer GP (2011) Genomic mapping of 5-hydroxymethylcytosine in the human brain. Nucleic Acids Res 39(12):5015–5024
95. Ehrlich M, Woods C, Yu M, Dubeau L, Yang F, Campan M, Weisenberger D, Long TI, Youn B, Fiala E, Laird P (2006) Quantitative analysis of association between DNA hypermethylation, hypomethylation, and DNMT RNA levels in ovarian tumors. Oncogene 25:2636–2645
96. Rodriguez J, Vives L, Jorda M, Morales C, Munoz M, Vendrell E, Peinado MA (2008) Genome-wide tracking of unmethylated DNA Alu repeats in normal and cancer cells. Nucleic Acids Res 36(3):770–784
97. Kim MJ, White-Cross JA, Shen L, Issa JP, Rashid A (2009) Hypomethylation of long interspersed nuclear element-1 in hepatocellular carcinomas. Mod Pathol 22(3):442–449
98. Roman AC, Gonzalez-Rico FJ, Molto E, Hernando H, Neto A, Vicente-Garcia C, Ballestar E, Gomez-Skarmeta JL, Vavrova-Anderson J, White RJ, Montoliu L, Fernandez-Salguero PM (2011) Dioxin receptor and SLUG transcription factors regulate the insulator activity of B1 SINE retrotransposons via an RNA polymerase switch. Genome Res 21(3):422–432
99. Wang J, Lunyak VV, Jordan IK (2011) Genome-wide prediction and analysis of human chromatin boundary elements. Nucleic Acids Res 40(2):511–29
100. Dante R, Dante-Paire J, Rigal D, Roizes G (1992) Methylation patterns of long interspersed repeated DNA and alphoid repetitive DNA from human cell lines and tumors. Anticancer Res 12(2):559–563
101. Jurgens B, Schmitz-Drager BJ, Schulz WA (1996) Hypomethylation of L1 LINE sequences prevailing in human urothelial carcinoma. Cancer Res 56(24):5698–5703
102. Florl AR, Lower R, Schmitz-Drager BJ, Schulz WA (1999) DNA methylation and expression of LINE-1 and HERV-K provirus sequences in urothelial and renal cell carcinomas. Br J Cancer 80(9):1312–1321
103. Schulz WA, Steinhoff C, Florl AR (2006) Methylation of endogenous human retroelements in health and disease. Curr Top Microbiol Immunol 310:211–250
104. Chan SW, Zilberman D, Xie Z, Johansen LK, Carrington JC, Jacobsen SE (2004) RNA silencing genes control de novo DNA methylation. Science 303(5662):1336
105. Narayan A, Ji W, Zhang X-Y, Marrogi A, Graff JR, Baylin SB, Ehrlich M (1998) Hypomethylation of pericentromeric DNA in breast adenocarcinomas. Int J Cancer 77:833–838
106. Qu G, Dubeau L, Narayan A, Yu M, Ehrlich M (1999) Satellite DNA hypomethylation vs. overall genomic hypomethylation in ovarian epithelial tumors of different malignant potential. Mut Res 423:91–101
107. Bollati V, Fabris S, Pegoraro V, Ronchetti D, Mosca L, Deliliers GL, Motta V, Bertazzi PA, Baccarelli A, Neri A (2009) Differential repetitive DNA methylation in multiple myeloma molecular subgroups. Carcinogenesis 30(8):1330–1335

108. Thoraval D, Asakawa J, Wimmer K, Kuick R, Lamb B, Richardson B, Ambros P, Glover T, Hanash S (1996) Demethylation of repetitive DNA sequences in neuroblastoma. Genes Chromosomes Cancer 17(4):234–244
109. Nagai H, Baba M, Konishi N, Kim YS, Nogami M, Okumura K, Emi M, Matsubara K (1999) Isolation of NotI clusters hypomethylated in HBV-integrated hepatocellular carcinomas by two-dimensional electrophoresis. DNA Res 6(4):219–225
110. Itano O, Ueda M, Kikuchi K, Hashimoto O, Hayatsu S, Kawaguchi M, Seki H, Aiura K, Kitajima M (2002) Correlation of postoperative recurrence in hepatocellular carcinoma with demethylation of repetitive sequences. Oncogene 21(5):789–797
111. Katargin AN, Pavlova LS, Kisseljov FL, Kisseljova NP (2009) Hypermethylation of genomic 3.3-kb repeats is frequent event in HPV-positive cervical cancer. BMC Med Genomics 2:30
112. Szpakowski S, Sun X, Lage JM, Dyer A, Rubinstein J, Kowalski D, Sasaki C, Costa J, Lizardi PM (2009) Loss of epigenetic silencing in tumors preferentially affects primate-specific retroelements. Gene 448(2):151–167
113. Choi SH, Worswick S, Byun HM, Shear T, Soussa JC, Wolff EM, Douer D, Garcia-Manero G, Liang G, Yang AS (2009) Changes in DNA methylation of tandem DNA repeats are different from interspersed repeats in cancer. Int J Cancer 125(3):723–729
114. Ehrlich M, Hopkins N, Jiang G, Dome JS, Yu MS, Woods CB, Tomlinson GE, Chintagumpala M, Champagne M, Diller L, Parham DM, Sawyer J (2003) Satellite hypomethylation in karyotyped Wilms tumors. Cancer Genet Cytogenet 141:97–105
115. Jackson K, Yu M, Arakawa K, Fiala E, Youn B, Fiegl H, Muller-Holzner E, Widschwendter M, Ehrlich M (2004) DNA hypomethylation is prevalent even in low-grade breast cancers. Cancer Biol Ther 3(12):1225–1231
116. Morey Kinney SR, Smiraglia DJ, James SR, Moser MT, Foster BA, Karpf AR (2008) Stage-specific alterations of DNA methyltransferase expression, DNA hypermethylation, and DNA hypomethylation during prostate cancer progression in the transgenic adenocarcinoma of mouse prostate model. Mol Cancer Res 6(8):1365–1374
117. Kerbel RS, Frost P, Liteplo R, Carlow DA, Elliott BE (1984) Possible epigenetic mechanisms of tumor progression: induction of high-frequency heritable but phenotypically unstable changes in the tumorigenic and metastatic properties of tumor cell populations by 5-azacytidine treatment. J Cell Physiol Suppl 3:87–97
118. Santourlidis S, Florl A, Ackermann R, Wirtz HC, Schulz WA (1999) High frequency of alterations in DNA methylation in adenocarcinoma of the prostate. Prostate 39(3):166–174
119. Itano O, Ueda M, Kikuchi K, Shimazu M, Kitagawa Y, Aiura K, Kitajima M (2000) A new predictive factor for hepatocellular carcinoma based on two- dimensional electrophoresis of genomic DNA. Oncogene 19(13):1676–1683
120. Grunau C, Sanchez C, Ehrlich M, van der Bruggen P, Hindermann W, Rodriguez C, Krieger S, De Sario A (2005) Frequent DNA hypomethylation in the human juxtacentromeric BAGE loci in cancer. Genes Chrom Cancer 43(1):11–24
121. Roman-Gomez J, Jimenez-Velasco A, Agirre X, Castillejo JA, Navarro G, San Jose-Eneriz E, Garate L, Cordeu L, Cervantes F, Prosper F, Heiniger A, Torres A (2008) Repetitive DNA hypomethylation in the advanced phase of chronic myeloid leukemia. Leuk Res 32(3): 487–490
122. Yegnasubramanian S, Haffner MC, Zhang Y, Gurel B, Cornish TC, Wu Z, Irizarry RA, Morgan J, Hicks J, DeWeese TL, Isaacs WB, Bova GS, De Marzo AM, Nelson WG (2008) DNA hypomethylation arises later in prostate cancer progression than CpG island hypermethylation and contributes to metastatic tumor heterogeneity. Cancer Res 68(21):8954–8967
123. Marchal R, Chicheportiche A, Dutrillaux B, Bernardino-Sgherri J (2004) DNA methylation in mouse gametogenesis. Cytogenet Genome Res 105(2–4):316–324
124. Zhang X-Y, Loflin PT, Gehrke CW, Andrews PA, Ehrlich M (1987) Hypermethylation of human DNA sequences in embryonal carcinoma cells and somatic tissues but not sperm. Nucleic Acids Res 15:9429–9449
125. Rubin CM, VandeVoort CA, Teplitz RL, Schmid CW (1994) Alu repeated DNAs are differentially methylated in primate germ cells. Nucleic Acids Res 22(23):5121–5127
126. Dupressoir A, Heidmann T (1997) Expression of intracisternal A-particle retrotransposons in primary tumors of oncogene-expressing transgenic mice. Oncogene 14(24):2951–2958

127. Smiraglia DJ, Szymanska J, Kraggerud SM, Lothe RA, Peltomaki P, Plass C (2002) Distinct epigenetic phenotypes in seminomatous and nonseminomatous testicular germ cell tumors. Oncogene 21(24):3909–3916
128. Netto GJ, Nakai Y, Nakayama M, Jadallah S, Toubaji A, Nonomura N, Albadine R, Hicks JL, Epstein JI, Yegnasubramanian S, Nelson WG, De Marzo AM (2008) Global DNA hypomethylation in intratubular germ cell neoplasia and seminoma, but not in nonseminomatous male germ cell tumors. Mod Pathol 21(11):1337–1344
129. Laird CD, Pleasant ND, Clark AD, Sneeden JL, Hassan KM, Manley NC, Vary JC Jr, Morgan T, Hansen RS, Stoger R (2004) Hairpin-bisulfite PCR: assessing epigenetic methylation patterns on complementary strands of individual DNA molecules. Proc Natl Acad Sci USA 101(1):204–209
130. Jiang G, Yang F, van Overveld PG, Vedanarayanan V, van der Maarel S, Ehrlich M (2003) Testing the position-effect variegation hypothesis for facioscapulohumeral muscular dystrophy by analysis of histone modification and gene expression in subtelomeric 4q. Hum Mol Genet 12:2909–2921
131. Zeng W, de Greef JC, Chen YY, Chien R, Kong X, Gregson HC, Winokur ST, Pyle A, Robertson KD, Schmiesing JA, Kimonis VE, Balog J, Frants RR, Ball AR Jr, Lock LF, Donovan PJ, van der Maarel SM, Yokomori K (2009) Specific loss of histone H3 lysine 9 trimethylation and HP1gamma/cohesin binding at D4Z4 repeats is associated with facioscapulohumeral dystrophy (FSHD). PLoS Genet 5(7):e1000559
132. Shao C, Lacey M, Dubeau L, Ehrlich M (2009) Hemimethylation footprints of DNA demethylation in cancer. Epigenetics 4(3):165–175
133. Lau S, Jardine K, McBurney MW (1999) DNA methylation pattern of a tandemly repeated LacZ transgene indicates that most copies are silent. Dev Dyn 215(2):126–138
134. Takai D, Jones PA (2002) Comprehensive analysis of CpG islands in human chromosomes 21 and 22. Proc Natl Acad Sci USA 99(6):3740–3745
135. Hu JL, Zhou BO, Zhang RR, Zhang KL, Zhou JQ, Xu GL (2009) The N-terminus of histone H3 is required for de novo DNA methylation in chromatin. Proc Natl Acad Sci USA 106(52):22187–22192
136. Schulte JH, Lim S, Schramm A, Friedrichs N, Koster J, Versteeg R, Ora I, Pajtler K, Klein-Hitpass L, Kuhfittig-Kulle S, Metzger E, Schule R, Eggert A, Buettner R, Kirfel J (2009) Lysine-specific demethylase 1 is strongly expressed in poorly differentiated neuroblastoma: implications for therapy. Cancer Res 69(5):2065–2071
137. Wang J, Hevi S, Kurash JK, Lei H, Gay F, Bajko J, Su H, Sun W, Chang H, Xu G, Gaudet F, Li E, Chen T (2009) The lysine demethylase LSD1 (KDM1) is required for maintenance of global DNA methylation. Nat Genet 41(1):125–129
138. Zampieri M, Passananti C, Calabrese R, Perilli M, Corbi N, De Cave F, Guastafierro T, Bacalini MG, Reale A, Amicosante G, Calabrese L, Zlatanova J, Caiafa P (2009) Parp1 localizes within the Dnmt1 promoter and protects its unmethylated state by its enzymatic activity. PLoS One 4(3):e4717
139. Ostler KR, Davis EM, Payne SL, Gosalia BB, Exposito-Cespedes J, Le Beau MM, Godley LA (2007) Cancer cells express aberrant DNMT3B transcripts encoding truncated proteins. Oncogene 26(38):5553–5563
140. Lopez de Silanes I, Gorospe M, Taniguchi H, Abdelmohsen K, Srikantan S, Alaminos M, Berdasco M, Urdinguio RG, Fraga MF, Jacinto FV, Esteller M (2009) The RNA-binding protein HuR regulates DNA methylation through stabilization of DNMT3b mRNA. Nucleic Acids Res 37(8):2658–2671
141. Shukla V, Coumoul X, Lahusen T, Wang RH, Xu X, Vassilopoulos A, Xiao C, Lee MH, Man YG, Ouchi M, Ouchi T, Deng CX (2010) BRCA1 affects global DNA methylation through regulation of DNMT1. Cell Res 20(11):1201–1215
142. Felle M, Joppien S, Nemeth A, Diermeier S, Thalhammer V, Dobner T, Kremmer E, Kappler R, Langst G (2011) The USP7/Dnmt1 complex stimulates the DNA methylation activity of Dnmt1 and regulates the stability of UHRF1. Nucleic Acids Res 39(19):8355–65
143. Sharma S, De Carvalho DD, Jeong S, Jones PA, Liang G (2011) Nucleosomes containing methylated DNA stabilize DNA methyltransferases 3A/3B and ensure faithful epigenetic inheritance. PLoS Genet 7(2):e1001286

144. Chen ZX, Riggs AD (2011) DNA methylation and demethylation in mammals. J Biol Chem 286(21):18347–18353
145. He YF, Li BZ, Li Z, Liu P, Wang Y, Tang Q, Ding J, Jia Y, Chen Z, Li L, Sun Y, Li X, Dai Q, Song CX, Zhang K, He C, Xu GL (2011) Tet-mediated formation of 5-carboxylcytosine and its excision by TDG in mammalian DNA. Science 333(6047):1303–1307
146. Kress C, Thomassin H, Grange T (2006) Active cytosine demethylation triggered by a nuclear receptor involves DNA strand breaks. Proc Natl Acad Sci USA 103(30):11112–11117
147. Bhutani N, Burns DM, Blau HM (2011) DNA demethylation dynamics. Cell 146(6): 866–872
148. Inoue A, Zhang Y (2011) Replication-dependent loss of 5-hydroxymethylcytosine in mouse preimplantation embryos. Science 334(6053):194
149. Holliday R, Pugh JE (1975) DNA modification mechanisms and gene activity during development. Science 187:226
150. Riggs AD (1975) X chromosome inactivation, differentiation and DNA methylation. Cytogenet Cell Genet 14:9–25
151. Jones PA, Liang G (2009) Rethinking how DNA methylation patterns are maintained. Nat Rev Genet 10(11):805–811
152. Otto SP, Walbot V (1990) DNA methylation in eukaryotes: kinetics of demethylation and de novo methylation during the life cycle. Genetics 124(2):429–437
153. Pfeifer GP, Steigerwald SD, Hansen RS, Gartler SM, Riggs AD (1990) Polymerase chain reaction-aided genomic sequencing of an X chromosome-linked CpG island: methylation patterns suggest clonal inheritance, CpG site autonomy, and an explanation of activity state stability. Proc Natl Acad Sci USA 87(21):8252–8256
154. Nicolas P, Kim KM, Shibata D, Tavare S (2007) The stem cell population of the human colon crypt: analysis via methylation patterns. PLoS Comput Biol 3(3):e28
155. Sontag LB, Lorincz MC, Georg Luebeck E (2006) Dynamics, stability and inheritance of somatic DNA methylation imprints. J Theor Biol 242(4):890–899
156. Genereux DP, Miner BE, Bergstrom CT, Laird CD (2005) A population-epigenetic model to infer site-specific methylation rates from double-stranded DNA methylation patterns. Proc Natl Acad Sci USA 102(16):5802–5807
157. Lacey M, Ehrlich M (2009) Modeling dependence in methylation patterns with application to ovarian carcinomas. Stat Appl Genet Mol Biol 8(1):40
158. Saluz HP, Jiricny J, Jost JP (1986) Genomic sequencing reveals a positive correlation between the kinetics of strand-specific DNA demethylation of the overlapping estradiol/glucocorticoid-receptor binding sites and the rate of avian vitellogenin mRNA synthesis. Proc Natl Acad Sci USA 83(19):7167–7171
159. Paroush Z, Keshet I, Yisraeli J, Cedar H (1990) Dynamics of demethylation and activation of the alpha-actin gene in myoblasts. Cell 63(6):1229–1237
160. Liang G, Chan MF, Tomigahara Y, Tsai YC, Gonzales FA, Li E, Laird PW, Jones PA (2002) Cooperativity between DNA methyltransferases in the maintenance methylation of repetitive elements. Mol Cell Biol 22(2):480–491
161. Chen T, Hevi S, Gay F, Tsujimoto N, He T, Zhang B, Ueda Y, Li E (2007) Complete inactivation of DNMT1 leads to mitotic catastrophe in human cancer cells. Nat Genet 39(3): 391–396
162. Gehring M, Reik W, Henikoff S (2009) DNA demethylation by DNA repair. Trends Genet 25(2):82–90
163. Zhu JK (2009) Active DNA demethylation mediated by DNA glycosylases. Annu Rev Genet 43:143–166
164. Bhutani N, Brady JJ, Damian M, Sacco A, Corbel SY, Blau HM (2010) Reprogramming towards pluripotency requires AID-dependent DNA demethylation. Nature 463(7284): 1042–1047
165. Kress C, Thomassin H, Grange T (2001) Local DNA demethylation in vertebrates: how could it be performed and targeted? FEBS Lett 494(3):135–140
166. Meilinger D, Fellinger K, Bultmann S, Rothbauer U, Bonapace IM, Klinkert WE, Spada F, Leonhardt H (2009) Np95 interacts with de novo DNA methyltransferases, Dnmt3a and Dnmt3b, and mediates epigenetic silencing of the viral CMV promoter in embryonic stem cells. EMBO Rep 10(11):1259–1264

167. Jeong S, Liang G, Sharma S, Lin JC, Choi SH, Han H, Yoo CB, Egger G, Yang AS, Jones PA (2009) Selective anchoring of DNA methyltransferases 3A and 3B to nucleosomes containing methylated DNA. Mol Cell Biol 29(19):5366–5376
168. Hervouet E, Lalier L, Debien E, Cheray M, Geairon A, Rogniaux H, Loussouarn D, Martin SA, Vallette FM, Cartron PF (2010) Disruption of Dnmt1/PCNA/UHRF1 interactions promotes tumorigenesis from human and mice glial cells. PLoS One 5(6):e11333
169. Frommer M, McDonald LE, Millar DS, Collis CM, Watt F, Grigg GW, Molloy PL, Paul CL (1992) A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. Proc Natl Acad Sci USA 89:1827–1831
170. Valinluck V, Sowers LC (2007) Endogenous cytosine damage products alter the site selectivity of human DNA maintenance methyltransferase DNMT1. Cancer Res 67(3):946–950
171. Burden AF, Manley NC, Clark AD, Gartler SM, Laird CD, Hansen RS (2005) Hemimethylation and non-CpG methylation levels in a promoter region of human LINE-1 (L1) repeated elements. J Biol Chem 280(15):14413–14419
172. Turker MS (2002) Gene silencing in mammalian cells and the spread of DNA methylation. Oncogene 21(35):5388–5393
173. Yan PS, Shi H, Rahmatpanah F, Hsiau TH, Hsiau AH, Leu YW, Liu JC, Huang TH (2003) Differential distribution of DNA methylation within the RASSF1A CpG island in breast cancer. Cancer Res 63(19):6178–6186
174. Nguyen C, Liang G, Nguyen TT, Tsao-Wei D, Groshen S, Lubbert M, Zhou JH, Benedict WF, Jones PA (2001) Susceptibility of nonpromoter CpG islands to de novo methylation in normal and neoplastic cells. J Natl Cancer Inst 93(19):1465–1472
175. Stirzaker C, Song JZ, Davidson B, Clark SJ (2004) Transcriptional gene silencing promotes DNA hypermethylation through a sequential change in chromatin modifications in cancer cells. Cancer Res 64(11):3871–3877
176. Homma N, Tamura G, Honda T, Matsumoto Y, Nishizuka S, Kawata S, Motoyama T (2006) Spreading of methylation within RUNX3 CpG island in gastric cancer. Cancer Sci 97(1): 51–56

Chapter 3
Ten Eleven Translocation Enzymes and 5-Hydroxymethylation in Mammalian Development and Cancer

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Abstract 5-Hydroxymethylcytosine (5hmC) is an oxidative product of 5-methylcytosine (5mC), catalyzed by the ten eleven translocation (TET) family of enzymes. Although 5hmC was discovered several decades ago, it was only after its recent identification in murine brain and stem cell DNA that it has become a major focus of epigenomic research. Part of the reason for this delay is due to the difficulty in detecting both global and locus-specific 5hmC levels. Several studies have addressed this issue with the development of novel techniques to locate and measure 5hmC, which led to multiple reports detailing 5hmC patterns in stem cells and global 5hmC levels during embryogenesis. Based on these studies of 5hmC levels and reports of tissue-specific TET expression, these enzymes are thought to play a role in mammalian development and differentiation. In addition, the TET enzymes are mutated in several types of cancer, affecting their activity and likely altering genomic 5hmC and 5mC patterns. Furthermore, oxidation of 5mC appears to be a step in several active DNA demethylation pathways, which may be important for normal processes, as well as global hypomethylation during cancer development and progression. Much has been revealed about this interesting DNA modification in recent years, but more research is needed for understanding the role of TET proteins and 5hmC in gene regulation and disease.

3.1 Discovery and History of 5-Hydroxymethylation

Methylation of cytosine residues at the 5-carbon position (5-methylcytosine, 5mC) has been studied as a stable epigenetic modification for decades [1]. However, oxidation of DNA has traditionally been considered a DNA damage event, which is readily removed by DNA repair pathways [2]. Recently, it was demonstrated that

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enzymatic oxidation of 5mC to 5hmC (5-hydroxymethylcytosine) may act as a stable modification of DNA and downstream removal of 5hmC may actually be part of a complex and intricate process of epigenetic gene regulation [3].

5-Hydroxymethylcytosine (5hmC) was first identified in T-even bacteriophages during early 1950s using paper chromatography and ultraviolet absorbance spectra [4]. This nucleotide is normally incorporated during DNA synthesis and then further glycosylated by phage encoded glucosyltransferases as a mechanism for protection of the phage DNA from bacterial restriction enzymes during infection [5, 6]. Later, during the 1970s, 5hmC was detected in genomic DNA purified from brain tissue of rats, mice, and frogs and, to a lesser extent, from liver tissue of rats [7]. The same group also observed an increase in 5hmC levels in the adult compared to newborn rat brain, as well as a decrease of 5hmC levels in brains from rats with low protein diets [8]. Unfortunately, these experiments could not be reproduced and this DNA modification was overlooked for several decades [9].

In 2009, 5hmC was rediscovered in mammalian DNA and shown to be present in substantial amounts (~10 to 20% of 5mC) in murine embryonic stem cells (ESCs) [10], Purkinje neurons, and granule cells [11]. These recent studies utilized more advanced analytical techniques, such as 2D thin layer chromatography (TLC) or high-pressure liquid chromatography (HPLC) coupled with mass spectrometry (MS), to confirm the existence of this DNA modification in mammalian DNA. In addition, 5hmC was recently identified in mammalian mitochondrial DNA [12]. As a result of these discoveries, there is a huge amount of interest in developing technologies for genome-wide mapping and site-specific quantification of 5hmC in an effort to decipher its possible role in development and disease.

3.2 TET Enzymes and Their Catalytic Activity

There are three known mammalian 5mC dioxygenases, which catalyze the conversion of 5mC to 5hmC [10]. These proteins belong to the family of ten eleven translocation (TET) enzymes, whose name is based on a common chromosomal translocation in some cancers (described in detail later in this chapter). TET1 was originally named leukemia-associated protein with a CXXC domain (LCX) when it was initially cloned in 2002 [13]. This gene was rediscovered in 2003 along with the two other members of the family and they were renamed ten eleven translocation, or TET, genes [14]. All three TET proteins share a similar catalytic domain structure to 2-oxoglutarate (2-OG) oxygenases. These types of enzymes can oxidize DNA and RNA that is methylated on either the nitrogen (N) or carbon (C) of the base by conversion of 2-OG and oxygen to carbon dioxide and succinate [15]. The TETs were identified based on their similarity to the JBP1 and JBP2 enzymes in trypanosome, which were originally named for their ability to bind to the unique nucleotide β-D-glucosylhydroxymethyl-uracil (base J) and then later were reported to hydroxylate thymine, the first step in the conversion of base J [16]. Proteins with similar

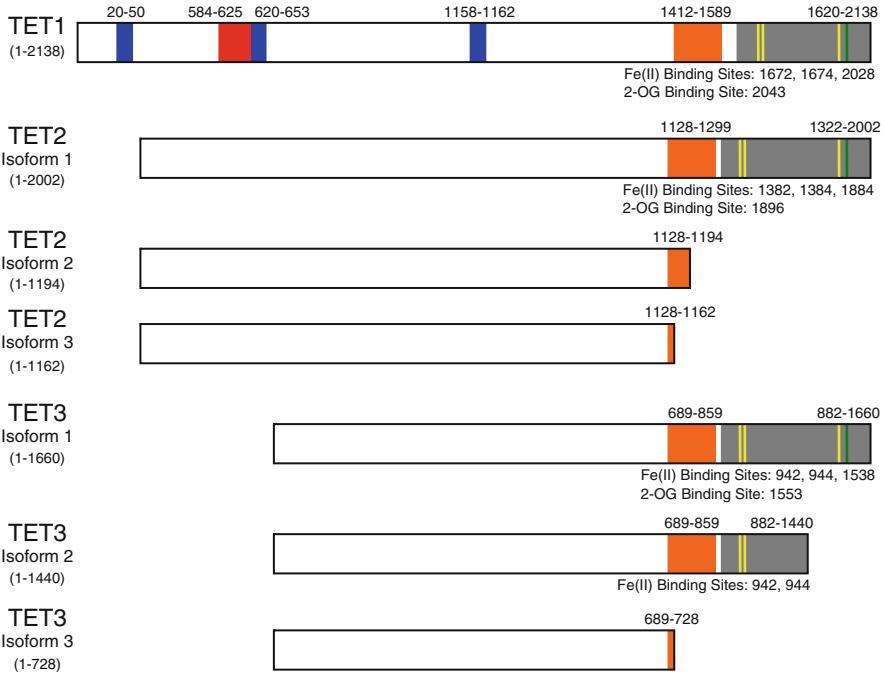

Fig. 3.1 Diagram of TET enzyme isoforms. TET1 is 2138 aa long with multiple putative Nuclear Localization Sequence (NLS), a CXXC motif, and cysteine-rich region N-terminal to the DSBH making up the core catalytic domain. There are three isoforms of TET2, the longest being 2002 aa long. TET2 does not contain any putative NLS or CXXC motif, but does have a core catalytic domain very similar to TET1. TET3 also has three isoforms, of 1660 aa or less in length. Similar to TET2, TET3 does not appear to have any other domains other than the core catalytic domain. Numbers in brackets represent length of proteins in aa or location of domains. *Blue bars* NLS; *red bar* CXXC motif; *orange* bar Cysteine-rich region; *Gray bar* DSBH; *yellow bars* Fe(II) binding sites; *green bar* 2-OG binding site

homology to the TET proteins are found in several lower eukaryote groups, including *Drosophila* [17].

The human TET1 gene is found at chromosomal location 10q21 and is approximately 134 kilobases (kb) long [18]. The resulting transcript contains 12 exons and is approximately 9.6 kb. The TET1 protein consists of ~2,136 amino acids encoding a 236 kilodalton (kDa) enzyme. TET1 is a multidomain protein containing several putative nuclear localization sequences, a binuclear Zn^{+2}-chelating CXXC domain, and a cysteine-rich region preceding the catalytic domain (Fig. 3.1). CXXC domains are frequently found in chromatin binding proteins, including DNA (cytosine-5) methyltransferase-1 (DNMT1), 5-methylcytosine binding proteins (MBDs), and mixed lineage leukemia (MLL) protein [19]. The CXXC domain of TET1 binds to CG-rich sequences of both methylated and unmethylated DNA, with some preference for unmethylated CpGs in cell free assays [19, 20].

The human TET2 gene is found on chromosome 4q24 and contains 11 exons, which can result in three known isoforms produced through alternative splicing. The longest form of TET2 is ~2,002 amino acids and similar to TET1 with approximately 70% homology in their C-terminal regions, including their catalytic domains [18]. There are two shorter isoforms of TET2 (1,164 and 1,194 amino acids long) that both lack catalytic domains due to truncation or introduction of stop codons (Fig. 3.1). The TET3 gene resides on chromosome 2p13. It is approximately 62 kb in length, with a transcript containing nine exons. Similar to TET2, the TET3 protein sequence shares approximately 70% sequence homology to TET1 in the regions surrounding the catalytic domain (Fig. 3.1). Three putative isoforms of TET3 have been identified using complementary DNA screening [18]. These include the full-length protein, as well as two shorter variants that are missing either a small portion, or most of the catalytic domain (Fig. 3.1).

TET2 and TET3 differ from TET1 in that they do not appear to contain any putative nuclear localization sequences or regions similar to a CXXC domain [18]. Interestingly, one study reported that the CXXC4 gene, at 4q22-24, is a very close neighbor to TET2 and may be the result of a chromosomal inversion of the TET2 CXXC domain followed by a translocation [17]. It has been proposed that interaction of CXXC4 and TET2 may be required for appropriate TET2 targeting and activity [17].

The catalytic domains of all 2-oxoglutarate (2-OG) oxygenases contain a double-stranded β-helix (DSBH) [10, 15]. The DSBH domain, in addition to the cysteine-rich region, of TET1 has been found to be both necessary and sufficient for catalytic activity [10]. Furthermore, the DSBH domain contains three Fe(II) binding sites and a 2-OG binding site (details in Fig. 3.1) [18]. Amino acid mutation studies have confirmed the requirement of these domains for TET catalytic activity [21].

The increased homology within the cysteine-rich region and the DSBH domain of TET1, TET2, and TET3 suggests that they have similar catalytic activity. Each protein of this family also contains unique regions indicating that they may have distinct binding affinities to chromatin and/or protein partners, resulting in the establishment of specific 5hmC patterns in various cell types and during different developmental stages. All three forms of the Tet enzymes are known to be catalytically active in cells [22] and tissue-specific expression of TET transcripts has also been reported [23, 24], supporting the above hypothesis.

Triple knockout (TKO, knockout of Dnmt1, Dnmt3a, and Dnmt3b) ESCs display decreased 5hmC levels although they have normal Tet expression. This confirms that the 5mC catalyzed by Dnmts is in fact the substrate for the Tet enzymes [22, 25]. In addition to the oxidation of 5mC to 5hmC, the TET enzymes have recently been reported to have the ability to further oxidize 5hmC to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) [26, 27]. Quantification of the three oxygenated forms of 5mC reveals unequal distribution with much more 5hmC than 5fC or 5caC in genomic DNA [27]. The function of these less frequent enzymatic products of TET enzymes is not well understood, but current knowledge

suggests they may be involved in the DNA demethylation process described later in this chapter [28].

3.3 Technologies and Advancements in 5hmC Detection

Identifying and quantifying 5hmC globally and at specific loci has been, and continues to be, quite a challenge. For example, the most accepted technique for 5mC detection and measurement, bisulfite sequencing, does not differentiate between 5mC and 5hmC or unmodified C and 5caC [28, 29]. Additionally, restriction enzymes have been used for years to specifically digest methylated or unmethylated DNA and recent data shows that many of these enzymes have different specificities or sensitivities for oxidized forms of 5mC or glucosylated 5hmC (5ghmC) [30–33]. Indeed, many of the 5mC-sensitive enzymes that have previously been used to measure DNA methylation are also sensitive to 5hmC [34]. Complicating matters further, 5mC-specific antibodies appear to have no cross reactivity with 5hmC, thus in the past oxidation of 5mC may have been mistaken for demethylation. Since the discovery of 5hmC in mammalian DNA there has been a flurry of new techniques reported to measure this elusive base, either globally or at a specific locus.

There are several techniques that have been shown to evaluate global 5hmC levels. Some are more qualitative than quantitative and each has its own range of sensitivity and accuracy. Initially, the existence of 5hmC (followed by 5fC and 5caC) in mammalian DNA was discovered using restriction enzyme-based TLC [7, 10, 11, 27, 28]. Dot blot of genomic DNA and immunofluorescence in mammalian cells using 5hmC-specific antibodies has also been used extensively to examine global 5hmC levels [22, 25, 35]. These antibodies appear to be sensitive but seem to require several proximal 5hmC sites for measurable binding to occur [36]. More recently, an antibody was developed targeting cytosine 5-methylenesulfonate (CMS), a product of sodium bisulfite treatment of hydroxymethylated DNA that can apparently detect as few as one 5hmC site on DNA [21]. Although these techniques are not truly quantitative, they offer more sensitivity as the input DNA could be as low as several nanograms. Currently, the most sensitive techniques for measuring global 5hmC, 5fC, and 5caC utilize HPLC coupled with mass spectrometry [27, 28, 37]. However, these techniques require unique expertise and complex analytical machinery. A simple, yet very accurate and sensitive, technique for measuring global 5hmC uses the T4 phage enzyme, β-glucosyltransferase (β-GT), and radioactive UDP-[^{3}H]-glucose [38, 39].

The 5hmC and CMS-specific antibodies mentioned above have also been utilized for hydroxymethylcytosine-DNA immunoprecipitation (hMe-DIP) followed by next generation sequencing, DNA array, or PCR [25, 36, 40, 41]. A second technique, (glucosylation, periodate oxidation, biotinylation, or GLIB) uses a glucosylation reaction to attach UDP-6-N3-glucose to 5hmC, which marks these sites with a reactive azide group. The azide group is further reacted with biotin using click

chemistry for subsequent pulldown with a streptavidin matrix [42, 43]. Although data derived from these techniques can be extremely useful in mapping the regions of 5hmC, it still does not offer single base resolution. Single-molecule real-time (SMRT) sequencing is a novel sequencing technique that can discriminate between unmodified cytosine, 5mC, and 5hmC due to variations in polymerase kinetics during the sequencing reaction [44]. It is also possible to differentiate 5mC from 5hmC with nanopore amperometry, as each modification causes unique breaks in current as synthetic DNA molecules are fed through nanopores [45]. Current research is focused towards optimizing the last two methods for genomic DNA samples and for high-throughput analysis, but these technologies are not yet commonly used.

Many restriction enzymes that can differentiate between 5mC and unmodified cytosine, as well as families of enzymes that target 5hmC or 5ghmC are being studied for unique properties that make them useful for measuring 5hmC [30, 33, 46]. For example, MspI and GlaI can fully digest 5mC or 5hmC in their respective target sequences, but after conversion of 5hmC to 5ghmC, digestion by both of these enzymes is blocked [32, 47]. Taq$^{\alpha}$I is a restriction enzyme that is not fully blocked by 5ghmC, but is blocked by biotin-N3-5gmC [31]. Therefore, tagging a 5hmC residue with glucose or a modified glucose may be a valuable tool for epigenetic studies. In contrast to restriction enzymes that are blocked by 5hmC or 5ghmC, but not by unmodified cytosine, another class known as PvuRts1I family show digestion preference for 5hmC or 5ghmC as compared to 5mC and cytosine [30, 33]. Using this class of enzymes for digestion followed by PCR amplification of a region of interest can reveal the level of 5hmC at a specific site. Alternatively, one could use the digested fragments for next generation sequencing for genome-wide mapping of 5hmC.

Novel and more accurate techniques for measuring 5hmC will be available in the near future as the epigenetics field progresses with reference to this modification. We must always consider how to normalize traditional techniques and any new ones that are developed to evaluate various DNA modifications when drawing conclusions about how epigenomic modification patterns relate to biological phenomenon.

3.4 Tet1 Binding and 5hmC in Embryonic Stem Cells

It is important to understand the normal function of TET enzymes and 5hmC in order to comprehend how and why they may be disrupted in disease. The study of mouse ESCs may allow us to gain some insight into these phenomena. Mouse ESCs are derived from the inner cell mass (ICM) of blastocysts and can be cultured in an undifferentiated state with use of leukemia inhibitory factor (LIF) [48]. ESCs can be differentiated into embryoid bodies (EB) with the removal of LIF or into other more specific lineages by addition or removal of cytokines and specific growth factors. As mentioned earlier, ESCs tend to have high levels of 5hmC as compared to other cell

types [10, 27]. It is thought that the TET enzymes and 5hmC may play a significant biological role in ESCs because epigenetic modifications and factors are important for both maintaining an undifferentiated state and for differentiation. *Tet1* and *Tet2* are expressed in ESCs and induced pluripotent stem (iPS) cells, while *Tet3* expression is quite low, suggesting that Tet1 and Tet2 are especially important for maintaining a pluripotent status [22, 49]. Furthermore, expression of *Tet1* and *Tet2* is repressed during differentiation and it appears that Oct4 [49], one of a few transcription factors that are required for ESC pluripotency and dedifferentiation of somatic cells, is involved in regulating Tet1 and Tet2 expression [50].

A number of reports describe Tet1 binding and/or 5hmC status throughout the genome of mouse ESCs and the relationship of these patterns to gene expression [25, 41, 42, 47, 51, 52]. Several techniques were utilized in these studies, including ChIP-seq, GLIB-Seq, hMeDIP-Seq, restriction enzyme-dependent genome-wide sequencing, and hMeDIP-Chip (with 5hmC and CMS-specific antibodies), as well as RNA-Seq and microarray analyses [25, 41, 42, 51, 52]. Even though there are some disagreements between these studies, their overall conclusions are similar. In general, Tet1 binds to CG-rich regions of the genome, which seems to be due, at least in part, to its CXXC domain. Tet1 binds to both active and inactive genes, with more binding in the gene bodies of active genes and increased binding in the promoters and transcriptional start sites (TSS) of inactive genes. Tet1 targeted genes are involved in many cellular pathways, including development, differentiation, and neural processes [22, 25, 49, 52]. Tet1 also appears to be enriched in regions containing the active H3K4me3 mark, as well as the bivalent H3K4me3 and H3K27me3 marks, and to a lesser extent at polycomb repressed genes exhibiting only H3K27me3 mark.

5hmC patterns in the genome are very similar to Tet1 binding. Both 5mC and its oxidative product 5hmC are commonly found in the gene bodies of active genes and in the promoters of inactive genes. Surprisingly, there are a number of Tet1 binding sites that do not appear to contain 5hmC. This suggests that Tet1 may have additional non-catalytic activities or that 5hmC is quickly removed specifically at these loci as part of a DNA demethylation/repair pathway. Several studies indicate that gene body 5hmC is more prevalent in exons than introns [25, 42, 51, 52]; however, results from another group indicated more enrichment in introns [41]. These ambiguities could be due to differences in the techniques utilized and will likely be sorted out in the future with base resolution mapping of the respective mammalian hydroxymethylome. Interestingly, 5hmC is enriched in and around the TSS, which is in contrast to a general reduction in 5mC at these locations [51]. Intergenic regions and repetitive elements appear to have less 5hmC than coding regions. Thus, 5hmC and 5mC coexist in some genomic regions, while also displaying unique patterns of genomic localization. Genome-wide 5hmC patterns have also been reported for human ESCs and they closely match with the description of mouse ESCs [36]. The patterning observed in both mouse and human ESCs suggests that 5hmC may have a more specific role in regulating transcription, while 5mC has additional roles in maintaining genomic integrity and transposon stability.

Upon knockdown of Tet1 expression or gene knockout, there are clear increases in both locus-specific and global 5mC with concomitant decreases in

5hmC globally and at Tet1 target sites [22, 47, 53]. In addition, loss or reduction of Tet1 consistently resulted in both increased and decreased gene expression with gene activation being associated with promoter hypo-hydroxymethylation [41, 51–53]. Tet1 enrichment occurs at almost two-thirds of all genes in mouse ESCs and thus overlaps with a number of chromatin modifying and transcriptional regulatory proteins, such as Suz12, Ezh2, Sin3a, Mbd3, and LIF activated Stat3 [41, 47, 51, 54]. Concomitantly, the binding of these proteins to the chromatin is reduced by Tet1 knockdown [41, 47, 51, 54]. It is not clear whether it is direct interaction with Tet1, possibly via other bridge proteins, or 5hmC that provides a platform for their recruitment to specific regions of the chromatin, except in the cases of Sin3a and Mbd3. These two proteins have been shown to either bind directly to Tet1 or in a complex with Tet1 by co-immunoprecipitation experiments. Mbd3 also appears to bind to 5hmC-modified DNA, which is thought to result in its recruitment to inactive genes [51, 54].

There is an overall enrichment of 5hmC at regulatory protein binding sites, such as gene promoters, enhancers, and insulators further supporting the hypothesis of 5hmC-specific binding proteins [25, 36]. In addition to transcriptional regulation by putative 5hmC binding proteins, active promoters bound by Tet1 may be maintained in an unmethylated state through constant oxidation of 5mC, allowing transcription factors and RNA polymerase to bind. Based on these observations, Tet enzymes can regulate the levels of both 5hmC and 5mC at specific gene sequences in order to direct the binding of transcriptional regulator proteins, resulting in both positive and/or negative effects on its expression.

3.5 Role of Tets and 5hmC in Early Mammalian Development and Embryonic Stem Cells

The mammalian paternal zygotic genome is thought to be actively demethylated upon fertilization of the egg and this demethylated state persists over the next several cell divisions, during which time the maternal genome undergoes passive demethylation [55]. At the blastocyst stage of development, both the maternal and paternal DNAs are remethylated by the de novo methyltransferases. The observation that the paternal genome is demethylated is based primarily on studies utilizing anti-5mC antibody staining and bisulfite sequencing of a small number of loci [56–59]. However, recent data suggests that the lack of staining of the paternal genome by the 5mC antibody is actually due to conversion of 5mC to 5hmC [35, 60]. High levels of 5hmC in the paternal genome persist for several genome replications suggesting that demethylation is not as extensive as was previously thought and may take place only at specific loci [35]. Technological advances that allow for the paternal and maternal DNA to be fully sequenced for epigenetic modifications will help in the future to resolve this important observation.

Tet3 is the most likely Tet family member that oxidizes the paternal DNA as it is expressed at high levels in oocytes and zygotes, but not at later developmental stages

[35, 60, 61]. *Tet1* seems to only be expressed at the two- and four-cell stages and in ESCs, and *Tet2* is only expressed at very low levels throughout fertilization and zygote development, except in ESCs where Tet2 expression is higher [35, 60]. Knockdown of *Tet3* by siRNA injection into the oocyte or conditional knockout of Tet3 in primordial germ cells (PGC) of mice significantly reduces oxidation of 5mC in the paternal genome [60, 61]. Furthermore, the Tet3 responsible for this process appears to be of maternal origin as wild-type (WT) females crossed with Tet3 conditionally null males did not exhibit this defect [61]. Finally, primordial germ cell gene 7 (PGC7) may be involved in protecting the maternal genome from demethylation [62] and knockout of this gene results in oxidation of the maternal genome [60]. It is not clear why only the paternal genome methylation specifically undergoes widespread oxidation during zygote development, but this process is likely involved in locus-specific 5mC erasure and epigenetic reprogramming of the chromatin.

There are several contradicting reports on whether knockdown or knockout of Tet genes alters growth and differentiation of ESCs. Two studies report that knockdown of *Tet1*, but not *Tet2* or *Tet3*, in mouse ESCs results in decreased alkaline phosphatase activity (a marker of healthy ESCs) and pluripotency associated genes, as well as an increase in differentiation markers and altered cell growth and morphology [22, 47]. It is suggested that this may be due to a decrease in *Nanog* expression as reintroduction of Nanog can rescue the phenotype. ChIP analysis shows that Tet1 binds to the Nanog promoter and depletion of Tet1 results in methylation and suppression of the *Nanog* gene. Furthermore, use of Dnmt TKO ES cells prevents the methylation and repression of *Nanog* [22].

In contrast, other studies did not report any effects on morphology or *Nanog* expression with *Tet1* knockdown or knockout in undifferentiated cells [49, 51, 53]. However, there was agreement amongst some reports that *Tet1* knockdown upregulates genes involved in trophectoderm and endoderm development and represses genes involved in neuroectoderm development [22, 49, 53]. Loss of Tet1 function in ESCs results in differentiation toward endoderm/mesoderm and trophoblast lineages. Based on this, and because *Tet1* is primarily expressed in the ICM (not the trophectoderm), it is thought that Tet1 participates in preventing the expression of trophectoderm developmental genes and maintaining proper cellular specification in embryos [22, 49]. *Tet2* knockdown did not seem to affect trophectoderm, endoderm, or mesoderm genes but did slightly increase neuroectoderm markers. In addition, knockdown of either *Tet1* or *Tet2* alters expression of unique subsets of genes suggesting that each enzyme has unique target regions in the genome [49]. *Tet3* knockdown in ESCs had minimal transcriptional effects on the differentiation genes that were examined.

Tet1 knockout ESCs are capable of producing live pups and loss of Tet1 has minimal effects on embryogenesis and mouse development, as Tet1 homozygous null mice maintain proper Mendelian ratios, appear healthy, and are fertile [53]. The only initial observations of aberrant development are that both male and female Tet1 null mice are born at lower body weight (although they are similar to WT mice as adults); they have slightly decreased neutrophil numbers, and smaller litter sizes when inter-crossed. These mice do not appear to have any myeloid or other disorders

[53]. Complete knockout of Tet2 has not yet been reported, but a mouse model has been developed that utilizes a Tet2-LacZ fusion to express an inactive Tet2 protein [63]. However, these mice maintain 20–50% of normal Tet2 transcripts, have no obvious reduction in 5hmC, are normal in overall appearance, and display expected Mendelian ratios. In spite of this, and unlike Tet1 null mice, Tet2 hypomorphs do appear to have aberrant hematopoiesis [63]. Although no changes in Tet gene expression have been reported, it is possible that the different members of the Tet family are compensating for the loss or reduction of Tet1 or Tet2 in these mouse models [53, 63].

Tet3 null mice are unique in that they exhibit neonatal lethality [61]. This lethality was overcome by creation of Tet3 conditional knockout mice [61]. As described above, the parental mice only lack Tet3 expression in PGC and thus are essentially normal, with only the females exhibiting reduced fecundity. The zygotes of these mice have decreased 5hmC levels and aberrant reprogramming of the paternal DNA, which is thought to disrupt prenatal development [61].

Tissue-specific expression of Dnmts and patterning of 5mC is known to be involved in gene regulation. It is hypothesized that Tet enzyme activity and 5hmC may be involved in specific biological functions in different tissues and organs as well. Indeed, TET enzymes display altered expression levels depending on the tissue or the stage of development [22–24, 35, 60]. A number of tissue types have been examined for TET expression, including but not limited to brain, lung, liver, heart, and kidney. *TET1* and *TET2* exhibit varied expression levels in different tissues examined [23] and isoforms 2 and 3 of *TET2* are expressed at a lower level than its isoform 1 [24]. Overall TET2 and its isoforms appear to be the most highly expressed amongst the TET enzymes in many tissues [22, 24]. *TET3* also tends to have consistently high expression across various tissues [23]. All TETs are highly expressed in hematopoietic cells, with *TET2* and *TET3* being the highest. Consistently, hypomorphic expression of Tet2 in mice has been shown to alter hematopoietic development [23, 24, 63].

Several studies have measured global 5hmC in DNA from various tissues using the techniques described above [26–28, 64]. Based on these analyses one would conclude that in addition to tissue-specific expression of TET enzymes, many tissues also display varied global 5hmC levels with some tissues having high, medium, or low levels of 5hmC. In general, tissues of the central nervous system have variable but overall high levels of global 5hmC [26, 27, 64]. Conversely, glandular tissues tend to have low 5hmC levels and the majority of key organs, such as heart, lung, and kidney tend to have midlevels of 5hmC in their genome [26–28]. This is in contrast with the stable global 5mC levels that are observed across most tissues [26]. However, it is important to note that in spite of stable global 5mC levels in various tissues there are locus-specific differences that are involved in maintaining proper tissue phenotype and function. These data suggest that high levels of 5hmC are not indicative of low 5mC levels on a genome-wide basis in somatic tissues, but that locus-specific shifts in the amount of unmodified, methylated, and hydroxymethylated cytosines are important for regulating gene expression in a tissue-specific manner. This is also supported by our work showing tissue-specific levels of 5hmC

at various loci in both mouse and human genomic DNA samples [32]. However, more detailed analysis of 5hmC patterning in various tissues and during development is required, which would help us to understand the roles of TET enzymes and 5hmC in differentiation and development.

3.6 Mutation of 5hmC Pathway Genes in Cancer and the Possible Consequences

TET1 is a common translocation partner of MLL histone methyltransferase at t(10;11)(q22;q23), in acute myeloid leukemia (AML) [13, 14]. The MLL-TET1 translocation has also been less commonly identified in acute lymphoblastic leukemia (ALL) [65]. Apart from the t(10;11)(q22;q23) translocation, no other mutations of TET1 have been reported. The MLL-TET1 fusion protein is predicted to have a molecular mass of approximately 204 kDa and is created by the fusion of the N-terminal part of MLL with the C-terminal part of TET1. The resulting protein contains the AT hooks, subnuclear localization domains, and the CXXC region of MLL fused to the core catalytic domain of TET1 [14]. The catalytic activity of the MLL-TET1 fusion protein is unknown, but it may be a gain of novel function of the fusion protein or loss of MLL and/or TET1 normal function that promotes oncogenesis. Regardless of the precise mechanism(s), MLL translocations correlate with a poor prognosis in ALL and AML patients [66–69].

Similar to TET1, it had been known that the 4q24 chromosomal region was commonly disrupted in hematologic malignancies, but the gene targeted within that region was not clear. It is now known that TET2 is the affected gene at 4q24 in many of these hematologic malignancies. TET2 mutations in myeloproliferative neoplasms (MPN) were identified recently [70–72]. Since then, mutations in TET2 have been observed in myelodysplastic syndrome (MDS), polycythemia vera, essential thrombocythemia, myelofibrosis, blastic plasmacytoid dendritic cell neoplasm (BPDCN), lymphomas, and different types of leukemia [23, 63, 70, 73–82]. Interestingly, certain TET2 mutations are found in specific subsets of these diseases [83].

TET2 mutations range from nonsense and missense mutations to frameshifts and deletions. Essentially all of these mutations are thought to result in loss of function of the TET2 enzyme and are generally somatic in nature. Several common mutations observed in MPN patients were tested for their effects on TET2 activity, including W1291R, E1318G, P1367S, I1873T, and G1913D [21]. All of these mutations are located in the cysteine-rich region or catalytic domain of human TET2. Overexpression of the mutant mouse counterpart of the W1291R (W1211R), P1367S (P1287S), and G1913D (C1834D) mutants in HEK293T cells results in reduced 5hmC as compared to overexpression of the WT Tet2 [21]. In addition, mutations of TET2 often occur on either one or both alleles suggesting that TET2 may either be haploinsufficient or gain an oncogenic function [70, 83]. These results indicate that TET2 functions as a tumor suppressor gene, especially in

hematopoietic cells. However, TET2 mutations may not be enough to cause transformation as it is commonly mutated along with genes in other important pathways, such as JAK and p53 [76, 84, 85].

Tet2 appears to have a direct role in myelopoiesis as *Tet2* knockdown alters differentiation of bone marrow stem cells when grown in the presence of specific cytokines [86]. Furthermore, conditional knockout or reduced expression of Tet2 in mice results in amplification of hematopoietic stem and progenitor cells with skewed numbers of differentiated myeloid and lymphoid lineages [63]. Several studies have attempted to evaluate the effect of TET2 mutations on patient prognosis, albeit in a limited number of samples. Mutations in TET2 correlate with reduced survival time in AML patients [77] and lower survival rate in patients with chronic myelomonocytic leukemia (CMML) [80]. Conversely, TET2 mutations in MDS patients appear to increase survival rate, as well as decrease progression to AML [79].

To date, there is only one report of a genetic aberration associated with TET3. A patient with refractory anemia with ringed sideroblasts (RARS), a specific form of MDS, and idiopathic myelofibrosis carried a deletion of 2p23 where the TET3 gene resides [87]. It is still unknown whether TET3 has a role in myeloproliferative diseases in a similar manner to TET1 and TET2. However, as TET3 is a catalytically active enzyme and has different tissue-specific expression patterns than TET1 and TET2, it remains a possibility that TET3 is involved in the development or progression of these and other diseases or disorders. Genetic studies will be required to test the functional role of TET enzymes in the development and progression of various diseases, including cancer.

As described above, the TET enzymes require cofactors for catalysis, one of which is 2-OG. Two enzymes that are involved in producing 2-OG are the cytosolic isocitrate dehydrogenase 1 (IDH1) and its mitochondrial homolog IDH2 [88]. Interestingly, IDH1 and IDH2 are commonly mutated in several diseases, including gliomas, astrocytomas, leukemias, and MPN [88], where 5hmC and TET expression are abundant. Furthermore, these mutations are not only mutually exclusive with each other but also with TET2 mutations in AML [88].

Mutations of IDH1 and IDH2 can result in a gain-of-function phenotype whereby 2-OG is further reduced by the mutant enzyme to 2-hydroxyglutarate (2-HG) [89, 90]. AML cells harboring mutations in IDH1, IDH2, or TET2 tend to have a hypermethylated phenotype (increased global and locus-specific methylation) and importantly a significant overlap of the genes that are hypermethylated [88]. Overexpression of mutant IDH enzyme results in a global increase in methylation and co-overexpression with TET2 does not result in increased 5hmC levels [88]. The above observation was confirmed in another study that showed inhibition of murine Tet1 and Tet2 in vitro by 2-HG and in vivo by mutant IDH1 [91]. In addition, glioma, astrocytoma, glioblastoma tissue samples harboring IDH1 mutations display decreased 5hmC staining and increased 5mC staining in immunohistological assays, as well as decreased 5hmC with LC-MS analysis [64, 91]. These studies suggest that alterations in 5hmC, either through directly disrupting the TET enzymes or changing availability of cofactors, may be involved in the development and progression of cancer and related diseases.

It is hypothesized that 5hmC is an intermediate in the process of demethylation (described fully in Sect. 3.7) and as a result disruption of the TET protein functions by translocation or mutation may result in a hypermethylated phenotype. Indeed, widespread locus-specific hypermethylation in AML patients with TET2 mutations has been reported [88]. Conversely, another study found that TET2 mutations in leukemia patients are associated with reduced 5hmC levels as expected, but also with global DNA hypomethylation [21]. Another recent report indicated that brain lesions, especially astrocytomas and glioblastomas, have significantly decreased global 5hmC with increasing tumor grade, although these samples did not display clear changes in 5mC levels [64]. Furthermore, several, tumor types appear to have decreased 5hmC when compared to matched normal tissue [39, 92, 93]. The mechanism of global hypo-hydroxymethylation in tumors and the relationship to mutations in TETs is not clear and may be dependent on tumor type and stage.

Hypomethylating agents were originally tested and approved for clinical use in MDS and leukemia patients [94]. The fact that these diseases have especially high rates of mutation in the TET proteins raises the question as to the correlation of TET mutations with treatment efficacy. One study on a very limited number of patients (two) did not confirm that TET2 mutations would improve the efficacy of DNMT inhibitors for the treatment of MDS [95]. In addition, a slightly larger study with AML patients reported that those with mutant TET2 had improved initial response, but did not yield better survival as compared to patients carrying the WT allele [96]. These results emphasize the necessity for studies to be completed using large cohorts of patients identify factors that categorize patients with myeloid disorders, harboring TET mutations, as likely or unlikely to benefit from treatment with demethylating agents. Finally, although TET mutations are clearly predominant in MPN it is still possible that they occur in any number of other diseases and this will likely be a focus of future research.

3.7 Demethylation Pathways of 5hmC and Possible Roles in Cancer Methylation

Reports of methylation cycling in the promoters of specific genes, active demethylation during certain stages of development, and global hypomethylation in tumors have left epigeneticists searching for a DNA demethylase [3]. Several possible demethylation mechanisms have been proposed in the past, including direct enzymatic removal of the methyl group by MBD2 [97], removal of the entire methylated base by a DNA glycosylase in a similar manner to the process of demethylation in plants [98], and deamination followed by base excision repair (BER), including deamination by DNMT3 enzymes in the presence of minimal *S*-adenosyl-L-methionine (AdoMet) [3, 99]. The stability of the carbon–carbon bond of the methyl group and the fifth carbon of the cytosine ring makes it unlikely that demethylation is due to direct removal of the methyl group from cytosine [3]. However, oxidation of methyl groups is a feasible mechanism for removal, especially as

histone demethylases function through oxidation to return histone proteins to an unmodified amino acid state [15].

Before 5hmC was found in mouse ESCs and brain DNA, several groups studied the effects of oxidation of 5mC on methyl binding proteins (MBD) and DNMT1 activity. For example, the MBD MeCP2 was shown to have decreased binding to 5hmC as compared to 5mC [100]. Altered binding of MeCP2 may have serious effects on transcriptional regulation, but would not lead to demethylation. However, DNMT1 was shown to have reduced catalytic activity when the DNA substrate was hemi-hydroxymethylated as opposed to the preferential hemi-methylated substrate [101]. This could have major effects on DNA methylation maintenance during replication, resulting in passive demethylation that is dependent on cell cycling. It is still unknown whether DNMT3a or DNMT3b expressed during S-phase is capable of methylating hemi-hydroxymethylated DNA.

Mammalian 5hmC glycosylases have been described as early as 1988 suggesting that this may be a possible mechanism for removal of this modified nucleobase [102]. Overexpression of TET genes causes increased 5hmC and then subsequent demethylation (based on digestion with methyl-sensitive restriction enzymes) of either endogenous or exogenous methylated DNA that requires a functional BER pathway [20, 103]. Additionally, overexpression of several of the Apobec family of cytidine deaminases causes further demethylation [103]. In fact, viral overexpression of Tet1 in the adult mouse dentate gyrus in the brain leads to substantial increases in global 5hmC, whereas viral overexpression of activation-induced deaminase (AID) in the same tissue causes a decrease in global 5hmC by more than 50%. Overexpression of either Tet1 or AID in adult mouse dentate granule cells results in demethylation and expression of neuronal genes known to display activity-induced DNA demethylation, but no demethylation occurs at non-neuronal promoters [103, 104]. Based on these findings, the following hypothesis has been proposed as one possible mechanism for 5hmC-stimulated demethylation: 5mC is first oxidized by TET enzymes to 5hmC, which is then deaminated by AID/APOBEC cytidine deaminases resulting in 5hmU, then 5hmU is targeted and removed by BER pathways (Fig. 3.2) [103].

Another possible mechanism of demethylation through 5hmC mimics the process of thymine conversion to uracil that is part of the thymidine salvage pathway in which successive oxidation of the 3-methyl group of thymine is completed to produce uracil by decarboxylation [3]. Previously, 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) (the further oxidized forms of 5hmC) could not be easily measured, but as more sensitive techniques were utilized it was clear that these forms of cytosine do exist in mammalian DNA (Fig. 3.2) [26–28]. Mouse ESC, mouse cortex DNA, and DNA from several other somatic tissues contain substantial amounts of each of these modifications, with 5caC being the lowest modified residue [27]. Interestingly, some tissue DNAs contained higher amounts of 5fC than 5hmC, such as liver and spleen [27]. The differences in the global amounts of each modified cytosine could be due to varied rate of conversion from one form to the next, as well as efficiency of removal for 5caC by thymine-DNA glycosylase (TDG) resulting in replacement with unmodified cytosine by

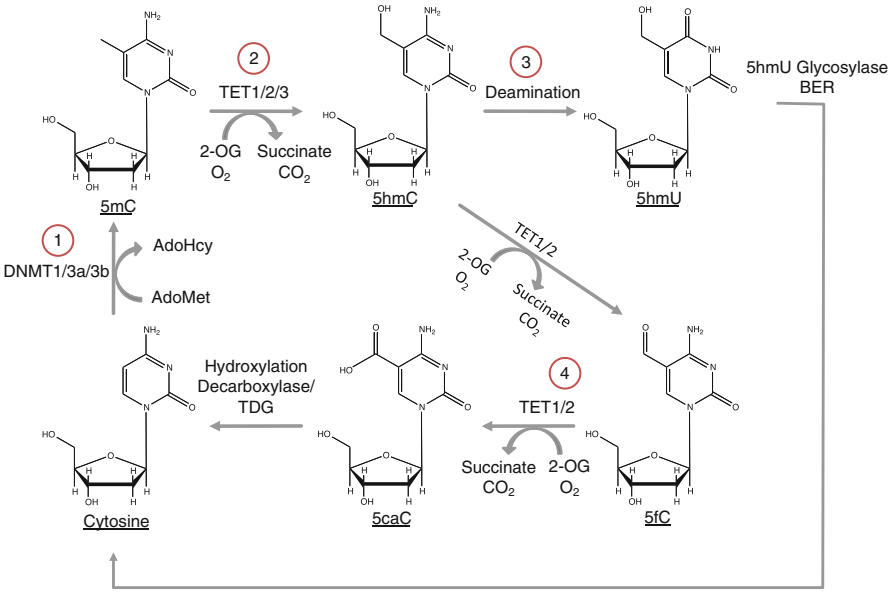

Fig. 3.2 5-Hydroxymethylcytosine and proposed demethylation pathways. (1) Cytosine in an unmodified state can be methylated by any of the three active DNMTs to 5-methylcytosine (5mC) to create the substrate for the TET enzymes. (2) 5mC can be oxidized by any of the three TET family enzymes to 5-hydroxymethylcytosine (5hmC). (3) 5hmC may then be deaminated by unknown enzymes to 5-hydroxymethyluracil (5hmU), which could then be removed by base excision repair pathway enzymes (BER). (4) 5hmC could also be further oxidized by the TET enzymes to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC), at which point the base can be removed by thymine-DNA glycosylase (TDG) or the carboxyl group can be removed by decarboxylases to produce unmethylated cytosine

DNA repair enzymes [28, 105]. Tet1 and Tet2 were both reported to oxidize 5hmC further to 5fC and 5caC both in vitro and in overexpression studies in cultured cells [27, 28].

The knowledge that 5hmC and its derivatives that are converted by the TET enzymes can result in demethylation provide some possible mechanisms for how aberrant methylation could occur in cancers. Loss-of-function mutations in TET2 correlate with hypermethylation and myeloid malignancies that commonly have TET mutations tend to be sensitive to hypomethylating agents [88, 94]. However, one study did correlate TET2 mutations with global hypomethylation in patients with myeloid malignancies [21]. For cancers that display hypomethylation, there are several potential explanations; one possibility is that hypomethylation by 5hmC is an earlier event during cancer progression than loss-of-function mutations that have been reported for TETs, or TET proteins (or other proteins involved in 5hmC-induced demethylation pathways) may be overexpressed or have gain-of-function mutations that are currently unknown. Clearly much research still needs to be done in this particular area to understand demethylation pathways of 5hmC and what enzymes are involved both in normal and disease states.

3.8 Future Perspectives

It was not long ago that the study of DNA methylation was uncharted territory, but now we have a basic understanding of how, when, and where DNA methylation occurs, as well as its role in many biological processes. The identification of 5hmC, and its oxidative products 5fC and 5caC, has complicated our understanding of this process, so now we have to tease out what past data (that may or may not include 5hmC, 5fC, and 5caC) means, and how to acquire more accurate data in the future. This has been and will continue to be a difficult process, but even in the short time since the identification of 5hmC, epigenetics research has moved forward by leaps and bounds, perhaps due to the past experiences with 5mC. Scientists have already developed several techniques to measure global and locus-specific 5hmC across the genome. It is known that there is tissue-specific expression of TETs and 5hmC levels, both globally and at specific loci, and that 5hmC may be involved in DNA demethylation pathways. Even so, there is certainly more research needed to determine the involvement of the TET enzymes and 5hmC in gene regulation, development, and disease.

3.9 Addendum

Two new methods have been reported that allow for single base resolution sequencing of 5hmC [106, 107]. Both techniques depend on the concept that 5fC and 5caC, unlike 5mC or 5hmC, are converted to uracil during sodium bisulfite treatment of the DNA. The first method utilizes potassium perruthenate (KRuO4) to chemically oxidize 5hmC to 5fC followed by rigorous bisulfite treatment and then sequencing of primarily CpG islands in mouse embryonic stem (ES) cell DNA [106]. The second method utilizes a three step process whereby the 5hmC sites are first glucosylated by beta-glucosyltransferase, which is followed by enzymatic oxidation of 5mC to 5caC by recombinant mouse Tet1 catalytic domain, and finally sodium bisulfite conversion and sequencing of human and mouse ES cell DNA. The glucosylated hydroxymethylcytosine residues are resistant to enzymatic oxidation and displayed as C in subsequent PCR based sequencing [107]. In both cases sequencing of both an oxidation pretreated DNA library and a control library must be completed to accurately map both 5mC and 5hmC sites across the genome. Considering that next generation sequencing analysis of bisulfite converted DNA is quite complicated, the data analysis for these methods could be especially difficult. However, these techniques should be useful for identification of 5mC and 5hmC at specific loci using a candidate gene approach in a similar manner to original bisulfite sequencing.

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References

1. Bird AP (1986) CpG-rich islands and the function of DNA methylation. Nature 321:209–213
2. Poulsen HE (2005) Oxidative DNA modifications. Exp Toxicol Pathol 57(Suppl 1):161–169
3. Wu SC, Zhang Y (2010) Active DNA demethylation: many roads lead to Rome. Nat Rev Mol Cell Biol 11:607–620
4. Wyatt GR, Cohen SS (1952) A new pyrimidine base from bacteriophage nucleic acids. Nature 170:1072–1073
5. Josse J, Kornberg A (1962) Glucosylation of deoxyribonucleic acid III alpha- and beta-glucosyl transferases from T4-infected Escherichia coli. J Biol Chem 237:1968–1976
6. Kornberg SR, Zimmerman SB, Kornberg A (1961) Glucosylation of deoxyribonucleic acid by enzymes from bacteriophage-infected Escherichia coli. J Biol Chem 236:1487–1493
7. Penn NW, Suwalski R, O'Riley C, Bojanowski K, Yura R (1972) The presence of 5-hydroxymethylcytosine in animal deoxyribonucleic acid. Biochem J 126:781–790
8. Penn NW (1976) Modification of brain deoxyribonucleic acid base content with maturation in normal and malnourished rats. Biochem J 155:709–712
9. Kothari RM, Shankar V (1976) 5-Methylcytosine content in the vertebrate deoxyribonucleic acids: species specificity. J Mol Evol 7:325–329
10. Tahiliani M, Koh KP, Shen Y, Pastor WA, Bandukwala H, Brudno Y, Agarwal S, Iyer LM, Liu DR, Aravind L, Rao A (2009) Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. Science 324:930–935
11. Kriaucionis S, Heintz N (2009) The nuclear DNA base 5-hydroxymethylcytosine is present in Purkinje neurons and the brain. Science 324:929–930
12. Shock LS, Thakkar PV, Peterson EJ, Moran RG, Taylor SM (2011) DNA methyltransferase 1, cytosine methylation, and cytosine hydroxymethylation in mammalian mitochondria. Proc Natl Acad Sci USA 108:3630–3635
13. Ono R, Taki T, Taketani T, Taniwaki M, Kobayashi H, Hayashi Y (2002) LCX, leukemia-associated protein with a CXXC domain, is fused to MLL in acute myeloid leukemia with trilineage dysplasia having t(10;11)(q22;q23). Cancer Res 62:4075–4080
14. Lorsbach RB, Moore J, Mathew S, Raimondi SC, Mukatira ST, Downing JR (2003) TET1, a member of a novel protein family, is fused to MLL in acute myeloid leukemia containing the t(10;11)(q22;q23). Leukemia 17:637–641
15. Loenarz C, Schofield CJ (2009) Oxygenase catalyzed 5-methylcytosine hydroxylation. Chem Biol 16:580–583
16. Yu Z, Genest PA, ter Riet B, Sweeney K, DiPaolo C, Kieft R, Christodoulou E, Perrakis A, Simmons JM, Hausinger RP, van Luenen HG, Rigden DJ, Sabatini R, Borst P (2007) The protein that binds to DNA base J in trypanosomatids has features of a thymidine hydroxylase. Nucleic Acids Res 35:2107–2115
17. Iyer LM, Tahiliani M, Rao A, Aravind L (2009) Prediction of novel families of enzymes involved in oxidative and other complex modifications of bases in nucleic acids. Cell Cycle 8:1698–1710
18. Mohr F, Dohner K, Buske C, Rawat VP (2011) TET genes: new players in DNA demethylation and important determinants for stemness. Exp Hematol 39:272–281
19. Frauer C, Rottach A, Meilinger D, Bultmann S, Fellinger K, Hasenoder S, Wang M, Qin W, Soding J, Spada F, Leonhardt H (2011) Different binding properties and function of CXXC zinc finger domains in Dnmt1 and Tet1. PLoS One 6:e16627
20. Zhang H, Zhang X, Clark E, Mulcahey M, Huang S, Shi YG (2010) TET1 is a DNA-binding protein that modulates DNA methylation and gene transcription via hydroxylation of 5-methylcytosine. Cell Res 20:1390–1393
21. Ko M, Huang Y, Jankowska AM, Pape UJ, Tahiliani M, Bandukwala HS, An J, Lamperti ED, Koh KP, Ganetzky R, Liu XS, Aravind L, Agarwal S, Maciejewski JP, Rao A (2010) Impaired hydroxylation of 5-methylcytosine in myeloid cancers with mutant TET2. Nature 468:839–843

22. Ito S, D'Alessio AC, Taranova OV, Hong K, Sowers LC, Zhang Y (2010) Role of Tet proteins in 5mC to 5hmC conversion, ES-cell self-renewal and inner cell mass specification. Nature 466:1129–1133
23. Langemeijer SM, Aslanyan MG, Jansen JH (2009) TET proteins in malignant hematopoiesis. Cell Cycle 8:4044–4048
24. Langemeijer SM, Kuiper RP, Berends M, Knops R, Aslanyan MG, Massop M, Stevens-Linders E, van Hoogen P, van Kessel AG, Raymakers RA, Kamping EJ, Verhoef GE, Verburgh E, Hagemeijer A, Vandenberghe P, de Witte T, van der Reijden BA, Jansen JH (2009) Acquired mutations in TET2 are common in myelodysplastic syndromes. Nat Genet 41:838–842
25. Ficz G, Branco MR, Seisenberger S, Santos F, Krueger F, Hore TA, Marques CJ, Andrews S, Reik W (2011) Dynamic regulation of 5-hydroxymethylcytosine in mouse ES cells and during differentiation. Nature 473:398–402
26. Globisch D, Munzel M, Muller M, Michalakis S, Wagner M, Koch S, Bruckl T, Biel M, Carell T (2010) Tissue distribution of 5-hydroxymethylcytosine and search for active demethylation intermediates. PLoS One 5:e15367
27. Ito S, Shen L, Dai Q, Wu SC, Collins LB, Swenberg JA, He C, Zhang Y (2011) Tet proteins can convert 5-methylcytosine to 5-formylcytosine and 5-carboxylcytosine. Science 333:1300–1303
28. He YF, Li BZ, Li Z, Liu P, Wang Y, Tang Q, Ding J, Jia Y, Chen Z, Li L, Sun Y, Li X, Dai Q, Song CX, Zhang K, He C, Xu GL (2011) Tet-mediated formation of 5-carboxylcytosine and its excision by TDG in mammalian DNA. Science 333:1303–1307
29. Huang Y, Pastor WA, Shen Y, Tahiliani M, Liu DR, Rao A (2010) The behaviour of 5-hydroxymethylcytosine in bisulfite sequencing. PLoS One 5:e8888
30. Wang H, Guan S, Quimby A, Cohen-Karni D, Pradhan S, Wilson G, Roberts RJ, Zhu Z, Zheng Y (2011) Comparative characterization of the PvuRts1I family of restriction enzymes and their application in mapping genomic 5-hydroxymethylcytosine. Nucleic Acids Res 39:9294–9305
31. Song CX, Yu M, Dai Q, He C (2011) Detection of 5-hydroxymethylcytosine in a combined glycosylation restriction analysis (CGRA) using restriction enzyme Taq(alpha)I. Bioorg Med Chem Lett 21:5075–5077
32. Kinney SM, Chin HG, Vaisvila R, Bitinaite J, Zheng Y, Esteve PO, Feng S, Stroud H, Jacobsen SE, Pradhan S (2011) Tissue-specific distribution and dynamic changes of 5-hydroxymethylcytosine in mammalian genomes. J Biol Chem 286:24685–24693
33. Szwagierczak A, Brachmann A, Schmidt CS, Bultmann S, Leonhardt H, Spada F (2011) Characterization of PvuRts1I endonuclease as a tool to investigate genomic 5-hydroxymethylcytosine. Nucleic Acids Res 39:5149–5156
34. Roberts RJ, Vincze T, Posfai J, Macelis D (2010) REBASE—a database for DNA restriction and modification: enzymes, genes and genomes. Nucleic Acids Res 38:D234–D236
35. Iqbal K, Jin SG, Pfeifer GP, Szabo PE (2011) Reprogramming of the paternal genome upon fertilization involves genome-wide oxidation of 5-methylcytosine. Proc Natl Acad Sci USA 08:3642–3647
36. Stroud H, Feng S, Morey Kinney S, Pradhan S, Jacobsen SE (2011) 5-Hydroxymethylcytosine is associated with enhancers and gene bodies in human embryonic stem cells. Genome Biol 12:R54
37. Le T, Kim KP, Fan G, Faull KF (2011) A sensitive mass spectrometry method for simultaneous quantification of DNA methylation and hydroxymethylation levels in biological samples. Anal Biochem 412:203–209
38. Szwagierczak A, Bultmann S, Schmidt CS, Spada F, Leonhardt H (2010) Sensitive enzymatic quantification of 5-hydroxymethylcytosine in genomic DNA. Nucleic Acids Res 38:e181
39. Terragni J, Bitinaite J, Zheng Y, Pradhan S (2012) Biochemical characterization of recombinant Beta-glucosyltransferase and analysis of global 5-hydroxymethylcytosine in unique genomes. Biochemistry 51:1009–1019
40. Wu H, D'Alessio AC, Ito S, Wang Z, Cui K, Zhao K, Sun YE, Zhang Y (2011) Genome-wide analysis of 5-hydroxymethylcytosine distribution reveals its dual function in transcriptional regulation in mouse embryonic stem cells. Genes Dev 25:679–684

41. Wu H, D'Alessio AC, Ito S, Xia K, Wang Z, Cui K, Zhao K, Sun YE, Zhang Y (2011) Dual functions of Tet1 in transcriptional regulation in mouse embryonic stem cells. Nature 473:389–393
42. Pastor WA, Pape UJ, Huang Y, Henderson HR, Lister R, Ko M, McLoughlin EM, Brudno Y, Mahapatra S, Kapranov P, Tahiliani M, Daley GQ, Liu XS, Ecker JR, Milos PM, Agarwal S, Rao A (2011) Genome-wide mapping of 5-hydroxymethylcytosine in embryonic stem cells. Nature 473:394–397
43. Song CX, Szulwach KE, Fu Y, Dai Q, Yi C, Li X, Li Y, Chen CH, Zhang W, Jian X, Wang J, Zhang L, Looney TJ, Zhang B, Godley LA, Hicks LM, Lahn BT, Jin P, He C (2011) Selective chemical labeling reveals the genome-wide distribution of 5-hydroxymethylcytosine. Nat Biotechnol 29:68–72
44. Flusberg BA, Webster DR, Lee JH, Travers KJ, Olivares EC, Clark TA, Korlach J, Turner SW (2010) Direct detection of DNA methylation during single-molecule, real-time sequencing. Nat Methods 7:461–465
45. Wanunu M, Cohen-Karni D, Johnson RR, Fields L, Benner J, Peterman N, Zheng Y, Klein ML, Drndic M (2010) Discrimination of methylcytosine from hydroxymethylcytosine in DNA molecules. J Am Chem Soc 133:486–492
46. Bair CL, Black LW (2007) A type IV modification dependent restriction nuclease that targets glucosylated hydroxymethyl cytosine modified DNAs. J Mol Biol 366:768–778
47. Freudenberg JM, Ghosh S, Lackford BL, Yellaboina S, Zheng X, Li R, Cuddapah S, Wade PA, Hu G, Jothi R (2011) Acute depletion of Tet1-dependent 5-hydroxymethylcytosine levels impairs LIF/Stat3 signaling and results in loss of embryonic stem cell identity. Nucleic Acids Res 40(8):3364–3377
48. Zhou GB, Meng QG, Li N (2010) In vitro derivation of germ cells from embryonic stem cells in mammals. Mol Reprod Dev 77:586–594
49. Koh KP, Yabuuchi A, Rao S, Huang Y, Cunniff K, Nardone J, Laiho A, Tahiliani M, Sommer CA, Mostoslavsky G, Lahesmaa R, Orkin SH, Rodig SJ, Daley GQ, Rao A (2011) Tet1 and Tet2 regulate 5-hydroxymethylcytosine production and cell lineage specification in mouse embryonic stem cells. Cell Stem Cell 8:200–213
50. Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126:663–676
51. Williams K, Christensen J, Pedersen MT, Johansen JV, Cloos PA, Rappsilber J, Helin K (2011) TET1 and hydroxymethylcytosine in transcription and DNA methylation fidelity. Nature 473:343–348
52. Xu Y, Wu F, Tan L, Kong L, Xiong L, Deng J, Barbera AJ, Zheng L, Zhang H, Huang S, Min J, Nicholson T, Chen T, Xu G, Shi Y, Zhang K, Shi YG (2011) Genome-wide regulation of 5hmC, 5mC, and gene expression by Tet1 hydroxylase in mouse embryonic stem cells. Mol Cell 42:451–464
53. Dawlaty MM, Ganz K, Powell BE, Hu YC, Markoulaki S, Cheng AW, Gao Q, Kim J, Choi SW, Page DC, Jaenisch R (2011) Tet1 is dispensable for maintaining pluripotency and its loss is compatible with embryonic and postnatal development. Cell Stem Cell 9:166–175
54. Yildirim O, Li R, Hung JH, Chen PB, Dong X, Ee LS, Weng Z, Rando OJ, Fazzio TG (2011) Mbd3/NURD Complex Regulates Expression of 5-Hydroxymethylcytosine Marked Genes in Embryonic Stem Cells. Cell 147:1498–1510
55. Reik W, Dean W, Walter J (2001) Epigenetic reprogramming in mammalian development. Science 293:1089–1093
56. Mayer W, Niveleau A, Walter J, Fundele R, Haaf T (2000) Demethylation of the zygotic paternal genome. Nature 403:501–502
57. Oswald J, Engemann S, Lane N, Mayer W, Olek A, Fundele R, Dean W, Reik W, Walter J (2000) Active demethylation of the paternal genome in the mouse zygote. Curr Biol 10:475–478
58. Santos F, Hendrich B, Reik W, Dean W (2002) Dynamic reprogramming of DNA methylation in the early mouse embryo. Dev Biol 241:172–182
59. Hajkova P, Jeffries SJ, Lee C, Miller N, Jackson SP, Surani MA (2010) Genome-wide reprogramming in the mouse germ line entails the base excision repair pathway. Science 329:78–82

60. Wossidlo M, Nakamura T, Lepikhov K, Marques CJ, Zakhartchenko V, Boiani M, Arand J, Nakano T, Reik W, Walter J (2011) 5-Hydroxymethylcytosine in the mammalian zygote is linked with epigenetic reprogramming. Nat Commun 2:241
61. Gu TP, Guo F, Yang H, Wu HP, Xu GF, Liu W, Xie ZG, Shi L, He X, Jin SG, Iqbal K, Shi YG, Deng Z, Szabo PE, Pfeifer GP, Li J, Xu GL (2011) The role of Tet3 DNA dioxygenase in epigenetic reprogramming by oocytes. Nature 477:606–610
62. Nakamura T, Arai Y, Umehara H, Masuhara M, Kimura T, Taniguchi H, Sekimoto T, Ikawa M, Yoneda Y, Okabe M, Tanaka S, Shiota K, Nakano T (2007) PGC7/Stella protects against DNA demethylation in early embryogenesis. Nat Cell Biol 9:64–71
63. Quivoron C, Couronne L, Della Valle V, Lopez CK, Plo I, Wagner-Ballon O, Do Cruzeiro M, Delhommeau F, Arnulf B, Stern MH, Godley L, Opolon P, Tilly H, Solary E, Duffourd Y, Dessen P, Merle-Beral H, Nguyen-Khac F, Fontenay M, Vainchenker W, Bastard C, Mercher T, Bernard OA (2011) TET2 inactivation results in pleiotropic hematopoietic abnormalities in mouse and is a recurrent event during human lymphomagenesis. Cancer Cell 20:25–38
64. Kraus TF, Globisch D, Wagner M, Eigenbrod S, Widmann D, Munzel M, Muller M, Pfaffeneder T, Hackner B, Feiden W, Schuller U, Carell T, Kretzschmar HA (2012) Low values of 5-hydroxymethylcytosine (5hmC), the "sixth base", are associated with anaplasia in human brain tumours. International journal of cancer. J Int Cancer Jan 10. doi: 10.1002/ijc.27429. [Epub ahead of print]
65. Burmeister T, Meyer C, Schwartz S, Hofmann J, Molkentin M, Kowarz E, Schneider B, Raff T, Reinhardt R, Gokbuget N, Hoelzer D, Thiel E, Marschalek R (2009) The MLL recombinome of adult CD10-negative B-cell precursor acute lymphoblastic leukemia: results from the GMALL study group. Blood 113:4011–4015
66. Pui CH, Chessells JM, Camitta B, Baruchel A, Biondi A, Boyett JM, Carroll A, Eden OB, Evans WE, Gadner H, Harbott J, Harms DO, Harrison CJ, Harrison PL, Heerema N, Janka-Schaub G, Kamps W, Masera G, Pullen J, Raimondi SC, Richards S, Riehm H, Sallan S, Sather H, Shuster J, Silverman LB, Valsecchi MG, Vilmer E, Zhou Y, Gaynon PS, Schrappe M (2003) Clinical heterogeneity in childhood acute lymphoblastic leukemia with 11q23 rearrangements. Leukemia 17:700–706
67. Pui CH, Gaynon PS, Boyett JM, Chessells JM, Baruchel A, Kamps W, Silverman LB, Biondi A, Harms DO, Vilmer E, Schrappe M, Camitta B (2002) Outcome of treatment in childhood acute lymphoblastic leukaemia with rearrangements of the 11q23 chromosomal region. Lancet 359:1909–1915
68. Liedtke M, Cleary ML (2009) Therapeutic targeting of MLL. Blood 113:6061–6068
69. Chou WC, Chou SC, Liu CY, Chen CY, Hou HA, Kuo YY, Lee MC, Ko BS, Tang JL, Yao M, Tsay W, Wu SJ, Huang SY, Hsu SC, Chen YC, Chang YC, Kuo KT, Lee FY, Liu MC, Liu CW, Tseng MH, Huang CF, Tien HF (2011) TET2 mutation is an unfavorable prognostic factor in acute myeloid leukemia patients with intermediate-risk cytogenetics. Blood 118:3803–3810
70. Delhommeau F, Dupont S, Della Valle V, James C, Trannoy S, Masse A, Kosmider O, Le Couedic JP, Robert F, Alberdi A, Lecluse Y, Plo I, Dreyfus FJ, Marzac C, Casadevall N, Lacombe C, Romana SP, Dessen P, Soulier J, Viguie F, Fontenay M, Vainchenker W, Bernard OA (2009) Mutation in TET2 in myeloid cancers. N Engl J Med 360:2289–2301
71. Tefferi A, Levine RL, Lim KH, Abdel-Wahab O, Lasho TL, Patel J, Finke CM, Mullally A, Li CY, Pardanani A, Gilliland DG (2009) Frequent TET2 mutations in systemic mastocytosis: clinical, KITD816V and FIP1L1-PDGFRA correlates. Leukemia 23:900–904
72. Tefferi A, Lim KH, Levine R (2009) Mutation in TET2 in myeloid cancers. N Engl J Med 361:1117
73. Tefferi A, Pardanani A, Lim KH, Abdel-Wahab O, Lasho TL, Patel J, Gangat N, Finke CM, Schwager S, Mullally A, Li CY, Hanson CA, Mesa R, Bernard O, Delhommeau F, Vainchenker W, Gilliland DG, Levine RL (2009) TET2 mutations and their clinical correlates in polycythemia vera, essential thrombocythemia and myelofibrosis. Leukemia 23:905–911
74. Tefferi A, Lim KH, Abdel-Wahab O, Lasho TL, Patel J, Patnaik MM, Hanson CA, Pardanani A, Gilliland DG, Levine RL (2009) Detection of mutant TET2 in myeloid malignancies other than myeloproliferative neoplasms: CMML, MDS, MDS/MPN and AML. Leukemia 23: 1343–1345

75. Makishima H, Jankowska AM, McDevitt MA, O'Keefe C, Dujardin S, Cazzolli H, Przychodzen B, Prince C, Nicoll J, Siddaiah H, Shaik M, Szpurka H, Hsi E, Advani A, Paquette R, Maciejewski JP (2011) CBL, CBLB, TET2, ASXL1, and IDH1/2 mutations and additional chromosomal aberrations constitute molecular events in chronic myelogenous leukemia. Blood 117:e198–e206
76. Jardin F, Ruminy P, Parmentier F, Troussard X, Vaida I, Stamatoullas A, Lepretre S, Penther D, Duval AB, Picquenot JM, Courville P, Capiod JC, Tilly H, Bastard C, Marolleau JP (2011) TET2 and TP53 mutations are frequently observed in blastic plasmacytoid dendritic cell neoplasm. Br J Haematol 153:413–416
77. Abdel-Wahab O, Mullally A, Hedvat C, Garcia-Manero G, Patel J, Wadleigh M, Malinge S, Yao J, Kilpivaara O, Bhat R, Huberman K, Thomas S, Dolgalev I, Heguy A, Paietta E, Le Beau MM, Beran M, Tallman MS, Ebert BL, Kantarjian HM, Stone RM, Gilliland DG, Crispino JD, Levine RL (2009) Genetic characterization of TET1, TET2, and TET3 alterations in myeloid malignancies. Blood 114:144–147
78. Jankowska AM, Szpurka H, Tiu RV, Makishima H, Afable M, Huh J, O'Keefe CL, Ganetzky R, McDevitt MA, Maciejewski JP (2009) Loss of heterozygosity 4q24 and TET2 mutations associated with myelodysplastic/myeloproliferative neoplasms. Blood 113:6403–6410
79. Kosmider O, Gelsi-Boyer V, Cheok M, Grabar S, Della-Valle V, Picard F, Viguie F, Quesnel B, Beyne-Rauzy O, Solary E, Vey N, Hunault-Berger M, Fenaux P, Mansat-De Mas V, Delabesse E, Guardiola P, Lacombe C, Vainchenker W, Preudhomme C, Dreyfus F, Bernard OA, Birnbaum D, Fontenay M (2009) TET2 mutation is an independent favorable prognostic factor in myelodysplastic syndromes (MDSs). Blood 114:3285–3291
80. Kosmider O, Gelsi-Boyer V, Ciudad M, Racoeur C, Jooste V, Vey N, Quesnel B, Fenaux P, Bastie JN, Beyne-Rauzy O, Stamatoulas A, Dreyfus F, Ifrah N, de Botton S, Vainchenker W, Bernard OA, Birnbaum D, Fontenay M, Solary E (2009) TET2 gene mutation is a frequent and adverse event in chronic myelomonocytic leukemia. Haematologica 94:1676–1681
81. Kohlmann A, Grossmann V, Klein HU, Schindela S, Weiss T, Kazak B, Dicker F, Schnittger S, Dugas M, Kern W, Haferlach C, Haferlach T (2010) Next-generation sequencing technology reveals a characteristic pattern of molecular mutations in 72.8% of chronic myelomonocytic leukemia by detecting frequent alterations in TET2, CBL, RAS, and RUNX1. J Clin Oncol 28:3858–3865
82. Nibourel O, Kosmider O, Cheok M, Boissel N, Renneville A, Philippe N, Dombret H, Dreyfus F, Quesnel B, Geffroy S, Quentin S, Roche-Lestienne C, Cayuela JM, Roumier C, Fenaux P, Vainchenker W, Bernard OA, Soulier J, Fontenay M, Preudhomme C (2010) Incidence and prognostic value of TET2 alterations in de novo acute myeloid leukemia achieving complete remission. Blood 116:1132–1135
83. Hellstrom-Lindberg E (2010) Significance of JAK2 and TET2 mutations in myelodysplastic syndromes. Blood Rev 24:83–90
84. Swierczek SI, Yoon D, Bellanne-Chantelot C, Kim SJ, Saint-Martin C, Delhommeau F, Najman A, Prchal JT (2011) Extent of hematopoietic involvement by TET2 mutations in JAK2V(1)F polycythemia vera. Haematologica 96:775–778
85. Rocquain J, Carbuccia N, Trouplin V, Raynaud S, Murati A, Nezri M, Tadrist Z, Olschwang S, Vey N, Birnbaum D, Gelsi-Boyer V, Mozziconacci MJ (2010) Combined mutations of ASXL1, CBL, FLT3, IDH1, IDH2, JAK2, KRAS, NPM1, NRAS, RUNX1, TET2 and WT1 genes in myelodysplastic syndromes and acute myeloid leukemias. BMC Cancer 10:401
86. Ko M, Bandukwala HS, An J, Lamperti ED, Thompson EC, Hastie R, Tsangaratou A, Rajewsky K, Koralov SB, Rao A (2011) Ten-Eleven-Translocation 2 (TET2) negatively regulates homeostasis and differentiation of hematopoietic stem cells in mice. Proc Natl Acad Sci USA 108:14566–14571
87. Gelsi-Boyer V, Trouplin V, Adelaide J, Bonansea J, Cervera N, Carbuccia N, Lagarde A, Prebet T, Nezri M, Sainty D, Olschwang S, Xerri L, Chaffanet M, Mozziconacci MJ, Vey N, Birnbaum D (2009) Mutations of polycomb-associated gene ASXL1 in myelodysplastic syndromes and chronic myelomonocytic leukaemia. Br J Haematol 145:788–800
88. Figueroa ME, Abdel-Wahab O, Lu C, Ward PS, Patel J, Shih A, Li Y, Bhagwat N, Vasanthakumar A, Fernandez HF, Tallman MS, Sun Z, Wolniak K, Peeters JK, Liu W, Choe

SE, Fantin VR, Paietta E, Lowenberg B, Licht JD, Godley LA, Delwel R, Valk PJ, Thompson CB, Levine RL, Melnick A (2010) Leukemic IDH1 and IDH2 mutations result in a hypermethylation phenotype, disrupt TET2 function, and impair hematopoietic differentiation. Cancer Cell 18:553–567
89. Dang L, White DW, Gross S, Bennett BD, Bittinger MA, Driggers EM, Fantin VR, Jang HG, Jin S, Keenan MC, Marks KM, Prins RM, Ward PS, Yen KE, Liau LM, Rabinowitz JD, Cantley LC, Thompson CB, Vander Heiden MG, Su SM (2009) Cancer-associated IDH1 mutations produce 2-hydroxyglutarate. Nature 462:739–744
90. Ward PS, Patel J, Wise DR, Abdel-Wahab O, Bennett BD, Coller HA, Cross JR, Fantin VR, Hedvat CV, Perl AE, Rabinowitz JD, Carroll M, Su SM, Sharp KA, Levine RL, Thompson CB (2010) The common feature of leukemia-associated IDH1 and IDH2 mutations is a neomorphic enzyme activity converting alpha-ketoglutarate to 2-hydroxyglutarate. Cancer Cell 17:225–234
91. Xu W, Yang H, Liu Y, Yang Y, Wang P, Kim SH, Ito S, Yang C, Xiao MT, Liu LX, Jiang WQ, Liu J, Zhang JY, Wang B, Frye S, Zhang Y, Xu YH, Lei QY, Guan KL, Zhao SM, Xiong Y (2011) Oncometabolite 2-hydroxyglutarate is a competitive inhibitor of alpha-ketoglutarate-dependent dioxygenases. Cancer Cell 19:17–30
92. Haffner MC, Chaux A, Meeker AK, Esopi DM, Gerber J, Pellakuru LG, Toubaji A, Argani P, Iacobuzio-Donahue C, Nelson WG, Netto GJ, De Marzo AM, Yegnasubramanian S (2011) Global 5-hydroxymethylcytosine content is significantly reduced in tissue stem/progenitor cell compartments and in human cancers. Oncotarget 2:627–637
93. Li W, Liu M (2011) Distribution of 5-hydroxymethylcytosine in different human tissues. J Nucleic Acids 2011:870726
94. Kantarjian H, O'Brien S, Cortes J, Wierda W, Faderl S, Garcia-Manero G, Issa JP, Estey E, Keating M, Freireich EJ (2008) Therapeutic advances in leukemia and myelodysplastic syndrome over the past 40 years. Cancer 113:1933–1952
95. Pollyea DA, Raval A, Kusler B, Gotlib JR, Alizadeh AA, Mitchell BS (2010) Impact of TET2 mutations on mRNA expression and clinical outcomes in MDS patients treated with DNA methyltransferase inhibitors. Hematol Oncol 29:157–160
96. Itzykson R, Kosmider O, Cluzeau T, Mansat-De Mas V, Dreyfus F, Beyne-Rauzy O, Quesnel B, Vey N, Gelsi-Boyer V, Raynaud S, Preudhomme C, Ades L, Fenaux P, Fontenay M (2011) Impact of TET2 mutations on response rate to azacitidine in myelodysplastic syndromes and low blast count acute myeloid leukemias. Leukemia 25:1147–1152
97. Detich N, Theberge J, Szyf M (2002) Promoter-specific activation and demethylation by MBD2/demethylase. J Biol Chem 277:35791–35794
98. Feng S, Jacobsen SE, Reik W (2010) Epigenetic reprogramming in plant and animal development. Science 330:622–627
99. Metivier R, Gallais R, Tiffoche C, Le Peron C, Jurkowska RZ, Carmouche RP, Ibberson D, Barath P, Demay F, Reid G, Benes V, Jeltsch A, Gannon F, Salbert G (2008) Cyclical DNA methylation of a transcriptionally active promoter. Nature 452:45–50
100. Valinluck V, Tsai HH, Rogstad DK, Burdzy A, Bird A, Sowers LC (2004) Oxidative damage to methyl-CpG sequences inhibits the binding of the methyl-CpG binding domain (MBD) of methyl-CpG binding protein 2 (MeCP2). Nucleic Acids Res 32:4100–4108
101. Valinluck V, Sowers LC (2007) Endogenous cytosine damage products alter the site selectivity of human DNA maintenance methyltransferase DNMT1. Cancer Res 67:946–950
102. Cannon SV, Cummings A, Teebor GW (1988) 5-Hydroxymethylcytosine DNA glycosylase activity in mammalian tissue. Biochem Biophys Res Commun 151:1173–1179
103. Guo JU, Su Y, Zhong C, Ming GL, Song H (2011) Hydroxylation of 5-methylcytosine by TET1 promotes active DNA demethylation in the adult brain. Cell 145:423–434
104. Ma DK, Jang MH, Guo JU, Kitabatake Y, Chang ML, Pow-Anpongkul N, Flavell RA, Lu B, Ming GL, Song H (2009) Neuronal activity-induced Gadd45b promotes epigenetic DNA demethylation and adult neurogenesis. Science 323:1074–1077

105. Maiti A, Drohat AC (2011) Thymine DNA glycosylase can rapidly excise 5-formylcytosine and 5-carboxylcytosine: Potential implications for active demethylation of CpG sites. J Biol Chem 286:35334–35338
106. Booth MJ, Branco MR, Ficz G, Oxley D, Krueger F, Reik W, Balasubramanian S. (2012) Quantitative sequencing of 5-methylcytosine and 5-hydroxymethylcytosine at single-base resolution. Science 336:934–937
107. Yu M, Hon GC, Szulwach KE, Song CX, Zhang L, Kim A, Li X, Dai Q, Shen Y, Park B, Min JH, Jin P, Ren B, He C (2012) Base-resolution analysis of 5-hydroxymethylcytosine in the mammalian genome. Cell 149:1368–1380

Chapter 4
Altered Histone Modifications in Cancer

Moray J. Campbell and Bryan M. Turner

Abstract In human health and disease the choreographed actions of a wide armory of transcription factors govern the regulated expression of coding and nonprotein coding genes. These actions are central to human health and are evidently aberrant in cancer. Central components of regulated gene expression are a variety of epigenetic mechanisms that include histone modifications. The post-translational modifications of histones are widespread and diverse, and appear to be spatial-temporally regulated in a highly intricate manner. The true functional consequences of these patterns of regulation are still emerging. Correlative evidence supports the idea that these patterns are distorted in malignancy on both a genome-wide and a discrete gene loci level. These patterns of distortion also often reflect the altered expression of the enzymes that control these histone states. Similarly gene expression patterns also appear to reflect a correlation with altered histone modifications at both the candidate loci and genome-

Chromatin-modifying enzymes: The nomenclature for enzymes involved in protein methylation, demethylation, and acetylation has recently been rationalized (Allis CD et al (2007) New nomenclature for chromatin-modifying enzymes. Cell 131:633–636). In this review, we use the new nomenclature for lysine methyltransferases (KMT), lysine demethylases (KDM), and lysine acetyltransferases (KAT). Histone deacetylases (HDACs) have retained their original nomenclature. To maintain a link between the new nomenclature and the literature, we use both the new designation and the original published designation(s), e.g., KDM5A/JARID1A/RBP2.

Histone modifications: We use the Brno nomenclature for histone modifications (Turner BM (2005) Reading signals on the nucleosome with a new nomenclature for modified histones. Nat Struct Mol Biol 12:110–112). For example, histone H3 tri-methylated at lysine 4 is shown as H3K4me3.

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wide level. Clarity is emerging in resolving these relationships between histone modification status and gene expression patterns. For example, altered transcription factor interactions with the key co-activator and co-repressors, which in turn marshal many of the histone-modifying enzymes, may distort regulation of histone modifications at specific gene loci. In turn these aberrant transcriptional processes can trigger other altered epigenetic events such as DNA methylation and underline the aberrant and specific gene expression patterns in cancer. Considered in this manner, altered expression and recruitment of histone-modifying enzymes may underline the distortion to transcriptional responsiveness observed in malignancy. Insight from understanding these processes addresses the challenge of targeted epigenetic therapies in cancer.

Abbreviations

AR	Androgen receptor
ChIP	Chromatin immunoprecipitation
CoA	Co-activator complex
E_2	Estradiol
ERα	Estrogen receptor alpha
ES	Embryonic stem cell
HDAC	Histone deacetylase
JMJD	Jumonji domain containing protein
JARID	Jumonji AT-rich interactive domain
KAT	Lysine acetyltransferase
KDM	Lysine demethylase
KMT	Lysine methyltransferase
LSD1	Lysine-specific demethylase 1
NCOR	Nuclear co-repressor
NR	Nuclear receptor
PSA	Prostate-specific antigen
SET	Su(var), enhancer of zeste and trithorax
TF	Transcription factor
TSA	Trichostatin A
TSS	Transcription start site

4.1 Altered Histone Modifications in Cancer

4.1.1 The Nucleosome and Its Modified Forms

Of the various protein–DNA interactions that are central to genome function, those between the histones and DNA are among the most intimate. A histone–DNA complex, the nucleosome, is the basic unit of chromatin structure in nearly

all eukaryotes, It comprises 146 bp of DNA wrapped in 1¾ superhelical turns around a core of eight histones, two each of H2A, H2B, H3, and H4. The structure of the nucleosome core particle has been defined in great detail by X-ray crystallography [3].

Despite its extreme conservation through evolution and its consistent crystal structure, the nucleosome in vivo is subject to a variety of enzyme-driven modifications that, potentially at least, alter its structure. Chromatin-modifying enzymes directly manipulate nucleosome structure or change nucleosome position along the DNA fiber [4]. DNA translocating enzymes such as polymerases, which pull and twist the DNA fiber as part of their normal activities, distort nucleosomes in their paths. Chromatin must deform reversibly in order to accommodate torsional and tensional stress generated by these enzymes ([5] and references therein). Nucleosome remodeling can dissociate the histone core, providing opportunities to enzymatically modify internal histone regions (see below), or to incorporate histone variants. All core histones, apart from H4, have nonallelic variant forms that differ in amino acid sequence and are associated with specific cellular and genomic functions [6].

4.1.1.1 Post-translational Modification of Histones

The most widespread and complex source of nucleosome variability is the enzyme catalyzed, post-translational modification of selected histone amino acids. All four core histones are subject to such changes, which include acetylation of lysines, methylation of lysines and arginines, phosphorylation of serines and threonines, and attachment of the small peptides ubiquitin and SUMO [7]. Advances in mass spectrometry and proteomics [8] have led to the identification of previously unsuspected chemical changes, including *O*-glycosylation of serines and threonines [9], formylation and crotonylation of lysines, and hydroxylation of serines [10]. They have also revealed that modifications occur both along the N-terminal tail domains, unstructured regions that are exposed on the nucleosome surface, and on residues in the globular internal regions that mediate histone–histone and histone–DNA interactions [11]. Histone modifications are put in place and removed by families (often large) of modifying and de-modifying enzymes and are consistently dynamic. The level of any particular modification reflects a steady-state balance between the actions of these two sets of enzymes.

The internal histone regions mediate the interactions that give the nucleosome its characteristic structure and their modification can, potentially, exert a direct structural effect. Yeast mutants with internal substitutions (some mimicking modifications) commonly cause functional changes, particularly altered gene silencing and increased sensitivity to DNA damaging agents [12, 13]. Acetylation of H3K56, on the lateral face of the nucleosome, is incorporated into chromatin at sites of DNA damage and repair [14, 15] and at replication forks [16]. These are all situations in which the nucleosome is partially dissociated, and during which internal residues will be accessible to modifying enzymes. Structural changes brought about by

H3K56 acetylation increase nucleosome mobility, thereby increasing DNA accessibility and, in the appropriate context, facilitating transcription [13, 17, 18].

Each core histone has an unstructured N-terminal tail domain that protrudes outside the nucleosomal DNA. These regions are not necessary for in vitro nucleosome assembly and crystallization [19] but contain many amino acids that are susceptible to post-translational modification [7]. How do these tail regions contribute to chromatin structure and function? Studies on the in vitro thermal mobility of nucleosomes [20] and earlier genetic and biochemical studies in yeast [21, 22] show that tails play a role in nucleosome mobility and higher order chromatin structure, but these roles are only revealed by removal of all, or most, of the tail, raising the question of how post-translational modifications could directly influence their function. Hyperacetylation of the tails of H2B, H3, and H4, each of which have 4–5 acetylatable lysines, will cause a significant loss of net positive charge and might influence higher order chromatin structures, even though the nucleosome itself is unaffected. An attempt to distinguish between the effects of lysine-specific and global acetylation of the H4 tail domain in yeast gave mixed results. For H4 lysines 5, 8, and 12, the level of acetylation (i.e., the number of lysines acetylated) seemed to be a more important determinant of transcription than the individual lysine involved, but H4 lysine 16 exerted independent effects [23]. Of course, methylation of lysines and arginines causes no change in net charge.

4.1.1.2 Chemical Signals on the Nucleosome Surface

An alternative explanation for the functional effects of histone tail modifications is that they act *indirectly* by generating, on the nucleosome surface, a variety of chemical signals that provide binding sites for nonhistone proteins. These binding proteins, in turn, regulate chromatin structure and function. This hypothesis was proposed 20 years ago [24, 25] and has since been extensively validated, not least by the identification of families of proteins carrying binding domains that recognize specific histone modifications [26, 27]. Bromodomains bind specifically to acetylated lysines, while chromodomains and several others bind to methylated lysines at selected positions on specific histones. Binding domains sometimes distinguish between lysines carrying one, two, or three methyl groups [26, 27].

A good example of how binding domains work is provided by the heterochromatin protein HP1, which is essential for heterochromatin formation in Drosophila and mammals. HP1 binds specifically, via its chromodomain, to H3 methylated at lysine 9 (H3K9me). H3K9me is located on heterochromatin in vivo and heterochromatin cannot form if the required methyl transferase is knocked out in mice [28]. Further, detailed studies of binding of HP1 to nucleosome arrays carrying methylated H3K9 provide likely mechanisms for both chromatin condensation and for the ability of heterochromatin to spread in vivo [29]. Other histone modifications have been associated with specific chromatin states. H4K36ac seems to be involved in the elongation phase of ongoing transcription [30], H4K20me3 is a marker for centric (constitutive) heterochromatin [31], and H3K27me3 is associated with long-term

gene silencing [32]. However, it is important to avoid oversimplifying a complex situation. Binding to any given modified residue will inevitably be influenced by modification at adjacent residues and functional outcomes are usually determined by the combinatorial action of different modifications. For example, phosphorylation of H3 serine 10 can displace HP1 bound to H3 methylated at lysine 9 [33]. Epigenomics approaches are beginning to reveal combinations of modifications that are consistently associated with functionally defined genomic regions, particularly promoters and enhancers [34–36].

The nucleosome can be seen as a gatekeeper that controls the access of transcription factors and other DNA binding proteins to DNA. Access is regulated by a variety of processes that change nucleosome structure, either directly (chromatin-remodeling enzymes, modification of internal amino acids) or indirectly (histone tail modifications). The enzyme families that carry out these processes are all susceptible to disruption, either through genetic mutation or environmental agents, triggering alterations in genome function that can sometimes precipitate changes in cell behavior and disease. Unraveling these complex chromatin-modifying enzyme systems will bring enormous benefits in the form of improved understanding of the etiology of diseases such as cancer and opening up new routes to therapy.

4.1.2 Histone Modification Status Is Regulated by Antagonistic Enzymes

Each histone modification is governed by antagonistic groups of enzymes that are able either to add or remove the modification in question. For example, histone acetyltransferases (KATs) catalyze the transfer of an acetyl group from acetyl-CoA to the ε-amino group of targeted lysine residues, and in this manner can neutralize the positive charge of the lysines. As a result the electrostatic interactions between histone and DNA are reduced. It is often suggested that this electrostatic effect can result in an open chromatin conformation that is more conducive to transcription [37, 38]. However, the role of the histone tails in maintaining higher order chromatin structure is not clear and while charge-mediated changes may be important in some contexts, they cannot provide a complete explanation for the functional affects of histone modifications. The actions of KATs are countered by HDACs. Broadly, acetylation is associated with gene activation and deacetylation with gene repression. However, for other modifications there is often not such a strict relationship between modification and function. For example, histone methyltransferases (KMTs) can either promote or inhibit transcription depending on the specific residue that is targeted and its genomic location relative to a gene's transcription start site (TSS). The functional identification of enzymes involved in setting and removing histone modifications has revealed an increasingly numerous battery of proteins and complexes. Many of these enzymes are either cofactors or binding partners for transcription factors (TF). Alternatively transcription regulatory factors can contain intrinsic histone-modifying capacity.

It is also apparent that at least some histone modifications can be regulated on a larger chromosomal scale or even globally, whereas other modifications have a much more restricted pattern. For example, H3 methylated at K79 (H3K79me3) is widely distributed across euchromatic regions in yeast and protects against the spreading of telomeric heterochromatin [39, 40] while H3K27me3, a mark put in place by the polycomb repressive complex, is spread across groups of genes (e.g., the HOX clusters) to bring about their coordinated silencing [41, 42]. Alternatively, marks such as H3K4me3 are closely associated with local genomic features, particularly promoters, enhancers, and TSSs [43, 44].

The KAT superfamily includes at least 20 different and diverse proteins including CLOCK and NCOA1. Several subfamilies exist including the P300/CBP family, e.g., p300; GCN5 family, e.g., KAT2A; the MYST family, e.g., MYST1; SRC/p160 nuclear receptor co-activator family, e.g., NCOA1. Eighteen HDAC are known in humans that are classified into four classes based on homology that include the HDAC1-11 and 7 SIRT members. Twenty-eight different KMT are known to act on histones, at least in vitro [1]. KMT are abundant and diverse reflecting the importance of the methylated state of key residues for the control of evolutionarily conserved transcriptional programs, for example, associated with development. There are at least 30 KMTs, including key families such as EZ, SUV39, and SET. At least 20 demethylases (KDM) are divided into two major groups that include the LSD family members, e.g., KDM1A/LSD1 and the Jumonji family, e.g., JHDM3 and JARID proteins containing ARID domains.

Two points are particularly important in considering the extent of redistribution and altered patterns of histone modifications in cancer. The first is that the steady-state level of each modification represents a dynamic balance between the effects of the modifying and de-modifying enzymes, with turnover likely to vary from one part of the genome to another, between cell types, and is intimately associated with cell cycle status, cell–cell interactions, and cell lineage commitment. Secondly, many, if not all, of the enzymes are either dependent upon, or influenced by, metabolites and components present in the intra- or extracellular environment. At the simplest level, many of these enzymes depend on cofactors such as acetyl CoA, NAD, and *S*-adenosyl methionine for their activity, and in turn these levels will depend on the metabolic and redox state of the cell. More subtle effects can be derived from metabolism. For example, naturally occurring inhibitors, such as short chain fatty acids (inhibitors of Class I HDACs) and nicotinamide (an inhibitor of the NAD-dependent deacetylase SIRT1) can be derived intrinsically within a cell or tissue and may naturally influence epigenetic status, for example, in the cell lining the lumen of the gut [45–47]. The effects of metabolic changes on gene expression are a strongly re-emergent area in cancer biology [48–50] and the generation of linked transcriptomic and metabolomics data is revealing the key functional associations in malignancy [51–53]. Thus the nucleosome, through the array of histone modifications it carries and the enzymes that put them in place, is a finely tuned sensor of the metabolic state of the cell and the composition of its environment. In this manner, nucleosome structure provides a platform through which external environmental and internal variables can influence genomic function.

4.2 Disruption of Histone Modifications in Cancer

Given that dynamic histone modifications are required for the precise control of DNA structure, during DNA repair and transcription, it is not surprising that there is significant evidence for the disruption of these events in malignancy. Understanding the differential recruitment and activity of proteins that govern histone modifications is key to understanding the roles that altered histone modifications can play in cancer initiation and development. Currently, a key focus in cancer biology is dissecting the mechanisms that alter the local and global recruitment and activity of histone-modifying complexes. It is anticipated that the insight generated will address the central challenge of separating which epigenetic processes directly drive cancer initiation and progression, from those that are merely a consequence of altered genomic structure such as mutation, copy number variation, and cytogenetic rearrangement. Insight into the contribution of altered histone functions to cancer progression can be gleamed by considering global and gene-loci specific alterations to histone modifications.

4.2.1 Global Distortions to Histone Modifications

A number of histone modifications are intimately associated with higher order chromatin structures and chromatin packaging and therefore changing the distribution of these global marks can have profound impact on the structure of chromatin in the nucleus. In turn such altered structures may be either more prone to aberrant DNA repair or promote genomic instability [54]. In prostate cancer, for example, quantifying global levels of five selected histone modifications in tissue sections by immunocytochemistry allowed discrimination between groups of patients with distinct risks of tumor recurrence [55, 56]. Quantitative analysis of just two modifications (H3K18ac and H3K4me2) was shown to provide useful prognostic information. The mechanisms underpinning these intriguing observations remain unknown.

The Polycomb complex (PcC) is a highly conserved inducer of repressive chromatin and sustains the H3K27me3 mark. This repression was shown to extend to multiple target genes associated with differentiation, often during development. Consequently, an emergent area in malignancy is the focus on aberrant PcC function to repress differentiation programs inappropriately. Increased H3K27me3 has been shown to have prognostic value in prostate and other cancers. These findings, however, reported the prognostic value to arise from the opposite patterns. Thus, increased levels of H3K27me3 are correlated with poor prognosis in esophageal cancer [57, 58], whereas in prostate cancer low levels have the poorer prognosis [59, 60].

The enzymes that control H3K27 methylation status are members of the enhancer of zeste homolog (EZH) that is the catalytic subunit of the polycomb repressive complex 2 [61]. These proteins are overexpressed in many cancers and in certain cases appear to correlate with poor prognosis or more aggressive disease.

However, although there are some correlations with increased H3K27me3 status, these are not universal in terms of the level of the mark. This may instead reflect the dynamics of turnover of the mark, and therefore the H3K27me3 status needs to be correlated with the enzymes that both add and subtract his mark.

Other modifications do appear to be altered in their global distribution in malignancy. For example, loss of H4K16 acetylation and H4K20me3 appears to be diminished globally in cancer cells, and indeed were some of the first histone marks to be characterized as being altered in malignancy [62, 63]. The consequence of these alterations probably reflect the role that certain modifications have in cross-talking with the mechanism of DNA methylation and indeed reduced levels of these marks were associated with DNA hypomethylation. Down-regulation of MYST1/MOF, one of the KAT that targets H4K16, may in part explain these altered patterns [64]. Reenforcing the concept of antagonistic enzymes, H4K16 is deacetylated by SIRT1 which is also up-regulated in several cancers and may have prognostic significance of its own [65]. Furthermore, the MYST family of KATs is associated with global changes in histone marks associated with chromatin packaging, DNA repair, and the control of developmental transcriptional programs (reviewed in [66]).

The control of lysine methylation states, however, is frequently more complex than acetylation states, and there are multiple enzymes controlling this modification. A major contributor to this complexity is the fact that the lysine epsilon amino group can accommodate one, two, or three methyl groups. All three methylation states are found in vivo and are often associated with distinct functional outcomes. Lysine methylation often proceeds in two steps, with mono and di-methylation governed by one class of enzyme and subsequent tri-methylation being regulated by a subsequent enzyme. For example, SET7 is able to catalyze the generation of H3K20me2, which then forms a substrate for the SUV class enzymes that generates the fully methylated state H3K20me3. Reflecting this, there is some evidence that levels of SUV family members are reduced in cancer in association with gene silencing [67, 68].

Further examples of a global alteration of histone status linked with cancer progression are those modifications that drive nucleosome movement. One of the key modifications in this regard is the internal lysine H3K56 that is targeted for acetylation by the KATs, CBP/p300 and GCN5, and has recently been shown to facilitate nucleosome disassembly and transcriptional activation. Inhibitor studies and expression profiling both suggest that the altered levels of H3K56ac distort the DNA damage response and maybe a trigger for genomic instability. Parallel studies have also revealed that H3K56ac is also involved in modulation of chromatin structure during DNA replication and repair; consequently, disruption to this process can also lead to genomic instability [18, 69–71]. Perhaps reflecting the importance of the regulation of this mark, multiple HDACs have been implicated in its control and include the NAD-dependent SIRTs.

Global changes in histone modifications have also been linked to stem cell differentiation. Undifferentiated embryonic stem (ES) cells show global enrichment in histone modifications associated with transcriptional activity and depletion in modifications associated with silent chromatin [72, 73]. By several criteria, ES cell

nuclear DNA is packaged in an unusual form of chromatin that appears to be more "open" than that in differentiated cells and is transcriptionally hyperactive [74]. How elevated histone modification levels are generated, and whether they are a cause or a consequence of open, hyperactive chromatin, remains to be determined.

Knocking down, individually, the histone demethylases KDM2A/JMJD1A and KDM4C/JMJD2C in mouse ES cells, globally increased the level of histone modifications usually associated with silent chromatin, namely, H3K9me2. In addition to their global effects, KDM2A/JMJD1A and KDM4C/JMJD2C were also shown to target, and regulate, specific genes, including *Tcl1*, a potential regulator of self-renewal, and *Nanog*, a key determinant of pluripotency [75]. Thus, key chromatin-modifying enzymes can exert both global and gene-specific effects that in turn influence differentiation. Intriguingly, both demethylase genes were themselves positively regulated by the key transcription factor Oct4, showing how a transcription factor might trigger a feed-forward signal to bring about a genome-wide change in the epigenetic landscape through regulation of genes encoding histone-modifying enzymes. In adult stem cell compartments, regulation of specific histone demethylating enzymes has also emerged as critical in activating differentiation programs, for example, the control of neural stem cell differentiation by the retinoic acid receptor, a member of the nuclear receptor (NR) superfamily [76]. A similar relationship between a transcription factor, global histone modifications, and adult stem cell differentiation is seen in studies of epidermal stem cells [77]. Quiescent stem cells are induced to leave their niche in the interfollicular epidermis and hair follicle bulge by activation of MYC, a process accompanied by globally increased H4 acetylation and di-methylation of H3K9 and H4K20. Together these studies illustrate how key transcription factors combine with environmental factors to influence and regulate the stem cell niche and control differentiation outputs.

Finally, the enzymes that govern histone methylation are also distorted in cancer with both loss and gain of function. Expression patterns of histone-modifying enzymes are even able to discriminate between tumor samples and their normal counterparts and cluster the tumor samples according to cell type [78]. This indicates that changes in the expression of histone-modifying enzymes have important and tumor-specific roles in cancer development. Thus, overexpression of G9a, an H3K9 KMT, occurs in lung and breast cancers and associates with aggressiveness [79]. Similarly enzymes that de-acetylate H3K9, and allow it to be methylated, are also overexpressed in cancers, including breast cancer. These enzymes may also be playing separate roles, and therefore expression is selected in malignancy on a different basis, for example, in gene regulation and DNA repair. It is possible that increases in HDAC levels are a homeostatic response in which the cell attempts to compensate for the aberrant increase in KAT activity (or vice versa). What is important from a functional point of view is not the absolute levels of KATs or HDACs, but the new steady-state levels of the (histone) modifications they regulate.

More precise specificity is dependent on the combination of both the enzyme *and* target gene(s). For example, mutation of KDM6A/UTX results in the inability to relieve H3K37me3 repression [80, 81]. Gain of function also occurs, for example, increased targeting of methyltransferases KMT1A/SUV39H1 to *CDKN1A* leads to

sustained H3K9me2 and transcriptional silencing that in turn can be targeted with the enzyme inhibitor, chaetocin [82, 83]. Similarly, the KMTs/MLLs are overexpressed in prostate cancer [84–86] and sustain levels of H3K27me3 at key targets such as DAP2IB, an RAS regulatory molecule, thereby leading to metastasis [87]. These observations illustrate deregulation of the enzymes that control histone lysine methylation is common but most likely highly targeted. This contextual nature is typified by KDM1A/LSD1 [88], which can target the demethylation of either H3K9me2 or H3K4me3 and thereby drive both gene activation [89, 90] and repression [91]. In this manner, KDM1A/LSD1 may mediate parallel repression and activation of target genes and play a key role in the malignant evolution of AR signaling in prostate cancer.

4.2.2 Altered Histone Modification Patterns at Discrete Gene Loci

Histone modifications therefore appear to operate at a level of restricted action, at discrete loci, exemplified by lysine methylation. Functional outcomes depend not only on which lysine on which histone is methylated, but also on whether the lysine carries one, two, or three methyl groups and its genomic position on a given loci with respect to the TSS. The different degrees of methylation are put in place, and removed, by a diverse group of enzymes. In particular, KDMs seem to have a particularly close association with key transcription factors that in turn are also implicated in malignancy such as MYC and members of the NR superfamily. Ligand binding or cofactor associations are able to influence the activity or even the specificity of these enzymes and thereby regulate functional outcomes (usually a change in gene expression) [92].

The modification of H3K9Ac and H3K9me2 serves to illustrate key concepts concerning histone status and specific gene expression. These marks are mutually exclusive and reciprocal, being associated with gene activation and repression, respectively. Loss of H3K9me2 is often associated with elevated gene expression. Recent studies have underscored the targeted changes in lysine methylation status and specifically illustrated that the KDM that targets H3K9me2 and the KMT that targets H3K4me at the gene TSS (to activate gene expression) are within the same complex associated with the ERα and therefore facilitate this two-step gene activation process [93]. Naturally, given that gene expression in cancer is uniformly neither up or down-regulated, the global expression of these marks is also not uniformly altered. Rather patterns are nuanced and suggest specific loci are deregulated.

Another example of this specificity emerges from considering KDM1A/LSD1 that can demethylate H3 mono- and di-methylated at either K4 or K9, and, remarkably, this specificity can be regulated in vitro by the protein cofactors, CoREST or BHC80, with which it is associated [94, 95]. Thus, KDM1A/LSD1 acts as an H3K4 demethylase (i.e., can remove a potentially activating modification) on NRSF targets and an H3K9 demethylase (i.e., can remove a potentially repressive modification)

on AR and ERα target promoters. Catalytic activity/specificity can also be regulated by adjacent histone modifications. H3K9 acetylation inhibits H3K4 demethylation (on the same tail) in vitro [96, 97]. Local patterns of modification are set by the combined actions of methylating and demethylating enzymes and the methylases too are influenced by other histone modifications. Further details of the gene-specific interactions have also emerged. JMJD2C demethylates H3K9me3, while KDM1A/LSD1 demethylates H3K9me2/me1 at promoters such as *PSA* and *KLK2* to remove H3K9 methylation associated with transcriptional silencing.

Therefore, the specific complex that KDM1A/LSD1 interacts with profoundly alters the transcriptional outputs, for example, of the AR, since demethylation of H3K9 has a gene activating effect, while demethylation of H3K4 has a gene silencing effect. The balance of these actions is in part controlled by the regulation of phosphorylation of H3 at threonine 6 (H3T6) by protein kinase C beta I. This prevents KDM1A/LSD1 from targeting H3K4me2 during AR-dependent gene activation and prevents it from limiting transcriptional activation. Also reflecting shared functions PKCbeta(I) co-localizes with AR and KDM1A/LSD1 on target gene promoters and phosphorylates H3T6 after androgen-induced gene expression. Therefore, it appears that androgen-dependent phosphorylation leads to the new chromatin mark H3T6ph, which in turn prevents removal of active methyl marks from H3K4 and forms a positive feed-forward loop of gene regulation [91]. More recently, KDM1A/LSD1 has been shown to drive AR-stimulated gene transrepression of the AR itself and thereby form a negative feedback loop of gene regulation [98]. Thus, the complex within which this one regulatory enzyme associates, its targeting to different genes, and the position of the response element, relative to the TSS, can all combine to determine how different H3K methylation states are governed.

4.2.3 Interplay Between Altered Transcriptional Signals and Epigenetic States

In normal cells a highly choreographed balance of histone modifications occurs during the dynamic regulation of coding and noncoding genes. These patterns are generated by the highly integrated actions of transcriptional networks [99] and are evident in many aspects of biology. For example, in development; in homeostasis to control the circadian rhythm [100], tissue self-renewal, and the response to hypoxia [83, 101]; in immune function to regulate inflammation [102]. Many of these processes are disrupted in malignancy and generally in cancer cells there is a loss of dynamic transcriptional patterns and signaling complexity is reduced [103]. Consequently, an area where altered histone modifications appear to associate with the cancer phenotype is in distortion of transcriptional control of key cellular processes.

Epigenetic events play a central role for transcriptional complexes and the various components in these multimeric complexes sequentially initiate, sustain, and

finally terminate transcription [104]. In this manner, transcription can work as a type of biological ratchet, with histone modifications being associated with the various states by generating chromatin states that are either receptive or resistant to transcription (reviewed in [27]). For example, different histone modifications can control the rate and magnitude of transcription (reviewed in [105]). These events are intertwined with low-level CpG methylation [106–108]. Thus, the histone modifications and other epigenetic events including DNA methylation processes combine during transcription to generate highly flexible chromatin states that are either transcriptionally receptive and resistant [101]. That is, the specific transcriptional potential of a gene is flexibly controlled by the combination of epigenetic events. These events are varied in space across the gene loci, and in time through the course of the transcriptional cycle. Current challenges in the field of cancer epigenetics, therefore, are to reveal how altered histone modifications directly drive distorted transcriptional programs, and what patterns exist on a genome-wide scale to distort networks of transcription. This will help to define how these altered histone states are genuine drivers in cancer progression.

Precisely how transcriptional programs evolve during malignancy is emerging. Genome-wide approaches are now allowing workers to ascribe broader views of the biology of transcription factor families, now that all members are known, and questions can be addressed in more detailed biological contexts. These findings suggest that the actions of the many key transcription factors are distilled through interactions with multiple cellular processes thereby generating an extremely flexible and integrated signaling module. In malignancy, however, these transcriptional choices and phenotypic outputs become restricted, for example, as seen with the emergence of a novel AR-transcriptome in androgen deprivation therapy-resistant prostate cancer [109].

Importantly, these epigenetic regulatory mechanisms operate in response to signals from the cellular microenvironment of the tumor, including signals from associated stromal (noncancerous) cells [110, 111]. The “niche” in which cells find themselves is an important determinant of their epigenetic properties [112] and raises the possibility that histone marks can be modified by environmental conditions that alter metabolic and redox status, leading to a heritable alteration in cell phenotype, an “epigenetic mutation.” Such lesions are not restricted to single nucleotides, but rather can be targeted to larger regions and therefore comparable to genetic deletions and amplifications. They can act alongside conventional genetic and cytogenetic alterations, either inherited or de novo, to cause the bi-allelic silencing of tumor suppressor genes that can be the first step in development of a cancer [113]. These concepts are illustrated by considering key transcription factor families implicated strongly in cancer initiation and progression.

4.2.3.1 The MYC/MAX/MAD Family

The MYC/MAX/MAD family forms heterodimeric complexes with MAX as the central partner to activate the expression of a diverse range of genes. Deregulated

and elevated expression of c-MYC has been documented in a wide range of human malignancies, associated frequently with aggressive and poorly differentiated tumors [114]. MYC has the potential to target a large proportion (11%) of all genes in the human genome [115], but the set of genes to which it actually binds in any particular cell is regulated by a variety of factors, including interacting proteins. For example, the MAD family of transcritpional repressors is , like MYC, able to bind MAX proteins and antagonize the activity of MYC by competing for MAX binding at E-box sequences in target gene promoters, actively repressing transcription of MYC target genes [116].

The specificity and affinity of MYC binding is influenced by the configuration of the chromatin packaging at potential binding sites, and particularly by patterns of histone modification [117]. MYC was found to bind E-boxes in regions enriched for several histone modifications generally associated with euchromatin, such as acetylated H3 (specifically H3K9ac, H3K14ac, and H3K18ac), but showed the strongest association with H3K4me3. Reciprocally, MYC was inversely correlated with the repressive polycomb group mark H3K27me3. On some promoters, MYC associated with both H3K4me3 and H3K27me3, a bivalent state that is common in ES cells but seems rarer in lineage committed cells [118]. Overall, it seems more likely that H3K4me3 recruits MYC rather than H3K27me3 excluding MYC binding. [117].

MYC function can be controlled interactions with JARID1A/RBP2 and JARID1B/PLU-1 [119, 120]. These enzymes are both specific for H3 methylated at lysine 4 (H3K4me1,2,3) and may help to regulate this modification at MYC binding sites. There is emerging evidence that this process is disrupted by increased association with histone demethylase NO66/MAPJD to alter the potential interactions with genes involved in proliferation of lung cancer cells [121]. A gene encoding a related protein, MINA53 (myc-induced nuclear antigen) is a MYC target that is overexpressed in lung cancer, for example [122, 123]. Together these findings suggest that the co-association of MYC with different histone-modifying enzymes, for example, through the consequence of altered enzyme expression, distorts and restricts the MYC transcriptome in malignancy.

In the light of these developments, MYC function has been reassessed to reveal the regulation of unexpected gene targets, some of which inhibit proliferation and induce programmed cell death [124], contrary to the accepted view of MYC as an oncogene promoting growth and survival. These findings suggest that the malignant function of MYC represents selection for a subset of its potential actions.

4.2.3.2 The NR Superfamily

The NR superfamily also illustrates the key concepts of distorted and selected transcription in cancer due to altered regulation of histone modifications. NRs are the largest superfamily of transcription factors in humans and generally form active heterodimers to control networks that regulate homeostasis, energy metabolism, and xenobiotic handling. These receptors are intimately associated with the control of self-renewal in a number of epithelial systems, notably the prostate and mammary

glands. For example, studies in the prostate have established that the androgen receptor (AR) cooperates with WNT and mTOR pathways [125, 126] to induce proliferation. Equally other receptors, such VDR, PPARs, and RARs, exert mitotic restraint, at least in part by antagonizing WNT signaling and activation of cell cycle arrest through regulation of gene targets such as *CDKN1A* (encodes $p21^{(waf1/cip1)}$) and *IGFBP3* [127–134].

Cancer is typified by the actions of individual receptors becoming selective and the NR network collectively displaying a loss of transcriptional plasticity. The AR transcriptional program evolves towards increased targeting of proliferative gene promoters and decreased targeting of pro-differentiation genes [135, 136]. Similarly, within breast cancer the transcriptional actions of the ERα appear to become increasingly selective for gene targets associated with proliferation and survival and away from targets associated with differentiation [137–139]. Equally in a range of solid tumors and myeloid leukemia, NRs that normally exert mitotic restraint, such as the VDR, RARs, and PPARs, become skewed, with selective silencing of antiproliferative target genes [129, 140–144]. Combined, oncogenic transcriptional rigidity reflects the simultaneous distorted regulation of target loci such that proliferative and survival signals are enhanced and antimitotic inputs are either limited or lost. This filtering of transcriptional choices during cancer progression has significant therapeutic implications. For example, the oncogenic actions of the TMPRSS2/ETS fusion, a common event in prostate cancer [145], are critical precisely because the *TMPRSS2* promoter is sustained in an AR-responsive state.

More recently, genome-wide ChIP approaches have revealed considerable variability in the networks of interactions capable of bringing about varied transcriptional responses [146–148]. For example, in prostate cancer, as the disease progresses, there are altered levels of H3K4me1 and 2 on gene enhancer regions in the so-called AR-independent state, where cells have evolved resistance to antiandrogen therapies. In this new state, the targeted increase of H3K4Me1 and 2 at different enhancer regions allows the cells to initiate a different AR transcriptional program [109].

4.2.3.3 Hypoxia-Inducible Factor-1 Alpha

The hypoxia response of hypoxia-inducible factor-1 alpha (HIF-1A) also illustrates how transcriptional actions are selectively distorted by epigenetic processes in cancer cells. Within a normal cell, the levels of oxygen are monitored sensitively by a transcriptional circuit that governs the function of HIF-1A. In normoxic conditions, HIF levels are kept low level by destruction by an E3 ubiquitin ligase containing the VHL tumor suppressor protein, where oxygen serves as a co-substrate. Also oxygen impedes the interactions of HIF1α with the KATs CBP/p300 thus limiting the capacity to initiate activating histone marks. In hypoxia, HIF-1α becomes stabilized and active, and promotes a stable interaction with CBP/p300 and therefore facilitates transcription [83]. Genome-wide analyses of HIF binding sites identified a number of KDMs as downstream targets, notably

JMJD1A and JMJD2B, thus providing the capacity to affect the epigenetic status of the cell. In part, this may contribute to maintenance of transcriptional activity under stress. It may also support the observed aberrant and selective HIF1α transcriptional responses [149].

Taken together these findings support the concept that the actions of major transcription factor families are selective at several levels to govern the expression of sub-transcriptomes that are phenotypically related. The flexibility of transcriptional actions includes the exact choice of target sequence, the timing, amplitude, and magnitude of transcription and integration with other transcriptional programs and signal transduction events. In malignancy, the dexterity of targeting and regulation is blunted and instead transcription factors become addicted to specific sub-transcriptomes, for example, those associated with blockade of programmed cell death and progression through the cell cycle.

4.2.4 Loss- and Gain-of-Function of Transcriptional Co-activators and Co-repressors

One means by which transcriptional actions are distorted is through the altered expression of associated cofactors that either have an intrinsic or associated capacity to regulate histone modifications. The diversity of co-activator and co-repressors is extreme and they have been the subject of numerous reviews [150–154]. Several examples are strongly illustrative of underlying mechanisms of transcriptional regulation. In essence, the altered expression and function of these key proteins alters the equilibrium of key histone modifications and thereby allowing the gene regulatory actions of a given transcription factor to become more or less pronounced.

Co-activators and co-repressors each display both loss and gain of function, and can result in similar phenotypes. Thus, the loss of a co-activator can lead to suppressed ability of a transcription factor to transactivate a given target. Similarly, the gain of function of co-repressors can limit transactivation ability and enhance transrepression. The opposite patterns will in turn enhance the transactivation function.

For example, NCOA3/SRC3 is situated within a common area of chromosomal amplification in breast cancer on chromosome 20q. Initially, cDNAs were isolated from this region that contained a putative target gene that was termed AIB1 (for "amplified in breast cancer-1"). Subsequently, this gene was found to be a member of the SRC co-activator family and was amplified and overexpressed in breast and ovarian cancer cell lines, as well as in breast cancer biopsies [155]. NCOA3/SRC3/AIB1 interacts with ERs in a ligand-dependent fashion and enhances the regulation of target genes. Specifically the protein has intrinsic KAT activity and also acts to recruit other CBP/p300 in an allosteric manner [156]. Therefore, increased expression increases the ability of the ERα to transactivate a given gene target. Subsequently, this protein was identified NCOA3 and shown to be a potent histone acetyl transferase able to enhance the function of multiple NRs [157–159].

Compared to their co-activator cousins, the co-repressors are somewhat under-explored. Again, these key proteins, originally identified for their repressive interactions with NR illustrate how deregulated functions can alter chromatin and thereby attenuate gene regulation. NCOR1 and NCOR2/SMRT were cloned in 1995 using NR as bait [160, 161], and both proteins exist in large multimeric complexes (~2.0 MDa) [162] with histone deactylases and other histone-modifying enzymes (reviewed in [153]). These complexes are recruited to many different transcription factors to repress gene activity during the transcriptional cycle. These transcription factors include: NR, MAD/MXI, MYOD, ETO, CBF, FOXP, AP-1, and NF-κB factors. The importance of targeted *basal repression* by co-repressors is evident in the lethality of the *Ncor1*$^{-/-}$ and *Ncor2/Smrt*$^{-/-}$ mice. These models reveal enhanced function of transcription factors, notably Pparγ in adipocytes [163] and FoxP in cardiomyocytes [164]. Dynamic mechanisms have also emerged whereby NCOR1 and NCOR2/SMRT complexes can be recruited to activate transcription factors leading to transrepression [165, 166]. Finally, an emerging theme is the pattern of active de-repression where loss of co-repressor association, following activated transcription factor, leads to up-regulation of target genes independently of the sustained presence of the transcription factor [167].

Well-established oncogenic roles for NCOR1 and NCOR2/SMRT have been elucidated in acute promyelocytic leukemia (PML) that results from a fusion between the NR, RARα, and either the PML or promyelocytic leukemia zinc finger (PLZF) genes [142]. Both chimeric proteins sustain NCOR1 interactions and consequently RARα-mediated cell differentiation is blocked, in part, as a result of maintaining a condensed chromatin structure around the promoters of RARα target genes that govern normal hematopoietic differentiation [168, 169]. In the PML-RAR fusion, this can be overcome by pharmacological dosing with retinoic acid. The PLZF-RAR fusion is resistant to retinoic acid alone and treatment with a combination of retinoic acid and HDAC inhibitors has shown promising results. Similarly, in acute myeloid leukemia (AML), the AML1/ETO fusion protein promotes leukemogenesis by recruiting NCOR1 and again impeding transcriptional regulation [170]. The importance of NCOR1 binding in the treatment of these disease states exemplifies the relevance of the co-repressors in firstly driving critical oncogenic events, but secondly providing a rational targeted strategy towards HDACs.

Expression profiling in solid tumors has revealed altered NCOR1 and NCOR2/SMRT expression and localization, for example, in breast, bladder, and prostate cancers [129, 141, 143, 171–173]. However, to date, uncertainty remains over their precise role in solid tumors, especially in the case of breast and prostate cancers where the etiology of disease is intimately driven by the actions of steroid hormone NRs. Indeed, the ability of the ligand-free NR to bind NCOR1 and NCOR2/SMRT is important to therapeutic exploitation with receptor antagonists such as Tamoxifen in the case of breast cancer. Therefore, ambiguity exists over the extent and timing of NCOR1 and NCOR2/SMRT expression changes, as they relate to initiation and progression of disease. Secondly, it remains unclear how changes in NCOR1 and NCOR2/SMRT expression relate to different NRs and other transcription factors that exert either pro- or antimitotic and survival effects. Resolving these ambiguities

has significant therapeutic implications in terms of targeting co-repressors as either epigenetic mono-therapies using HDAC inhibitors or in combinations with transcription factor targeting.

In prostate cancer cells, elevated levels of NCOR2/SMRT have been detected and suppress VDR responsiveness [129]. Similarly, PPAR actions are disrupted and can be targeted selectively by using HDAC inhibitor co-treatments [174, 175]. More specifically, elevated NCOR1, and to a lesser extent NCOR2/SMRT correlated with, and functionally drove, the selective insensitivity of PPARα/γ receptors towards dietary derived and therapeutic ligands [175] most clearly in androgen-independent disease. Similar roles for NCOR1 and NCOR2/SMRT appear in the development of breast cancer and Tamoxifen resistance [171]. Elevated levels of NCOR1 occur in ERα negative disease and in turn attenuate antimitotic actions of VDR. Again, this molecular lesion can be targeted in ERα negative breast cancer cell lines with co-treatments of VDR ligand (e.g., $1\alpha,25(OH)_2D_3$) plus HDAC inhibitors resulting in selective re-expression of VDR target genes, notably *VDUP1* and *GADD45A* [143]. Together, the studies in breast and prostate cancer suggest that NR show specificity in their interactions with co-repressors. NCOR1 appears to be involved in the regulation of receptors such as the VDR and PPARs and NCOR2/SMRT with steroid hormone receptors, reflecting the emergent specificities of NR interactions in the murine knockout models.

4.3 Consequences of Altered Histone Modification States

4.3.1 Higher Order Chromatin Interactions Associated with Transcription

Another theme that has emerged concerning epigenetic regulation of transcription is higher order chromosomal interactions. It seems that large-scale chromatin rearrangement, through looping, is frequent and widespread. Loops can be inter- or intra-chromosomal and are guided by transcription factors, key pioneer factors, and chromatin-modifying enzymes [176, 177]. Improved microscopy techniques have recently shown nascent RNA on the surface of protein dense transcription factories ("gene hubs") that seem to correspond to structures previously termed "nuclear speckles" [178].

A clear example of these interactions has been illustrated in the transcriptional responses of B-cells where translocation of genes occurs from separate chromosomes and nuclear regions to common sites referred to as transcription factories. These sites contain significant levels of RNA Pol II, and other proteins, including factors required for elongation, chromatin remodeling, capping, splicing, and nonsense-mediated decay. Recruitment of genes to transcription factories is highly selective, with certain genes and chromosome regions co-localizing far more frequently than expected by chance. Intriguingly, sites of chromosome translocation

associated with various cancers often co-localize. For example, *Myc* and *Igh* tend to co-localize and their fusion, in human lymphoid cells, is a common cause of Burkitt's Lymphoma. These rapid movements are associated with movements of the nuclear architecture and involve ATP-dependent mechanisms that involve a chromosome locus usually located at the nuclear periphery being rapidly translocated to the interior in a direction perpendicular to the nuclear membrane [179].

Again, the NR superfamily illustrates these aspects of the deregulation of epigenetic states. NRs appear to interact with more dominant more widely binding pioneer factors. For example, ERα interacts with pioneer factors and KDMs. This interaction is involved with micro-chromatin reorganization at response elements, and also with higher order chromatin reorganization. Active ATP-dependent transport mechanisms have recently been shown to be an essential intermediate step in gene activation by ERα and act to move discrete chromosomal regions together into interchromatin hubs. These granules are subsequently joined to the surface of nuclear structures rich in splicing and transcriptional machinery that may reflect the previously termed "nuclear speckles" [180].

This suggests a role for KDM1A/LSD1 in directing docking of the ERα-gene hub complex with the nuclear speckles, but the exact function of KDM1/LSD1 in this process remains unclear. If this role is catalytic rather than purely structural, it is possible that the substrate involved is a nonhistone protein. It will also be of interest to determine whether KDM1/LSD1 or related enzymes play a role in directing *MYC* and *IGH* alleles to transcription factories. The recent development of improved microscopy techniques which has shown nascent RNA appearing on the surface of protein dense transcription factories should aid in clarifying this situation [178], as well as further work investigating the relationship between nuclear speckles and transcription factories.

4.3.2 Directing DNA Methyltransferase Specificity and Stable Gene Silencing

There is compelling evidence that histone and DNA methylation processes disrupt transcriptional actions, both alone and together. For example, one consequence of NCOR1 and NCOR2/SMRT association at target genes is the loss of H3K9ac and accumulation of H3K9me2, allowing the potential for hypermethylation at adjacent CpG regions. Further links exist between NCOR1 and DNA methylation through its interaction with KAISO [181]. Correlative studies reveal that a number of key AR and VDR target genes are silenced by increased CpG methylation [182, 183]. At high density regions of CpG methylation, spanning hundreds of base pairs, the entire region acquires H3K9 and -K27 methylation, loses H3K4 methylation, and recruits heterochromatin binding protein 1 (HP1) [101]. The recruitment of HP1 through interaction with MBD1 leads to recruitment of both an H3K9 methylase (KMT1A/SUV39H1) [184] and DNA methyltransferases (DNMTs) [185]; enzymes that add repressive methylation marks to histones and CpG.

DNMT3L and UHFR1 also provide potential links between DNA methylation and absence of H3K4 methylation and presence of H3K9 methylation, respectively (reviewed in [186]).

Thus, these processes become self-reinforcing. It is not precisely clear, however, in mammalian cells whether either the H3K9 methylation or the high density of CpG methylation is required first to set up this heterochromatic structure. However in *Neurospora crassa*, loss of HP1 (which requires H3K9 methylation for binding to chromatin) leads to loss of DNA methylation [187]. This situation describes stable heterochromatic silencing of genomic regions and is in contrast to the dynamic changes at a locus with active epigenetic regulation of transcription in response to NR activation. However, even in such actively regulated regions, dynamic changes in DNA methylation appear to occur. For example, these have been measured in response to NR actions [106–108].

This differential regulation of histone methylation has profound implications for transcriptional control. DNA methylation and H3K4 methylation are mutually exclusive, while H3K9 methylation is strongly associated with DNA methylation, for example, through the formation of heterochromatin by HP1 binding and histone deacetylation. In the absence of DNA methylation, these inter-relationships are highly dynamic, with target gene promoters often poised to be subsequently pushed towards a fully active, or a more stably repressed state. For example, CpG island promoter regions of non-expressed genes do in fact show low-level RNA POLII association and modest transcriptional initiation. It seems that the presence of H3K4me3 methylation holds these promoters in a chromatin structure that is accessible to the transcriptional machinery, poised to recruit specific transcription factors to drive high level, efficient transcription. In turn this prevents H3K9me2 and DNA methylation. Aberrant DNA methylation of these CpG islands in cancer cells reduces this plasticity and coincides with loss of H3K4 methylation, gain of H3K9 methylation along with other heterochromatin marks, and stable transcriptional silencing [101].

The distributions of these histone modifications and DNA methylation patterns in cell line models are being organized by research consortia, for example, ENCODE [188]. Again, these genome-wide datasets also appear to support the idea that these histone marks are strongly associated with features of genomic architecture, such as gene regions, TSS, and enhancer regions where regulatory transcription factors can bind.

The links between sustained repressive histone modifications in the enhancer or promoter regions of a gene locus and altered DNA methylating events are targets for exploitation. Importantly, these epigenetic lesions are individually highly targetable with clinically available small molecular weight inhibitors targeted to specific histone deacetylation events and more recently this has been extended to include histone methylation events [189], coupled with agents that target CpG methylation (reviewed in [190]). Thus, comprehensive understanding of the key co-repressors in malignancy, delineating the key transcription factors interactions and the critical targets that are thereby dysregulated, may have considerable prognostic utility, specifically through the capacity to stratify patients for specific tailored epigenetic therapies.

References

1. Allis CD et al (2007) New nomenclature for chromatin-modifying enzymes. Cell 131: 633–636
2. Turner BM (2005) Reading signals on the nucleosome with a new nomenclature for modified histones. Nat Struct Mol Biol 12:110–112
3. Luger K, Mader AW, Richmond RK, Sargent DF, Richmond TJ (1997) Crystal structure of the nucleosome core particle at 2.8 A resolution. Nature 389:251–260
4. Flaus A, Owen-Hughes T (2011) Mechanisms for ATP-dependent chromatin remodelling: the means to the end. FEBS J 278:3579–3595
5. Recouvreux P et al (2011) Linker histones incorporation maintains chromatin fiber plasticity. Biophys J 100:2726–2735
6. Talbert PB, Henikoff S (2010) Histone variants—ancient wrap artists of the epigenome. Nat Rev Mol Cell Biol 11:264–275
7. Kouzarides T (2007) Chromatin modifications and their function. Cell 128:693–705
8. Ngara R, Ndimba R, Borch-Jensen J, Jensen ON, Ndimba B (2012) Identification and profiling of salinity stress-responsive proteins in Sorghum bicolor seedlings. J Proteomics 75: 4139–4150
9. Zhang S, Roche K, Nasheuer HP, Lowndes NF (2011) Modification of histones by sugar beta-N-acetylglucosamine (GlcNAc) occurs on multiple residues, including histone H3 serine 10, and is cell cycle-regulated. J Biol Chem 286:37483–37495
10. Tan M et al (2011) Identification of 67 histone marks and histone lysine crotonylation as a new type of histone modification. Cell 146:1016–1028
11. Cosgrove MS (2007) Histone proteomics and the epigenetic regulation of nucleosome mobility. Expert Rev Proteomics 4:465–478
12. Hyland EM et al (2005) Insights into the role of histone H3 and histone H4 core modifiable residues in Saccharomyces cerevisiae. Mol Cell Biol 25:10060–10070
13. Xu F, Zhang K, Grunstein M (2005) Acetylation in histone H3 globular domain regulates gene expression in yeast. Cell 121:375–385
14. Masumoto H, Hawke D, Kobayashi R, Verreault A (2005) A role for cell-cycle-regulated histone H3 lysine 56 acetylation in the DNA damage response. Nature 436:294–298
15. Vempati RK (2012) DNA damage in the presence of chemical genotoxic agents induce acetylation of H3K56 and H4K16 but not H3K9 in mammalian cells. Mol Biol Rep 39:303–308
16. Clemente-Ruiz M, Gonzalez-Prieto R, Prado F (2011) Histone H3K56 acetylation, CAF1, and Rtt106 coordinate nucleosome assembly and stability of advancing replication forks. PLoS Genet 7:e1002376
17. Watanabe S et al (2010) Structural characterization of H3K56Q nucleosomes and nucleosomal arrays. Biochim Biophys Acta 1799:480–486
18. Xie W et al (2009) Histone h3 lysine 56 acetylation is linked to the core transcriptional network in human embryonic stem cells. Mol Cell 33:417–427
19. Luger K, Richmond TJ (1998) The histone tails of the nucleosome. Curr Opin Genet Dev 8:140–146
20. Ferreira H, Somers J, Webster R, Flaus A, Owen-Hughes T (2007) Histone tails and the H3 alphaN helix regulate nucleosome mobility and stability. Mol Cell Biol 27:4037–4048
21. Lenfant F, Mann RK, Thomsen B, Ling X, Grunstein M (1996) All four core histone N-termini contain sequences required for the repression of basal transcription in yeast. EMBO J 15:3974–3985
22. Ling X, Harkness TA, Schultz MC, Fisher-Adams G, Grunstein M (1996) Yeast histone H3 and H4 amino termini are important for nucleosome assembly in vivo and in vitro: redundant and position-independent functions in assembly but not in gene regulation. Genes Dev 10:686–699
23. Dion MF, Altschuler SJ, Wu LF, Rando OJ (2005) Genomic characterization reveals a simple histone H4 acetylation code. Proc Natl Acad Sci USA 102:5501–5506

24. Turner BM (1993) Decoding the nucleosome. Cell 75:5–8
25. Turner BM, Birley AJ, Lavender J (1992) Histone H4 isoforms acetylated at specific lysine residues define individual chromosomes and chromatin domains in Drosophila polytene nuclei. Cell 69:375–384
26. Kutateladze TG (2011) SnapShot: histone readers. Cell 146:842–842.e1
27. Taverna SD, Li H, Ruthenburg AJ, Allis CD, Patel DJ (2007) How chromatin-binding modules interpret histone modifications: lessons from professional pocket pickers. Nat Struct Mol Biol 14:1025–1040
28. Liu Z et al (2010) Jmjd1a demethylase-regulated histone modification is essential for cAMP-response element modulator-regulated gene expression and spermatogenesis. J Biol Chem 285:2758–2770
29. Canzio D et al (2011) Chromodomain-mediated oligomerization of HP1 suggests a nucleosome-bridging mechanism for heterochromatin assembly. Mol Cell 41:67–81
30. Kolasinska-Zwierz P et al (2009) Differential chromatin marking of introns and expressed exons by H3K36me3. Nat Genet 41:376–381
31. Kourmouli N et al (2004) Heterochromatin and tri-methylated lysine 20 of histone H4 in animals. J Cell Sci 117:2491–2501
32. Ringrose L, Paro R (2007) Polycomb/trithorax response elements and epigenetic memory of cell identity. Development 134:223–232
33. Mateescu B, England P, Halgand F, Yaniv M, Muchardt C (2004) Tethering of HP1 proteins to chromatin is relieved by phosphoacetylation of histone H3. EMBO Rep 5:490–496
34. Ernst J et al (2011) Mapping and analysis of chromatin state dynamics in nine human cell types. Nature 473:43–49
35. Kharchenko PV et al (2011) Comprehensive analysis of the chromatin landscape in Drosophila melanogaster. Nature 471:480–485
36. Rada-Iglesias A et al (2011) A unique chromatin signature uncovers early developmental enhancers in humans. Nature 470:279–283
37. Korolev N, Lyubartsev AP, Nordenskiold L (2006) Computer modeling demonstrates that electrostatic attraction of nucleosomal DNA is mediated by histone tails. Biophys J 90: 4305–4316
38. Perico A, La Penna G, Arcesi L (2006) Electrostatic interactions with histone tails may bend linker DNA in chromatin. Biopolymers 81:20–28
39. Takahashi YH et al (2011) Dot1 and histone H3K79 methylation in natural telomeric and HM silencing. Mol Cell 42:118–126
40. Jones B et al (2008) The histone H3K79 methyltransferase Dot1L is essential for mammalian development and heterochromatin structure. PLoS Genet 4:e1000190
41. Lee MG et al (2007) Demethylation of H3K27 regulates polycomb recruitment and H2A ubiquitination. Science 318:447–450
42. Agger K et al (2007) UTX and JMJD3 are histone H3K27 demethylases involved in HOX gene regulation and development. Nature 449:731–734
43. Pekowska A et al (2011) H3K4 tri-methylation provides an epigenetic signature of active enhancers. EMBO J 30:4198–4210
44. Robertson AG et al (2008) Genome-wide relationship between histone H3 lysine 4 mono- and tri-methylation and transcription factor binding. Genome Res 18:1906–1917
45. Zeissig S et al (2007) Butyrate induces intestinal sodium absorption via Sp3-mediated transcriptional up-regulation of epithelial sodium channels. Gastroenterology 132:236–248
46. Augenlicht LH et al (2002) Short chain fatty acids and colon cancer. J Nutr 132: 3804S–3808S
47. Tanaka Y, Bush KK, Klauck TM, Higgins PJ (1989) Enhancement of butyrate-induced differentiation of HT-29 human colon carcinoma cells by 1,25-dihydroxyvitamin D3. Biochem Pharmacol 38:3859–3865
48. Cuezva JM et al (2002) The bioenergetic signature of cancer: a marker of tumor progression. Cancer Res 62:6674–6681

49. Racker E, Spector M (1981) Warburg effect revisited: merger of biochemistry and molecular biology. Science 213:303–307
50. Hsu PP, Sabatini DM (2008) Cancer cell metabolism: Warburg and beyond. Cell 134:703–707
51. Cavill R et al (2011) Consensus-phenotype integration of transcriptomic and metabolomic data implies a role for metabolism in the chemosensitivity of tumour cells. PLoS Comput Biol 7:e1001113
52. Su G, Burant CF, Beecher CW, Athey BD, Meng F (2011) Integrated metabolome and transcriptome analysis of the NCI60 dataset. BMC Bioinformatics 12(Suppl 1):S36
53. Sharon D, Chen R, Snyder M (2010) Systems biology approaches to disease marker discovery. Dis Markers 28:209–224
54. Misteli T, Soutoglou E (2009) The emerging role of nuclear architecture in DNA repair and genome maintenance. Nat Rev Mol Cell Biol 10:243–254
55. Seligson DB et al (2005) Global histone modification patterns predict risk of prostate cancer recurrence. Nature 435:1262–1266
56. Kurdistani SK (2007) Histone modifications as markers of cancer prognosis: a cellular view. Br J Cancer 97:1–5
57. He LR et al (2009) Prognostic impact of H3K27me3 expression on locoregional progression after chemoradiotherapy in esophageal squamous cell carcinoma. BMC Cancer 9:461
58. Tzao C et al (2009) Prognostic significance of global histone modifications in resected squamous cell carcinoma of the esophagus. Mod Pathol 22:252–260
59. Yu J et al (2007) A polycomb repression signature in metastatic prostate cancer predicts cancer outcome. Cancer Res 67:10657–10663
60. Wei Y et al (2008) Loss of trimethylation at lysine 27 of histone H3 is a predictor of poor outcome in breast, ovarian, and pancreatic cancers. Mol Carcinog 47:701–706
61. Hansen KH et al (2008) A model for transmission of the H3K27me3 epigenetic mark. Nat Cell Biol 10:1291–1300
62. Fraga MF et al (2005) Loss of acetylation at Lys16 and trimethylation at Lys20 of histone H4 is a common hallmark of human cancer. Nat Genet 37:391–400
63. Tryndyak VP, Kovalchuk O, Pogribny IP (2006) Loss of DNA methylation and histone H4 lysine 20 trimethylation in human breast cancer cells is associated with aberrant expression of DNA methyltransferase 1, Suv4-20h2 histone methyltransferase and methyl-binding proteins. Cancer Biol Ther 5:65–70
64. Pfister S et al (2008) The histone acetyltransferase hMOF is frequently downregulated in primary breast carcinoma and medulloblastoma and constitutes a biomarker for clinical outcome in medulloblastoma. Int J Cancer 122:1207–1213
65. Bosch-Presegue L, Vaquero A (2011) The dual role of sirtuins in cancer. Genes Cancer 2:648–662
66. Rius M, Lyko F (2011) Epigenetic cancer therapy: rationales, targets and drugs. Oncogene Dec 19. doi:10.1038/onc.2011.601
67. Pogribny IP et al (2006) Histone H3 lysine 9 and H4 lysine 20 trimethylation and the expression of Suv4-20h2 and Suv-39h1 histone methyltransferases in hepatocarcinogenesis induced by methyl deficiency in rats. Carcinogenesis 27:1180–1186
68. Lakshmikuttyamma A, Scott SA, DeCoteau JF, Geyer CR (2010) Reexpression of epigenetically silenced AML tumor suppressor genes by SUV39H1 inhibition. Oncogene 29:576–588
69. Das C, Lucia MS, Hansen KC, Tyler JK (2009) CBP/p300-mediated acetylation of histone H3 on lysine 56. Nature 459:113–117
70. Williams SK, Truong D, Tyler JK (2008) Acetylation in the globular core of histone H3 on lysine-56 promotes chromatin disassembly during transcriptional activation. Proc Natl Acad Sci USA 105:9000–9005
71. Adkins MW, Williams SK, Linger J, Tyler JK (2007) Chromatin disassembly from the PHO5 promoter is essential for the recruitment of the general transcription machinery and coactivators. Mol Cell Biol 27:6372–6382

72. Meshorer E, Misteli T (2006) Chromatin in pluripotent embryonic stem cells and differentiation. Nat Rev Mol Cell Biol 7:540–546
73. Spivakov M, Fisher AG (2007) Epigenetic signatures of stem-cell identity. Nat Rev Genet 8:263–271
74. Meshorer E, Gruenbaum Y (2008) Gone with the Wnt/Notch: stem cells in laminopathies, progeria, and aging. J Cell Biol 181:9–13
75. Loh YH, Zhang W, Chen X, George J, Ng HH (2007) Jmjd1a and Jmjd2c histone H3 Lys 9 demethylases regulate self-renewal in embryonic stem cells. Genes Dev 21:2545–2557
76. Jepsen K et al (2007) SMRT-mediated repression of an H3K27 demethylase in progression from neural stem cell to neuron. Nature 450:415–419
77. Frye M, Fisher AG, Watt FM (2007) Epidermal stem cells are defined by global histone modifications that are altered by Myc-induced differentiation. PLoS One 2:e763
78. Ozdag H et al (2006) Differential expression of selected histone modifier genes in human solid cancers. BMC Genomics 7:90
79. Watanabe H et al (2008) Deregulation of histone lysine methyltransferases contributes to oncogenic transformation of human bronchoepithelial cells. Cancer Cell Int 8:15
80. Wang JK et al (2010) The histone demethylase UTX enables RB-dependent cell fate control. Genes Dev 24:327–332
81. van Haaften G et al (2009) Somatic mutations of the histone H3K27 demethylase gene UTX in human cancer. Nat Genet 41:521–523
82. Cherrier T et al (2009) p21(WAF1) gene promoter is epigenetically silenced by CTIP2 and SUV39H1. Oncogene 28:3380–3389
83. Pollard PJ et al (2008) Regulation of Jumonji-domain-containing histone demethylases by hypoxia-inducible factor (HIF)-1alpha. Biochem J 416:387–394
84. Scharer CD et al (2009) Genome-wide promoter analysis of the SOX4 transcriptional network in prostate cancer cells. Cancer Res 69:709–717
85. Varambally S et al (2002) The polycomb group protein EZH2 is involved in progression of prostate cancer. Nature 419:624–629
86. Kondo Y et al (2008) Gene silencing in cancer by histone H3 lysine 27 trimethylation independent of promoter DNA methylation. Nat Genet 40:741–750
87. Min J et al (2010) An oncogene-tumor suppressor cascade drives metastatic prostate cancer by coordinately activating Ras and nuclear factor-kappaB. Nat Med 16:286–294
88. Shi Y et al (2004) Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. Cell 119:941–953
89. Wissmann M et al (2007) Cooperative demethylation by JMJD2C and LSD1 promotes androgen receptor-dependent gene expression. Nat Cell Biol 9:347–353
90. Metzger E et al (2005) LSD1 demethylates repressive histone marks to promote androgen-receptor-dependent transcription. Nature 437:436–439
91. Metzger E et al (2010) Phosphorylation of histone H3T6 by PKCbeta(I) controls demethylation at histone H3K4. Nature 464:792–796
92. Shi Y (2007) Histone lysine demethylases: emerging roles in development, physiology and disease. Nat Rev Genet 8:829–833
93. Shi L et al (2011) Histone demethylase JMJD2B coordinates H3K4/H3K9 methylation and promotes hormonally responsive breast carcinogenesis. Proc Natl Acad Sci USA 108:7541–7546
94. Lee MG, Wynder C, Cooch N, Shiekhattar R (2005) An essential role for CoREST in nucleosomal histone 3 lysine 4 demethylation. Nature 437:432–435
95. Shi YJ et al (2005) Regulation of LSD1 histone demethylase activity by its associated factors. Mol Cell 19:857–864
96. Forneris F, Binda C, Vanoni MA, Battaglioli E, Mattevi A (2005) Human histone demethylase LSD1 reads the histone code. J Biol Chem 280:41360–41365
97. Forneris F et al (2006) A highly specific mechanism of histone H3-K4 recognition by histone demethylase LSD1. J Biol Chem 281:35289–35295

98. Cai C et al (2011) Androgen receptor gene expression in prostate cancer is directly suppressed by the androgen receptor through recruitment of lysine-specific demethylase 1. Cancer Cell 20:457–471
99. Yosef N, Regev A (2011) Impulse control: temporal dynamics in gene transcription. Cell 144:886–896
100. Alenghat T et al (2008) Nuclear receptor corepressor and histone deacetylase 3 govern circadian metabolic physiology. Nature 456(7224):997–1000
101. Mohn F, Schubeler D (2009) Genetics and epigenetics: stability and plasticity during cellular differentiation. Trends Genet 25:129–136
102. De Santa F et al (2007) The histone H3 lysine-27 demethylase Jmjd3 links inflammation to inhibition of polycomb-mediated gene silencing. Cell 130:1083–1094
103. Cui Q et al (2007) A map of human cancer signaling. Mol Syst Biol 3:152
104. Dobrzynski M, Bruggeman FJ (2009) Elongation dynamics shape bursty transcription and translation. Proc Natl Acad Sci USA 106(8):2583–2588
105. Goldberg AD, Allis CD, Bernstein E (2007) Epigenetics: a landscape takes shape. Cell 128:635–638
106. Le May N et al (2010) NER factors are recruited to active promoters and facilitate chromatin modification for transcription in the absence of exogenous genotoxic attack. Mol Cell 38:54–66
107. Metivier R et al (2008) Cyclical DNA methylation of a transcriptionally active promoter. Nature 452:45–50
108. Kangaspeska S et al (2008) Transient cyclical methylation of promoter DNA. Nature 452:112–115
109. Wang Q et al (2009) Androgen receptor regulates a distinct transcription program in androgen-independent prostate cancer. Cell 138:245–256
110. Hu M et al (2005) Distinct epigenetic changes in the stromal cells of breast cancers. Nat Genet 37:899–905
111. Orimo A, Weinberg RA (2006) Stromal fibroblasts in cancer: a novel tumor-promoting cell type. Cell Cycle 5:1597–1601
112. Li L, Neaves WB (2006) Normal stem cells and cancer stem cells: the niche matters. Cancer Res 66:4553–4557
113. Feinberg AP, Ohlsson R, Henikoff S (2006) The epigenetic progenitor origin of human cancer. Nat Rev Genet 7:21–33
114. Vita M, Henriksson M (2006) The Myc oncoprotein as a therapeutic target for human cancer. Semin Cancer Biol 16:318–330
115. Fernandez PC et al (2003) Genomic targets of the human c-Myc protein. Genes Dev 17:1115–1129
116. Adhikary S, Eilers M (2005) Transcriptional regulation and transformation by Myc proteins. Nat Rev Mol Cell Biol 6:635–645
117. Guccione E et al (2006) Myc-binding-site recognition in the human genome is determined by chromatin context. Nat Cell Biol 8:764–770
118. Bernstein BE et al (2006) A bivalent chromatin structure marks key developmental genes in embryonic stem cells. Cell 125:315–326
119. Secombe J, Li L, Carlos L, Eisenman RN (2007) The Trithorax group protein Lid is a trimethyl histone H3K4 demethylase required for dMyc-induced cell growth. Genes Dev 21:537–551
120. Secombe J, Eisenman RN (2007) The function and regulation of the JARID1 family of histone H3 lysine 4 demethylases: the Myc connection. Cell Cycle 6:1324–1328
121. Suzuki C et al (2007) Identification of Myc-associated protein with JmjC domain as a novel therapeutic target oncogene for lung cancer. Mol Cancer Ther 6:542–551
122. Ogasawara S et al (2010) Accelerated expression of a Myc target gene Mina53 in aggressive hepatocellular carcinoma. Hepatol Res 40:330–336
123. Komiya K et al (2010) Mina53, a novel c-Myc target gene, is frequently expressed in lung cancers and exerts oncogenic property in NIH/3T3 cells. J Cancer Res Clin Oncol 136:465–473

124. Watt FM, Frye M, Benitah SA (2008) MYC in mammalian epidermis: how can an oncogene stimulate differentiation? Nat Rev Cancer 8:234–242
125. Li H, Kim JH, Koh SS, Stallcup MR (2004) Synergistic effects of coactivators GRIP1 and beta-catenin on gene activation: cross-talk between androgen receptor and Wnt signaling pathways. J Biol Chem 279:4212–4220
126. Yang X et al (2006) Complex regulation of human androgen receptor expression by Wnt signaling in prostate cancer cells. Oncogene 25(24):3436–3444
127. Campbell MJ, Elstner E, Holden S, Uskokovic M, Koeffler HP (1997) Inhibition of proliferation of prostate cancer cells by a 19-nor-hexafluoride vitamin D3 analogue involves the induction of p21waf1, p27kip1 and E-cadherin. J Mol Endocrinol 19:15–27
128. Degenhardt T, Matilainen M, Herzig KH, Dunlop TW, Carlberg C (2006) The insulin-like growth factor binding protein 1 gene is a primary target of peroxisome proliferator-activated receptors. J Biol Chem 281(51):39607–39619
129. Khanim FL et al (2004) Altered SMRT levels disrupt vitamin D(3) receptor signalling in prostate cancer cells. Oncogene 23:6712–6725
130. Kubota T et al (1998) 19-nor-26,27-bishomo-vitamin D3 analogs: a unique class of potent inhibitors of proliferation of prostate, breast, and hematopoietic cancer cells. Cancer Res 58:3370–3375
131. Liu M, Lee MH, Cohen M, Bommakanti M, Freedman LP (1996) Transcriptional activation of the Cdk inhibitor p21 by vitamin D3 leads to the induced differentiation of the myelomonocytic cell line U937. Genes Dev 10:142–153
132. Palmer HG et al (2004) The transcription factor SNAIL represses vitamin D receptor expression and responsiveness in human colon cancer. Nat Med 10:917–919
133. Saramaki A, Banwell CM, Campbell MJ, Carlberg C (2006) Regulation of the human p21(waf1/cip1) gene promoter via multiple binding sites for p53 and the vitamin D3 receptor. Nucleic Acids Res 34:543–554
134. Thorne J, Campbell MJ (2008) The vitamin D receptor in cancer. Proc Nutr Soc 67:115–127
135. Hendriksen PJ et al (2006) Evolution of the androgen receptor pathway during progression of prostate cancer. Cancer Res 66:5012–5020
136. Taneja SS et al (2004) ART-27, an androgen receptor coactivator regulated in prostate development and cancer. J Biol Chem 279:13944–13952
137. Ross-Innes CS et al (2012) Differential oestrogen receptor binding is associated with clinical outcome in breast cancer. Nature 481(7381):389–393
138. Ceschin DG et al (2011) Methylation specifies distinct estrogen-induced binding site repertoires of CBP to chromatin. Genes Dev 25:1132–1146
139. Welboren WJ et al (2009) ChIP-Seq of ERalpha and RNA polymerase II defines genes differentially responding to ligands. EMBO J 28:1418–1428
140. Rashid SF et al (2001) Synergistic growth inhibition of prostate cancer cells by 1 alpha,25 Dihydroxyvitamin D(3) and its 19-nor-hexafluoride analogs in combination with either sodium butyrate or trichostatin A. Oncogene 20:1860–1872
141. Abedin SA et al (2009) Elevated NCOR1 disrupts a network of dietary-sensing nuclear receptors in bladder cancer cells. Carcinogenesis 30(3):449–456
142. Lin RJ et al (1998) Role of the histone deacetylase complex in acute promyelocytic leukaemia. Nature 391:811–814
143. Banwell CM et al (2006) Altered nuclear receptor corepressor expression attenuates vitamin D receptor signaling in breast cancer cells. Clin Cancer Res 12:2004–2013
144. Ting HJ, Bao BY, Reeder JE, Messing EM, Lee YF (2007) Increased expression of corepressors in aggressive androgen-independent prostate cancer cells results in loss of 1alpha,25-dihydroxyvitamin D3 responsiveness. Mol Cancer Res 5:967–980
145. Tomlins SA et al (2005) Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. Science 310:644–648
146. Anderson SP et al (2004) Overlapping transcriptional programs regulated by the nuclear receptors peroxisome proliferator-activated receptor {alpha}, retinoid X receptor and liver X receptor in mouse liver. Mol Pharmacol 66(6):1440–1452

147. Bookout AL et al (2006) Anatomical profiling of nuclear receptor expression reveals a hierarchical transcriptional network. Cell 126:789–799
148. Handschin C, Meyer UA (2005) Regulatory network of lipid-sensing nuclear receptors: roles for CAR, PXR, LXR, and FXR. Arch Biochem Biophys 433:387–396
149. Xia X et al (2009) Integrative analysis of HIF binding and transactivation reveals its role in maintaining histone methylation homeostasis. Proc Natl Acad Sci USA 106:4260–4265
150. Malik S, Roeder RG (2010) The metazoan Mediator co-activator complex as an integrative hub for transcriptional regulation. Nat Rev Genet 11:761–772
151. Xu J, Wu RC, O'Malley BW (2009) Normal and cancer-related functions of the p160 steroid receptor co-activator (SRC) family. Nat Rev Cancer 9:615–630
152. Taatjes DJ, Marr MT, Tjian R (2004) Regulatory diversity among metazoan co-activator complexes. Nat Rev Mol Cell Biol 5:403–410
153. Perissi V, Jepsen K, Glass CK, Rosenfeld MG (2010) Deconstructing repression: evolving models of co-repressor action. Nat Rev Genet 11:109–123
154. Battaglia S, Maguire O, Campbell MJ (2010) Transcription factor co-repressors in cancer biology: roles and targeting. Int J Cancer 126:2511–2519
155. Anzick SL et al (1997) AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer. Science 277:965–968
156. Demarest SJ et al (2002) Mutual synergistic folding in recruitment of CBP/p300 by p160 nuclear receptor coactivators. Nature 415:549–553
157. Zhao C et al (2003) Elevated expression levels of NCOA3, TOP1, and TFAP2C in breast tumors as predictors of poor prognosis. Cancer 98:18–23
158. Doi M, Hirayama J, Sassone-Corsi P (2006) Circadian regulator CLOCK is a histone acetyltransferase. Cell 125:497–508
159. Esteyries S et al (2008) NCOA3, a new fusion partner for MOZ/MYST3 in M5 acute myeloid leukemia. Leukemia 22:663–665
160. Horlein AJ et al (1995) Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor. Nature 377:397–404
161. Chen JD, Evans RM (1995) A transcriptional co-repressor that interacts with nuclear hormone receptors. Nature 377:454–457
162. Li J et al (2000) Both corepressor proteins SMRT and N-CoR exist in large protein complexes containing HDAC3. EMBO J 19:4342–4350
163. Yu C et al (2005) The nuclear receptor corepressors NCoR and SMRT decrease PPARgamma transcriptional activity and repress 3T3-L1 adipogenesis. J. Biol, Chem
164. Jepsen K, Gleiberman AS, Shi C, Simon DI, Rosenfeld MG (2008) Cooperative regulation in development by SMRT and FOXP1. Genes Dev 22:740–745
165. Tiefenbach J et al (2006) SUMOylation of the corepressor N-CoR modulates its capacity to repress transcription. Mol Biol Cell 17:1643–1651
166. Surjit M et al (2011) Widespread negative response elements mediate direct repression by agonist-liganded glucocorticoid receptor. Cell 145:224–241
167. Heikkinen S et al (2011) Nuclear hormone 1{alpha},25-dihydroxyvitamin D3 elicits a genome-wide shift in the locations of VDR chromatin occupancy. Nucleic Acids Res 39(21):9181–9193
168. Muller-Tidow C et al (2010) Profiling of histone H3 lysine 9 trimethylation levels predicts transcription factor activity and survival in acute myeloid leukemia. Blood 116:3564–3571
169. Hoemme C et al (2008) Chromatin modifications induced by PML-RARalpha repress critical targets in leukemogenesis as analyzed by ChIP-Chip. Blood 111:2887–2895
170. Minucci S et al (2000) Oligomerization of RAR and AML1 transcription factors as a novel mechanism of oncogenic activation. Mol Cell 5:811–820
171. Girault I et al (2003) Expression analysis of estrogen receptor alpha coregulators in breast carcinoma: evidence that NCOR1 expression is predictive of the response to tamoxifen. Clin Cancer Res 9:1259–1266
172. Zhang Z et al (2005) NCOR1 mRNA is an independent prognostic factor for breast cancer. Cancer Lett 237(1):123–129

173. Kim JY, Son YL, Lee YC (2009) Involvement of SMRT corepressor in transcriptional repression by the vitamin D receptor. Mol Endocrinol 23:251–264
174. Chang TH, Szabo E (2002) Enhanced growth inhibition by combination differentiation therapy with ligands of peroxisome proliferator-activated receptor-gamma and inhibitors of histone deacetylase in adenocarcinoma of the lung. Clin Cancer Res 8:1206–1212
175. Battaglia S et al (2010) Elevated NCOR1 disrupts PPAR signaling in prostate cancer and forms a targetable epigenetic lesion. Carcinogenesis 31(9):1650–1660
176. Bau D et al (2011) The three-dimensional folding of the alpha-globin gene domain reveals formation of chromatin globules. Nat Struct Mol Biol 18:107–114
177. Li Q, Barkess G, Qian H (2006) Chromatin looping and the probability of transcription. Trends Genet 22:197–202
178. Eskiw CH, Rapp A, Carter DR, Cook PR (2008) RNA polymerase II activity is located on the surface of protein-rich transcription factories. J Cell Sci 121:1999–2007
179. Mitchell JA, Fraser P (2008) Transcription factories are nuclear subcompartments that remain in the absence of transcription. Genes Dev 22:20–25
180. Hu Q et al (2008) Enhancing nuclear receptor-induced transcription requires nuclear motor and LSD1-dependent gene networking in interchromatin granules. Proc Natl Acad Sci USA 105:19199–19204
181. Yoon HG, Chan DW, Reynolds AB, Qin J, Wong J (2003) N-CoR mediates DNA methylation-dependent repression through a methyl CpG binding protein Kaiso. Mol Cell 12:723–734
182. Yegnasubramanian S et al (2004) Hypermethylation of CpG islands in primary and metastatic human prostate cancer. Cancer Res 64:1975–1986
183. Asatiani E et al (2005) Deletion, methylation, and expression of the NKX3.1 suppressor gene in primary human prostate cancer. Cancer Res 65:1164–1173
184. Fujita N et al (2003) Methyl-CpG binding domain 1 (MBD1) interacts with the Suv39h1-HP1 heterochromatic complex for DNA methylation-based transcriptional repression. J Biol Chem 278:24132–24138
185. Esteve PO et al (2006) Direct interaction between DNMT1 and G9a coordinates DNA and histone methylation during replication. Genes Dev 20:3089–3103
186. Cheng X, Blumenthal RM (2010) Coordinated chromatin control: structural and functional linkage of DNA and histone methylation. Biochemistry 49:2999–3008
187. Freitag M, Hickey PC, Khlafallah TK, Read ND, Selker EU (2004) HP1 is essential for DNA methylation in Neurospora. Mol Cell 13:427–434
188. Birney E et al (2007) Identification and analysis of functional elements in 1% of the human genome by the ENCODE pilot project. Nature 447:799–816
189. Schulte JH et al (2009) Lysine-specific demethylase 1 is strongly expressed in poorly differentiated neuroblastoma: implications for therapy. Cancer Res 69:2065–2071
190. Graham JS, Kaye SB, Brown R (2009) The promises and pitfalls of epigenetic therapies in solid tumours. Eur J Cancer 45:1129–1136

Chapter 5
Nucleosome Occupancy and Gene Regulation During Tumorigenesis

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Abstract Nucleosomes are the basic structural units of eukaryotic chromatin. In recent years, it has become evident that nucleosomes and their position, in concert with other epigenetic mechanisms (such as DNA methylation, histone modifications, changes in histone variants, as well as small noncoding regulatory RNAs) play essential roles in the control of gene expression. Here, we discuss the mechanisms and factors that regulate nucleosome position and gene expression in normal and cancer cells.

5.1 Introduction

Nucleosomes are the basic units of eukaryotic chromatin, each one containing ~146 bp of DNA wrapped around an octamer of histone core proteins (H3, H4, H2A, and H2B), which in turn are separated by linker DNA of variable length [1]. At least five epigenetic mechanisms have been shown to act in concert to regulate gene expression by modifying chromatin structure, namely DNA methylation, histone modifications, nucleosome remodeling, and changes in histone variants as well as small noncoding regulatory RNAs [2]. In addition to playing a pivotal role in chromatin structure, nucleosomes display differential occupancy at promoter regions, thereby regulating gene expression by altering DNA accessibility. For instance, a nucleosome-depleted region (NDR) at transcriptional start sites correlates with gene expression, whereas the positioning of a nucleosome over the transcriptional start site results in gene repression [2, 3]. The position of nucleosomes is determined and influenced by a number of factors, including DNA sequence, DNA methylation, histone modifications and histone variants, chromatin remodelers, and

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transcription factor binding [4]. We discuss how these factors act in normal cells and how abnormalities in these factors impact nucleosome occupancy and gene expression in cancer cells.

5.2 Regulation of Nucleosome Position in Normal Cells

5.2.1 DNA Sequence Preferences

The sequences that regulate nucleosome position fall into two categories: motifs that are preferred (included within the nucleosome) and motifs that avoided (excluded from the nucleosome) [5]. Preferred sequences were originally characterized as particular dinucleotides, including CG and GC dinucleotides, occurring with approximately 10 bp periodicity, although nucleosomes may also prefer longer DNA motifs [4, 6]. The sequences that are disfavored by nucleosomes include various 5-mers and long tracts of As (10–20 bp or more), possibly due to their resistance to the structural distortions required for DNA wrapping and nucleosome formation [4, 7]. Such organization helps restrict nucleosome access to those regions to ensure proper gene expression pattern [7]. An example of regions containing both preferred and disfavored sequences with restricted nucleosome positioning are the Alu repeats [6, 8]. More recently, however, the concept of intrinsically DNA-encoded positioning as an organizational determinant of the 5′ end of genes has been challenged. In this regard, studies showed that the majority of the human genome displays great flexibility in nucleosome positioning, although DNA sequence can strongly drive the organization of nucleosomes at specific sites [9]. It has also been shown that these intrinsic signals can be overridden, confirming that additional factors are involved in nucleosome organization [9, 10].

5.2.2 Nucleosomes and DNA Methylation

DNA methylation in mammals occurs at CpG dinucleotides, which are distributed along the genome in clusters (CpG islands) or in regions containing high concentration of repeat sequences, and acts as a relatively stable gene silencing mechanism [2]. The majority of isolated CpGs tend to be methylated in mammals. In contrast, the majority of the CpG islands, which represent 60% of all human promoters, remain largely unmethylated [2, 3]. However, a number of CpG island promoters, such as those of imprinted genes, are methylated resulting in monoallelic gene expression in normal cells [3]. CpG islands can also be found within or in between transcriptional units (orphan CpG islands) [3] and can be associated with novel promoter regions and to be active in a tissue-specific manner [3]. DNA methylation also appears to be important for the regulation of non-CpG island promoters and the

tissue-specific expression of the genes that they control including *MASPIN*, *OCT-4*, *LAMB3*, and *RUNX3* promoter 1 [11–14]. Methylation is also observed in repetitive genomic sequences, which include transposable elements and noncoding DNA, where it helps maintain genomic stability [15, 16]. DNA methylation is established by the activity of three DNA methyltransferases (DNMTs): DNMT1, which preferentially methylates hemimethylated DNA during replication, and DNMT3A and DNMT3B, which are replication-independent, have been shown to establish de novo DNA methylation. Furthermore, DNMT3A and 3B were shown to be recruited to sites methylated by DNMT1 thereby contributing to propagate the methylated state [17, 18].

CpG DNA methylation causes steric interference in the formation of nucleosomes in vitro, suggesting that methylation may play a role in nucleosome occupancy [4]. However, more recent in vivo studies demonstrate that the nucleosome architecture plays a role in the shaping of DNA methylation patterns [19]. This is in agreement with studies from our laboratory showing that nucleosomes are required for stable DNMT3A/3B anchoring [17, 18, 20] and that nucleosome occupancy precedes de novo DNA methylation in vivo [14]. While the direction of the relationship is still under investigation, it is clear that nucleosome position and methylation are interrelated.

5.2.3 Nucleosomes and Histone Modifications

The N-terminus of histones can undergo a variety of modifications in specific residues, including acetylation, methylation, ubiquitination, sumoylation, and phosphorylation [21]. Histone modifications work in a combinatorial fashion to alter chromatin accessibility by disrupting interactions between nucleosomes or by regulating the recruitment of nonhistone proteins [4, 22]. Specific patterns of histone modifications characterize genomic regions. For instance, active promoter regions are enriched in trimethylated H3 at lysine 4 (H3K4me3), whereas inactive promoters are enriched in trimethylated H3 at lysine 27 and trimethylated H3 at lysine 9 (H3K27me3 and H3K9me3), and regulatory enhancers are enriched in monomethylated H3 at lysine 4 (H3K4me1) [21]. Such patterns are dynamic and regulated by enzymes that can add or remove the modifications. These include histone methyltransferases (HMTs) and demethylases (HDMTs), which introduce and remove methyl groups, respectively, and histone acetyltransferases (HATs) and deacetylases (HDACs), which introduce and remove acetyl groups, respectively. Histone acetylation is an important marker of transcriptional activity; for instance, acetylated histone H3 (acH3) can also be found at well-positioned nucleosomes flanking the AR binding site of 20% of AR enhancers, upon hormone stimulation [23]. In addition, acH4K16 can be found at well-positioned nuclesomes flanking unmethylated CpG islands at the promoter regions of some tumor-suppressor genes [24]. In addition, although histone modifications themselves are not likely to have a direct impact in nucleosome

positioning, their ability to recruit chromatin remodeler proteins and other factors may have a substantial impact in nucleosome organization [4].

5.2.4 ATPase-Dependent Chromatin Remodelers and Histone Variants

ATP-dependent chromatin remodelers can be grouped in families based on subunit composition and activity: the SWI/SNF family includes the SWI/SNF, INO80, and SWR1 complexes; the ISWI family comprises the RSF, ACF/CHRAC, WICH, and NURF complexes; and the CHD family which includes NURD complexes [25, 26]. These complexes directly affect nucleosome positioning by actively mobilizing nucleosomes or introducing histone variants.

5.2.4.1 SWItch/Sucrose Non-fermenting

These complexes consist of 9–12 subunits, which include one of two ATPases: Brahma homologue (BRM/SMARCA2) or Brahma-related gene 1 (BRG1/SMARCA4), a set of "core" subunits, including SNF5 and BAF53a/b, and a number of variable subunits [27]. A number of the variable subunits are mutually exclusive; for example, AT-rich interactive proteins (ARID) 1A and ARID1B (BAF250a and BAF250b) [25, 27] do not coexist in the same complex and Polybromo 1 (PBRM1 or BAF180), bromodomain-containing 7 (BRD7), and BAF200 are only present in complexes lacking ARID1 proteins [27]. Complexes containing ARID1 proteins are named BAF whereas complexes containing PBRM1 are known as PBAF [27]. The variety of subunits allows for a combinatorial assemblage that leads to functional diversity as evidenced by the developmental stage-specific composition of SWItch/sucrose non-fermenting (SWI/SNF) complexes [25]. SWI/SNF complexes remodel chromatin by sliding or by ejecting or inserting nucleosomes thereby contributing to either transcriptional activation or repression [27, 28]; interestingly, they are primarily enriched at distal regulatory regions rather than at promoters [25]. SWI/SNF complexes also associate and act in concert with histone modifying complexes, including HDACs, HATs, and protein arginine methyltransferases (PRMT4/CARM1 and 5), to regulate gene expression [27, 29, 30].

5.2.4.2 INO80 and SWR1

These complexes consist of core proteins (the ATPase, helicases, and actin-related proteins) and additional subunits [31]. INO80 complexes contain the INO80 ATPase [31] whereas the SWR1 complexes (SRCAP and TRAAP/Tip60) contain the ATPases SRCAP or p400 and share a number of subunits [31]. The INO80 complex displays helicase activity and catalyzes nucleosome sliding *in cis*, and is involved in

chromosome segregation [32], the DNA, and damage repair response, and facilitates recombination-mediated events [25, 33, 34]. INO80 recruitment to damaged sites has been recently shown to depend on actin-related protein 8 [35]. SRCAP complex directs the incorporation of H2A.Z into nucleosomes by exchange of H2A/H2B dimers for H2A.Z/H2B dimers in a replication-independent manner [36, 37]. SRCAP-mediated deposition of H2A.Z is required for gene reactivation in colon cancer cells treated with the DNA methylase inhibitor Azacitidine [38]. P400-containing complexes play a role in DNA repair by destabilizing nucleosomes and promoting chromatin ubiquitination [39]. It has been suggested that TRAAP/Tip60 (p400) complexes are involved in the deposition of H2A.Z into chromatin in an acetylation-dependent manner. P400-mediated H2A.Z deposition is important for estrogen receptor-mediated gene expression [40] whereas SRCAP appears to be important for the androgen receptor-stimulated expression of Kallikrein 3/prostate specific antigen (KLK3/PSA) and cell proliferation in prostate cancer cells [41].

H2A.Z deposition is associated with several nucleosomes surrounding the transcriptional start site of active and poised promoters, and nucleosomes and H2A.Z are lost preferentially at the −1 nucleosome upon gene activation [42]. In addition, enrichment in H2A.Z, and also the histone H3 variant histone H3.3, has been found at distal regulatory regions such as enhancers [42, 43]. During mitosis, the H2A.Z-containing +1 nucleosome of active genes shift upstream to occupy the transcriptional start site of genes silenced during mitosis, significantly reducing NDRs [44]. Interestingly, H2A.Z has also been shown to play an inhibitory role in cell cycle arrest, providing evidence that H2A.Z localization at regulatory regions may contribute to the positive or negative regulation of gene transcription [42]. Differential H2A.Z acetylation patterns at promoters may contribute to the opposing functions of H2A.Z, as the presence of acetylated H2A.Z has been shown to correlate with gene activation in prostate cancer cells [45] and to be anti-correlated with DNA methylation [46, 47].

5.2.4.3 ISWI Complexes

Similar to SWI/SNF complexes, the combinatorial assembly of subunits allows for a multiplicity of ISWI complexes that display specific functions, including transcriptional repression, DNA replication, and heterochromatin formation. The remodeling spacing factor (RSF), ATP-utilizing chromatin assembly and remodeling factor (ACF), chromatin accessibility (CHRAC), and WICH complexes share the hSNF2H ATPase [25], while the nucleosome remodeling factor (NURF) complexes contain the hSNF2L ATPase. All ISWI complexes display ATPase and nucleosome spacing and remodeling activities and RSF, in particular, promotes regular spacing between nucleosomes and stimulates transcriptional activation [25]. In addition, WICH complexes are important for DNA replication of pericentromeric heterochromatin and the WSTF subunit of this complex binds and stabilizes H2A.X by phosphorylation after DNA damage [25]. NURF complexes have also been shown to play a role in the regulation of chromatin barriers; for example, the

transcription factor USF1 (upstream stimulatory factor 1) recruits NURF and an HMT to the insulator of the beta-globin gene to retain its active configuration [48].

5.2.4.4 NURD Complexes

These complexes are formed by the CHD ATPases CHD3 or CHD4 (or Mi-2a or Mi2b), HDACs, and additional subunits and contain both HDAC and remodeling activity [25]. NURD complexes play a role in transcription, cell differentiation, cell cycle checkpoint control, and metastasis, and are recruited to sites of DNA damage by poly-ADP-ribose polymerase (PARP) [25, 49, 50]. The methyl CpG binding domain 2/3 (MBD2 and 3) subunits of these complexes are thought to be involved in protein–protein interaction and are mutually exclusive, whereas the metastasis associated gene 1 and 2 (MTA1 and 2) subunits bind to specific transcription factors thereby targeting the complex to different genomic loci [50].

5.2.5 Transcription Factor Binding

The position of nucleosomes can be directly affected by transcription factors as they compete for DNA access [4]. Transcription factors often bind at NDRs. For example, OCT-4 is required for establishing and maintaining of an NDR at the distal OCT-4 enhancer and the proximal NANOG promoter regions, which are necessary for gene expression [14]. We have recently reported that a percentage of androgen receptor (AR) enhancers show a NDR in the absence of ligand, and that androgen treatment and subsequent AR recruitment increase the number of enhancers with NDRs without changes in footprint [51]. The pioneering factor GATA-2 is required for the maintenance of the NDR at the AR enhancer of TMPRSS2 in the absence of ligand [51]. The presence of GATA-2 at the enhancer may facilitate AR binding, as proposed by the model of transcription factor cooperativity of Segal and Widom [4]. In contrast, other transcription factors are frequently bound to nucleosome occupied regions; for instance, P53 binding occurs preferentially to regions with high intrinsic nucleosome occupancy [52]. Thus, the relationship between nucleosome occupancy and transcription factor binding is context-specific.

5.3 Aberrant Epigenetic Regulation and Epigenetic Switching in Cancer Cells

Genetic and epigenetic changes play important roles in cancer initiation and progression [53, 54]. During tumorigenesis, the cell epigenome undergoes global changes, including a genome-wide reduction in DNA methylation, an increase in localized DNA methylation at CpG island promoters, and changes in histone

modification profiles [55]; in addition, cancer cells display aberrant expression of chromatin-modifying enzymes [56]. The events leading to these epigenetic abnormalities are still not fully understood. Epigenetic changes are mitotically inherited and may promote tumorigenesis by either silencing tumor suppressor genes [57] or by activating oncogenes [2].

Because of the interaction amongst chromatin remodeling complexes [58] and between these complexes and DNMTs [59, 60], genetic mutations in enzymes or other subunits of chromatin remodeling complexes may lead to profound epigenetic changes, including aberrant nucleosome position, DNA methylation, histone composition, and/or histone modifications [2]. In addition, deregulated expression of proteins involved in the recruitment of remodeling complexes to specific loci may alter nucleosome localization and/or retention at such sites, contributing to the propagation of abnormal epigenetic states [2]. All these changes will in turn lead to aberrant gene expression patterns and genomic instability, which ultimately may predispose or give rise to disease [2]. The mechanisms contributing to the altered epigenetic landscape of cancer cells are discussed below.

5.3.1 Mutations in DNA Methylation Enzymes

CpG island methylation at gene promoters affects gene expression and abnormal patterns of DNA methylation have been implicated in carcinogenesis [53, 54]. Hypomethylation of retrotransposons may lead to their reactivation and genomic translocation or to the activation of alternative transcripts. These DNA methylation changes have also been shown to correlate with changes in nucleosome occupancy [2]. For instance, LINE-1 is hypomethylated and nucleosome depleted in colon cancer [61] and bladder cancer, where it induced the expression of an alternate transcript of the *MET* oncogene [16]. Hypomethylation of centromeric regions and/or of pericentromeric satellite sequences may lead to abnormal chromosome segregation and genomic instability [62]. Perhaps the best example of chromosome instability is a germ line mutation in DNMT3B, which underlies a chromosome instability and immunodeficiency syndrome [63]. In addition, DNA hypomethylation may lead to loss of imprinting (LOI), resulting in biallelic expression of a monoallelic gene [2, 64], which often occurs in a variety of cancer types [64]. Re-expression of normally silenced genes or microRNAs (miRNA) can also occur due to DNA hypomethylation; examples of these events are *R-RAS*, *MASPIN*, and *Cyclin D2* in gastric cancer; *MAGE* in melanoma; *HPV16* (human papillomavirus 16) in cervical cancer; *S100A4* in colon cancer; and the *let-7a-3* miRNA in lung adenocarcinomas [2, 62].

Site-specific hypermethylation and silencing of tumor suppressor genes has also reported in cancer and correlates with changes in nucleosome occupancy [65]. Genes that regulate cell cycle progression, and DNA repair, such as *RB* (retinoblastoma), *MLH1* (endometrial cancer), *p16* (glioma, lymphoma, multiple myeloma), and *p15* (lymphoma and multiple myeloma), *BRCA1* and *BRCA2* (lung and ovarian cancer), *APC* (lung, breast, and colorectal cancer), *PTEN* (brain and thyroid gland

cancers), *XRCC5* (lung and ovarian cancer), and estrogen receptor (prostate cancer) have all been reported to be hypermethylated in cancer [2, 62]. DNA hypermethylation can also indirectly inactivate other genes by silencing transcription factors that control their expression. For example, hypermethylation has been found at the *RUNX3* promoter in esophageal cancer and at the *GATA-4* and *-5* promoters in colorectal and gastric cancers [2, 62]. In addition, inactivation of miRNAs by hypermethylation has been observed in a variety of cancer types including bladder and prostate (mir-127), endometrial (mir-152, mir-129-2), pancreatic (mir-132), oral (mir-137 and miR-193a), gastrointestinal (mir-34b/c), and colorectal (mir-137) cancers, and in ALL (mir-124a), and other hematological malignancies (mir-124-1) [66–75]. A new class of noncoding RNA (mirtrons) has been also shown to be susceptible to epigenetic silencing in urothelial cell carcinoma [73]. DNMT1 mutations have been described in colorectal cancer and DNMT3A mutations and decreased protein levels have been shown to occur in myelodysplastic syndromes (MDS) and AML, and in primary prostate tumors, respectively [76–80], *DNMT1*, *DNMT3A,* and *DNMT3B* appear to be largely overexpressed in a variety of cancer types and may contribute to ectopic hypermethylation [81].

Recent studies have pointed to the existence of both passive and active mechanisms of DNA demethylation [82]. Active demethylation occurs during early embryogenesis and is mediated by the formation of cytosine intermediaries, for instance 5-hydroxy-methyl cytosine or 5-methyl uracil, via the action of enzymes such as ten-eleven-translocation (TET) or activation-induced cytidine deaminase (AID), respectively [82].

TET1 translocations have been reported to occur in AML [83] and TET2 mutations have been frequently found in myelodysplasia and in myeloid malignancies [84–90]. In addition, TET2 promoter hypermethylation was observed in a fraction of gliomas [91].

AID promotes somatic hypermutation and class switch recombination of immunoglobulin (Ig) genes in germinal center (GC) B cells and aberrant AID expression has been implicated in the progression of chronic myeloid leukemia (CML) into fatal blast crisis [92].

Because DNA methylation stabilizes nucleosome occupancy, mutations in DNMTs and in enzymes involved in DNA demethylation are likely to cause large-scale epigenetic alterations in cancer cells; in addition, de novo functions generated by fusion with their translocation partners may also contribute to tumorigenesis [93].

5.3.2 Mutations in Genes Encoding Histone Modifiers

Genome-wide analyses of histone modifications in cancer cells have revealed global changes in various histone marks [2]. These changes may affect the recruitment of transcription factors and chromatin remodeler complexes to specific genomic loci, thereby affecting nucleosome positioning.

5.3.2.1 HATs and HDACs

In cancer cells, there is a global reduction in the active acH4K16 and H3K4me3 marks, and in the repressive H4K20me3 mark [94] as well as a gain in the repressive H3K27me3 mark [95]. Acetylation patterns are disrupted in colon, uterus, lung tumors, and in leukemias as a result of translocations or mutations in the genes that encode some of the HATs and HDACs (for instance, HDAC2) or due to mistargeting of the fusion products [94]. HDAC overexpression has also been observed; for example, the levels of the dedicated H4K16 HDAC SIRT1 were found to be high in hepatocellular carcinoma [96] and colon cancer [97, 98].

5.3.2.2 HMTs and HDMTs

Alterations in HMTs and HDMTs have also been shown to be involved in tumorigenesis. Mixed lineage leukemia (MLL) 1–4, SETD1A, and SETD1B are H3K4 HMTs that exist as multiprotein complexes that contain core subunits and various unique subunits including HATs, tumor suppressor gene products, mRNA-processing factors, and nuclear hormone receptors. MLLs play critical roles during development and in adult tissues; they regulate gene transcription directly by introducing the active H3K4me3 mark, and indirectly via their partnership with other chromatin remodeling complexes and co-regulators [99]. In addition, a potential role for MLL complexes in alternative splicing has been proposed [99]. Mutations in MLL1 and MLL3 genes have been reported in 59% of bladder cancer patients [100]. Chromosomal rearrangements in the *MLL1* gene occur preferentially in hematopoietic cells [101] and result in a multiplicity of fusion proteins with new properties and binding partners that contribute to the development of hematological malignancies [101]. Mutations in *MLL2* [102, 103] and MLL2 decreased expression levels as well as mutations and deletions in *MLL3* have also been reported (Table 5.1) [79, 104–106]. Deletions in MLL5, a member of the MLL family that lacks the HMT and DNA binding domains [107], have been shown in leiomyomata (benign uterine fibroids) [108] and low expression of MLL5 was associated with poorer outcome in acute myeloid leukemia (AML) patients [109]. Genomic alterations in other HMTs have also been reported; for instance, mutations in SETD2, an H3K36 HMT, were found in renal clear cell carcinoma [110].

Members of the polycomb group (Pc-G) of repressor proteins have been shown to be deregulated in cancer. The Pc-G HMT EZH2 (enhancer of zeste homologue 2), a subunit of the polycomb repressor complexes (PRC) 2 and PRC3, is not expressed in adult tissues [111]. However, it is overexpressed in several tumor types (Table 5.1) [112, 113]. EZH2 has been shown to interact with DNMTs in human cell lines, suggesting that it may also play a role in controlling DNA methylation [114]. Overexpression of BMI-1 (B lymphoma Mo-MLV insertion region 1 homolog), a component of PRC1, was also observed in a variety of tumors (Table 5.1) [115–117].

Table 5.1 Summary of epigenetic abnormalities found in cancer

Gene	Function	Alteration	Tumor type	Reference
DNMT1	DNA methylase	Mutation	7% of colorectal	[78]
DNMT3A	DNA methylase	Mutation	MDS; 22% AML	[74–77]
TET1	5'methylcytosine hydroxylase	Chromosome translocation	AML	[81]
TET2	5'methylcytosine hydroxylase	Mutation	MDS, myeloid malignancies	[82–88]
		Silencing	Gliomas	[89]
AID	5'cytidine deaminase	Aberrant expression	CML	[90]
SIRT1	H4K16 HDAC	Overexpression	HCC, colon	[94–96]
MLL1	H3K4 HMT	Mutation	Bladder TCC	[98]
		Chromosome translocation	Hematopoietic	[99]
MLL2	H3K4 HMT	Mutation	Non-Hodgkin lymphoma, B-cell lymphoma	[100, 101]
		Low expression	Prostate (primary)	[77]
MLL3	H3K4 HMT	Mutation	Bladder TCC, glioblastoma, breast, pancreas	[98, 102–104]
		Deletion	Colon	[99]
		Low expression	Prostate (primary)	[77]
MLL5	tumor suppressor gene	Deletion	Uterine fibroids	[106]
		Low expression	AML	[107]
SETD2	H3K36 HMT	Mutation	RCCC	[108]
EZH2	H3K27 HMT	Overexpression	Breast, prostate, bladder, colon, pancreas, liver, gastric, uterine tumors, melanoma, lymphoma, myeloma, and Ewing's sarcoma	[110, 111]
BMI-1	PRC1 subunit	Overexpression	Ovarian, mantle cell lymphomas, and Merkel cell carcinomas	[113–115]
NSD1	H3K36/H4K20 HMT	Chromosome rearrangement	AML	[116]
		Amplification	Lung	[117]
		Silencing	Neuroblastoma	[118]
		Low expression	Prostate (primary)	[77]

G9a	H3K9 HMT	Overexpression	HCC	[111]
LSD1	H3K4/H3K9 HDMT	Mutation	Prostate	[119]
UTX	H3K27 HDMT	Mutation	Bladder, breast, kidney, lung, pancreas, esophagus, colon, uterus, brain, hematological malignancies	[98, 108, 120]
JARID1C	H3K4 HDMT	Mutation	RCCC	[108]
JMJD2C	H3K9/H3K36 HDMT	Overexpression	Esophageal cancer	[121]
JARID1B	H3K4 HDMT	Overexpression	Testicular and breast	[121]
GASC1	H3K9	Amplification	Squamous cell carcinoma	[122]
SNF5	BAF subunit	Mutation	Kidney malignant rhabdoid tumors, atypical rhabdoid/teratoid tumors (extra-renal), epithelioid sarcomas, small cell hepatoblastomas, extraskeletal myxoid chondrosarcomas, and undifferentiated sarcomas	[130–134]
		Loss of expression	Renal medullary carcinoma, metastatic melanoma	[135]
BRG1	ATPase of BAF	Mutations	Lung, rhabdoid, medulloblastoma	[138–141]
BRM	ATPase of BAF	Low expression	Prostate	[142]
		Mutations	Basal cell carcinoma	[143, 144]
ARID1A	Subunit of BAF	Mutations	50% of ovarian clear cell carcinomas, 30% of endometrioid carcinomas, endometrial carcinomas	[150–154]
		Deletions	33% of primary pancreatic adenocarcinomas	[155]
		Genomic rearrangements	Bladder TCC	[98]
		Low expression	56% of ER-/PR-/HER2-breast tumors	[156]
ARID2	PBAF subunit	Mutation	HCC	[159]
BRD7	PBAF subunit	Mutations and deletions	Breast	[161]
		Hypermethylation	Nasopharyngeal	[165]
PBRM1	PBAF subunit	Mutations	41% RCCC, breast	[166, 167]
SRCAP	ATPase of SWR1	Overexpression	Prostate (primary)	[77]
		Low expression	Prostate (metastatic)	[77]

(continued)

Table 5.1 (continued)

Gene	Function	Alteration	Tumor type	Reference
Tip60	Acetylase of SWR1	Low expression (monoallelic expression loss)	Lymphomas, head and neck, breast	[170]
		Low expression	Colon	[171]
P400	ATPase of SWR1	Low expression	Colon	[172]
hSNF2	ATPase of RSF, ACF, CHRAC, WICH and NURF	Overexpression	AML	[175]
BPTF	NURD subunit	Amplification	55% of neuroblastomas, 27% lung tumors	[173, 174]
RBBP7/RbAp46	Helicase	Overexpression	79% of breast tumors	[175]
RBBP4/RbAp48	Helicase	Overexpression	AML	[176]
CHD4	ATPase of NURD	Mutation with loss of expression	55.7% colorectal and 56.4% gastric cancer	[178]
MTA1	NURD subunit	Overexpression	Breast, colorectal, gastric, pancreatic, ovarian, prostate, esophageal, endometrial, NSCLC, HCC, and diffuse B cell lymphoma	[49]
MTA3	NURD subunit	Loss of expression	Advanced breast carcinoma	[49]
CHD5	ATP-dependent helicase	Deletion and mutations	Ovarian, prostate, neuroblastoma, hematopoietic	[178–181]
		Hypermethylation	Gliomas, laryngeal squamous carcinoma, colon, gastric, ovarian, and breast	[178, 182–185]
CHD7	ATP-dependent helicase	Mutations	Gastric and colorectal	[177]

HCC Hepatocellular carcinoma; *NSCLC* non-small cell lung carcinoma, *AML* acute myeloid leukemia; *MDS* myelodysplastic syndromes; *CML* chronic myeloid leukemia; *TCC* transitional cell carcinoma; *RCCC* renal clear cell carcinoma; *ER* estrogen receptor; *PR* progesterone receptor; *HER2* human epidermal growth factor receptor 2; *HMT* histone methyltransferase; *HDMT* histone demethylase; *DNMT* DNA methylase, *PRC1* polycomb repressor complex 1

Other HMTs have been shown to display aberrant expression patterns or chromosome rearrangements. Nuclear receptor binding SET domain protein 1 (NSD1) has been reported to undergo chromosome rearrangements in pediatric AML [118], to be amplified in some lung cancer cases [119] and to be silenced by DNA methylation in neuroblastomas [120]. In addition, the H3K9me3 HMT G9a was found to be upregulated in hepatocellular carcinoma [113].

Lysine-specific histone demethylases, such as LSD1, lysine (K)-specific demethylase 6A (KDM6A/UTX), and Jumonji C-domain containing proteins (JARID1A-D), have been implicated in cancer progression (Table 5.1). For instance, mutations in LSD1 have been reported in prostate cancer [121], whereas KDM6A/UTX was found mutated in many tumors (Table 5.1) [100, 110, 122]. Mutations in KDM5C/JARID1C were observed in renal cell carcinoma lacking VHL [110]. In addition, overexpression of KDM4C/JMJD2C and JARID1B/PLU-1 was found in esophageal cancer and in breast and testicular tumors, respectively, whereas genomic amplification of GASC1 was observed in squamous cell carcinoma [123, 124]. Thus, mutations and aberrant expression of histone modifiers may alter or block the recruitment of chromatin remodelers and transcription factors to specific loci, thereby affecting nucleosome positioning and gene expression patterns.

5.3.3 Mutations in Genes Encoding Subunits of Chromatin Remodeler Complexes

ATPase-dependent chromatin remodeler complexes directly control the position of nucleosomes or alter their stability by introducing histone variants. Thus, aberrant expression of their subunits will cause changes in nucleosome composition, location, and stability.

5.3.3.1 SWItch/Sucrose Non-fermenting

Because of their important role in controlling fundamental processes such as cell proliferation, migration, and differentiation [27], the aberrant expression of SWI/SNF components will have profound effects on cell function. Indeed, mutations in several subunits have been recently identified in tumors of various origins. Since genomic instability is largely absent in tumors harboring defective SWI/SNF complexes, it is likely that perturbations in nucleosome positioning, misslocalization, and excessive formation of complexes with opposing functions contribute to the development of these aggressive cancers [27].

The SWI/SNF subunit SNF5 helps recruit this complex to specific genomic sites and is required for the expression of genes associated with cell proliferation, including *P53* and the cell cycle inhibitor *p16INK4a* [125–127], adipocyte differentiation [128], and inhibition of cell migration [129]. *SNF5* loss, however, does not result in genome instability [130] nor does it inactivate SWI/SNF complexes completely, as

tumorigenesis in the absence of *SNF5* is dependent on BRG1 activity [131]. Thus, it is thought that tumorigenesis arises from aberrant activity of the remaining complexes [131]. *SNF5* mutations have been found in rhabdoid and other tumors (Table 5.1) [132–136]. Loss of the SNF5 protein was also observed in renal medullary carcinomas and in advanced and metastatic melanomas, where it correlated with poor survival rates [137].

Although complexes containing the catalytic subunits BRM or BRG1 display some functional redundancy, they also play distinct roles [27, 28]. *BRG1* mutations have been shown to occur in cancer cell lines of various origins [138, 139] and in primary lung tumors [140, 141], medulloblastoma [142], and rhabdoid tumors [143]. Reduced BRM protein levels occur in prostate tumors [144], and mutations have been found in basal cell carcinoma [145, 146]. In addition, BRM has been shown to be postranslationally regulated in cancer cell lines [28].

BAF250A/ARID1A binds to DNA without sequence specificity [147, 148] and its recruitment represses the expression of cell cycle-related genes in differentiated mouse calvaria cells [149, 150]. In addition, BAF250A/ARID1A is required for normal cell cycle arrest in senescent human fibroblasts [151]. *ARID1A/BAF250a* mutations have been recently described in ovarian clear cell [152–154] and endometrioid carcinomas (Table 5.1) [153]. Frequent mutations in low- and high grade endometrial carcinomas have also been observed [155, 156]. Heterozygous deletions and mutations in *ARID1A/BAF250a* have been reported to exist in 33% of primary pancreatic adenocarcinomas [157]. Genetic aberrations in *ARID1A* were recently reported in transitional cell carcinoma (TCC) of the bladder [100] and low ARID1A expression was found to be significantly associated with larger tumor size and grade and the ER-/PR-/HER2-phenotype in breast cancer cases (Table 5.1) [158]. ARID1A/BAF250a expression was also found to be severely reduced in breast (T47D), renal clear cell (Caki-1 and Caki-2), and cervical (C33A) cancer cell lines [159]. BAF250b/ARID1B containing complexes include components of an E3 ubiquitin ligase that was found to target H2BK20 for monoubiquitination in a nucleosomal context, an upstream event for trimethylation of H3K4 and gene activation [160]. BAF250b/ARID1B and BAF250a/ARID1A have also been shown to play opposing roles in the control of cell cycle genes in osteoblast differentiation in mice [149, 150]; however, no mutations in human *BAF250b/ARID1B* have been described to date. In contrast, inactivating mutations in *ARID2*, which encodes a component of PBAF that facilitates transcriptional activation by nuclear receptors, have been reported in four subtypes of hepatocellular carcinomas (HCC) [161].

BRD7 and BAF180/PBRM1 are regulators of replicative senescence in human cells by controlling P53 transcriptional activity towards a subset of its target genes required for replicative and oncogenic stress senescence induction [162]. BRD7 has also been shown to either activate or repress the expression of a number of genes by protein–protein interaction. BRD7 physically interacts with P53 and the acetylase P300 [162, 163], disheveled-1 [164], and TRIM24 [165], as well as with BRCA1 thereby regulating genes involved in DNA repair [166]. *BRD7* deletions and reduced expression levels have been observed in breast tumors [163] (Table 5.1). In addition, the *BRD7* promoter has also been shown to be silenced by DNA methylation in

nasopharyngeal cancer cell lines and tumors [167]. Mutations in *BAF180/PBRM1* have been recently described in renal clear cell carcinomas [168] and breast tumors (Table 5.1) [169]. BAF57 is required to maintain the proper subunit composition of the PBAF complex and to regulate the transcription of a subset of cell cycle-related genes in Hela cells [170]. Thus far, loss of *BAF57* has only been reported in the breast cancer cell line BT-549 [171]. Thus, aberrant expression of SWI/SNF subunits is a frequent event in a variety of cancer types. Although SWI/SNF complexes control nucleosome positioning, the extent of the changes caused by the mutation of specific subunits remains to be elucidated.

5.3.3.2 INO80 and SWR1

Deregulated expression of the subunits of these complexes may affect H2A.Z deposition and nucleosome dynamics as well as nucleosome position and DNA repair. SRCAP deregulated expression has been found in primary and metastatic prostate cancer, although the mechanisms underlying such dysregulation are unclear [79]. Monoallelic loss of the acetylase Tip60 (a subunit of TRAAP/Tip60/p400) has been reported in lymphomas, and head-and-neck and mammary carcinomas, with decreased mRNA and protein expression levels, suggesting that critical levels of Tip60 are required for normal cell function [172]. Tip60 and P400 expression is also decreased in colorectal tumors compared to normal colon, although no mutations were found in these two genes [173]. Finally, single nucleotide polymorphisms (SNPs) in Tip49a/RUVBL1 have been recently associated with higher risk of serous epithelial ovarian cancer [174].

5.3.3.3 RSF, ACF, CHRAC, WICH, and NURF

To date no mutations in the ATPase subunits of ISWI complexes have been described. However, genomic amplification of bromodomain PHD finger transcription factor (BPTF), a subunit of NURD, has been reported in neuroblastomas and lung cancer cases (Table 5.1) [175, 176]. In addition, increased expression of other subunits of the NURF complex, including Retinoblastoma-related protein 46 (RBBP7/RbAp46), as well as Retinoblastoma-related protein 48 (RBBP4/RbAp48) and hSNF2 have been reported in breast carcinomas [177] and in AML [178], respectively (Table 5.1).

5.3.3.4 NURD

Mutations and loss of expression of the CHD4 ATPase subunit have been recently described in colorectal and gastric cancers (Table 5.1) [179]. MTA1 expression is high in a number of cancer types (Table 5.1) [50]. In contrast, MTA3 expression is lost in advanced breast epithelial carcinoma (Table 5.1) [50].

5.3.3.5 Mutations in Other CHD Proteins

Recent studies have identified the helicase CHD5 as a tumor suppressor involved in the transactivation of *p16Ink4a/p19arf* and deleted or mutated in ovarian and prostate cancer [180, 181], neuroblastomas [182], and hematopoietic malignancies [183]. Silencing of the *CHD5* promoter by DNA hypermethylation has also been observed in various tumor types (Table 5.1) [180, 184–187]. CHD7 plays a role in pluripotency [25] and mutations in CHD7 have been found in more than 50% of the cases of CHARGE syndrome, which is characterized by nonrandom congenital abnormalities in several tissues [188, 189]. In addition, gastric and colorectal cancers also showed mutations in *CHD7* [179].

5.4 Epigenetic Switching

The gene silencing events that take place during tumorigenesis as a consequence of aberrant DNA methylation or histone modification result in a reduction of cellular plasticity. A subset of genes becomes repressed by the action of Pc-G proteins through the establishment of the H3K27me3 mark at their promoters when stem cells differentiate into developmental lineages [2]. After differentiation, this mark and, thus, the repressive state are maintained by the action of EZH2. In cancer cells, H3K27me3 is replaced by de novo DNA methylation likely through the recruitment of DNMTs [114, 190–192]. This process is termed "epigenetic switching" and results in permanent silencing of genes that may be implicated in tumorigenesis by locking nucleosome positions.

5.5 Epigenetic Therapy and Gene Reactivation

Epigenetic therapy aims to reverse epigenetic aberrations that occur in cancer in order to restore a more normal epigenetic state [55]. The first characterized DNA methylation inhibitors, namely 5-Azacitidine (5′-aza-CR, Azacitidine) and 5-aza-2-deoxy-cytidine (5′-aza-CdR, Decitabine) [193], are incorporated into the DNA of proliferating cells during DNA replication and inhibit DNA methylation by trapping DNMTs onto the DNA, leading to their depletion [2, 56]. The resulting DNA hypomethylation causes nucleosome depletion at the promoters of tumor suppressor genes that were silenced during tumorigenesis, leading to gene reactivation and growth arrest [2, 65]. Azacitidine and decitabine have been approved by the FDA for the treatment of myelodysplastic syndromes and have shown great promise in the treatment of AML and myeloid leukemia [194]. Decitabine has also been tested in clinical trials for the treatment of epithelial ovarian cancer, alone or in combination with chemotherapy [195]. These studies have shown that combination therapies are more effective, particularly in patients with platinum resistance, likely due to re-sensitization [195]. Clinical applications for Zebularine, a newer generation

DNMT inhibitor that can be orally administered, are currently under investigation [196]. Alternative approaches include small molecule DNMT inhibitors, such as SGI110, RG108, and MG98, which block DNMT enzyme activity or target regulatory messenger RNA sequences [2].

Loss of histone acetylation at promoter regions occurs concomitant to DNA hypermethylation, and therefore HDAC inhibitors (HDACI) have also been tested as potential therapeutic agents. HDACIs induce growth arrest, apoptosis, cell differentiation, and tumor suppressor gene reactivation. Suberoylanilide hydroxamic acid (SAHA, Vorinostat) has been recently approved for the treatment of T-cell cutaneous lymphoma [197]; however, it was not successful for the treatment of recurrent ovarian cancer [195]. Treatment with another HDACI, belinostat (PDX, 101), has shown to lead to disease stabilization in patients with different malignancies, including sarcoma, renal cancer, thymoma and melanoma, and ovarian cancer [195]. Other HDACIs are currently under investigation [2, 197]. The lysine HMT inhibitors described to date, chaetocin, DZNep, and BIX-01294, have shown some antitumor properties in vitro [197]. Combined epigenetic therapies have also been tested; for instance, chemotherapeutic agents have been successfully used in combination with HDAC, SIRT, DNMT inhibitors [197]. Thus, epigenetic drugs currently in use or under investigation target histone modifiers or DNMTs to restore chromatin plasticity, thereby affecting nucleosome positioning in an indirect manner. Targeting subunits of the ATPase-dependent chromatin remodeler complexes may provide a more efficient and direct way to restore nucleosome position and composition.

5.6 Challenges and Future Prospects

In recent years, high-throughput technologies have been successfully applied to the field of epigenetics allowing for the mapping of histone modifications, proteins binding to DNA, nucleosome positioning, and DNA methylation. The emerging picture is that nucleosome positioning and occupancy is determined by the combined action of DNA sequence, transcription factors, and chromatin remodelers, and that the resulting nucleosome configuration has direct effects in sequence accessibility and gene transcription (Fig. 5.1). Recent studies show that the genes more frequently mutated in various types of cancers encode for subunits of chromatin remodeler complexes [197], further highlighting the relevance of nucleosome positioning in tumorigenesis (Fig. 5.1). As most of these genes regulate multiple cellular processes, they are likely to be important therapeutic targets.

Although the wealth of information generated by epigenomic studies has greatly improved our understanding of chromatin regulation, the integration of epigenetic, genetic, and transcriptional changes will be essential to advance our knowledge of cancer development and progression. Several challenges lay ahead as we explore further the development of epigenetic therapies, although a combinatorial approach holds promise. Key issues to be resolved include type of agent combinations and optimal doses, agent specificity, the sequence of agent delivery, and the method of

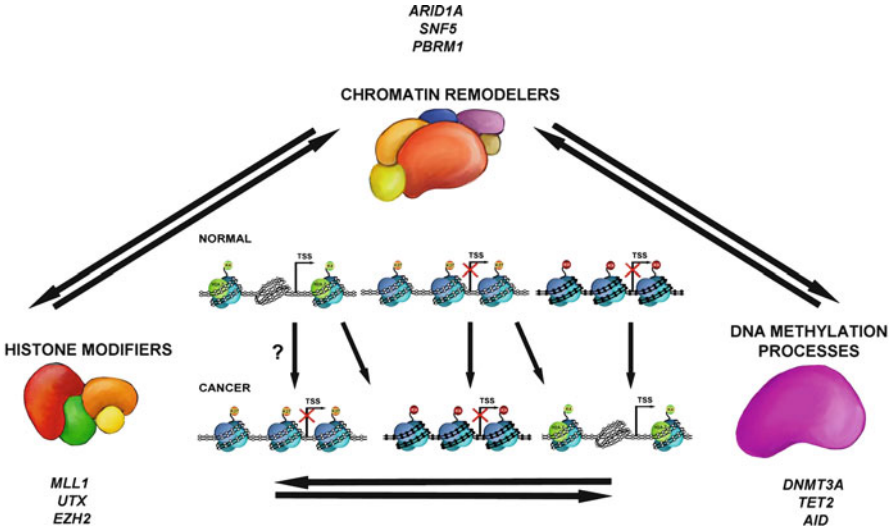

Fig. 5.1 The emerging picture is that nucleosome positioning and occupancy, which is influenced by chromatin remodelers, histone modifiers and DNA methylating enzymes, has direct effects in sequence accessibility and gene transcription and that. In normal cells, gene promoter regions exist in three epigenetic states: open (left), which shows nucleosome depletion at the transcriptional start site (TSS) and contains active histone marks (e.g. H3K4me3) and the histone variant H2A.Z; repressed (center), which shows nucleosome occupancy at the TSS and contains repressive histone marks (e.g. H3K27me3); or silenced (right), which shows nucleosome occupancy at the TSS and DNA methylation, and contains silencing histone marls (e.g.H3K9me3). These epigenetic states correlate with transcriptional activity (left) or lack thereof (center and right). In cancer, epigenetic states are altered, and active promoters may become silenced by DNA methylation, or, potentially, repressed; repressed promoters may become reactivated or silenced by DNA methylation; and silenced promoters may become reactivated, thereby causing profound changes in gene expression patterns. Recent studies show that the genes more frequently mutated in various types of cancers encode for subunits of chromatin remodeler complexes (e.g. *ARID1A, SNF5, PBRM1*), histone modifying enzymes (e.g. *MLL1, UTX, EZH2*) or enzymes involved in the DNA methylation pathway (e.g. *DNMT3A, TET2, AID*). These studies provide evidence for a link between genetic mutation and epigenetic alterations

delivery. Given the current multi-institutional and multinational efforts to map the human epigenome in all cancer types, it is likely that therapeutic development will be significantly advanced in the near future.

References

1. Luger K, Mader AW, Richmond RK, Sargent DF, Richmond TJ (1997) Crystal structure of the nucleosome core particle at 2.8 A resolution. Nature 389:251–260
2. Sharma S, Kelly TK, Jones PA (2010) Epigenetics in cancer. Carcinogenesis 31:27–36
3. Deaton AM, Bird A (2011) CpG islands and the regulation of transcription. Genes Dev 25:1010–1022

4. Segal E, Widom J (2009) What controls nucleosome positions? Trends Genet 25:335–343
5. Sadeh R, Allis CD (2011) Genome-wide "re"-modeling of nucleosome positions. Cell 147:263–266
6. Bettecken T, Frenkel ZM, Trifonov EN (2011) Human nucleosomes: special role of CG dinucleotides and Alu-nucleosomes. BMC Genomics 12:273
7. Tillo D et al (2010) High nucleosome occupancy is encoded at human regulatory sequences. PLoS One 5:e9129
8. Tanaka Y, Yamashita R, Suzuki Y, Nakai K (2010) Effects of Alu elements on global nucleosome positioning in the human genome. BMC Genomics 11:309
9. Valouev A et al (2011) Determinants of nucleosome organization in primary human cells. Nature 474:516–520
10. Zhang Z et al (2011) A packing mechanism for nucleosome organization reconstituted across a eukaryotic genome. Science 332:977–980
11. Han H et al (2011) DNA methylation directly silences genes with non-CpG island promoters and establishes a nucleosome occupied promoter. Hum Mol Genet 20(22):4299–4310
12. Hattori N et al (2004) Epigenetic control of mouse Oct-4 gene expression in embryonic stem cells and trophoblast stem cells. J Biol Chem 279:17063–17069
13. Futscher BW et al (2002) Role for DNA methylation in the control of cell type specific maspin expression. Nat Genet 31:175–179
14. You JS et al (2011) OCT4 establishes and maintains nucleosome-depleted regions that provide additional layers of epigenetic regulation of its target genes. Proc Natl Acad Sci USA 108:14497–14502 [AU4]
15. Taberlay PC, Jones PA (2010) DNA methylation and cancer. Prog Drug Res 67:1–23
16. Wolff EM et al (2010) Hypomethylation of a LINE-1 promoter activates an alternate transcript of the MET oncogene in bladders with cancer. PLoS Genet 6:e1000917
17. Sharma S, De Carvalho DD, Jeong S, Jones PA, Liang G (2011) Nucleosomes containing methylated DNA stabilize DNA methyltransferases 3A/3B and ensure faithful epigenetic inheritance. PLoS Genet 7:e1001286
18. Jones PA, Liang G (2009) Rethinking how DNA methylation patterns are maintained. Nat Rev Genet 10:805–811
19. Chodavarapu RK et al (2010) Relationship between nucleosome positioning and DNA methylation. Nature 466:388–392
20. Jeong S et al (2009) Selective anchoring of DNA methyltransferases 3A and 3B to nucleosomes containing methylated DNA. Mol Cell Biol 29:5366–5376
21. Mills AA (2010) Throwing the cancer switch: reciprocal roles of polycomb and trithorax proteins. Nat Rev Cancer 10:669–682
22. Bell O, Tiwari VK, Thoma NH, Schubeler D (2011) Determinants and dynamics of genome accessibility. Nature reviews 12:554–564
23. Berman BP, Frenkel B, Coetzee GA, Jia L (2010) Androgen receptor responsive enhancers are flanked by consistently-positioned H3-acetylated nucleosomes. Cell Cycle 9:2249–2250
24. Kapoor-Vazirani P, Kagey JD, Powell DR, Vertino PM (2008) Role of hMOF-dependent histone H4 lysine 16 acetylation in the maintenance of TMS1/ASC gene activity. Cancer Res 68:6810–6821
25. Hargreaves DC, Crabtree GR (2011) ATP-dependent chromatin remodeling: genetics, genomics and mechanisms. Cell Res 21:396–420
26. Clapier CR, Cairns BR (2009) The biology of chromatin remodeling complexes. Annu Rev Biochem 78:273–304
27. Wilson BG, Roberts CW (2011) SWI/SNF nucleosome remodellers and cancer. Nat Rev Cancer 11:481–492
28. Reisman D, Glaros S, Thompson EA (2009) The SWI/SNF complex and cancer. Oncogene 28:1653–1668
29. Pal S, Sif S (2007) Interplay between chromatin remodelers and protein arginine methyltransferases. J Cell Physiol 213:306–315

30. Choi HK et al (2007) The functional role of the CARM1-SNF5 complex and its associated HMT activity in transcriptional activation by thyroid hormone receptor. Exp Mol Med 39:544–555
31. Morrison AJ, Shen X (2009) Chromatin remodelling beyond transcription: the INO80 and SWR1 complexes. Nat Rev Mol Cell Biol 10:373–384
32. Hur SK et al (2010) Roles of human INO80 chromatin remodeling enzyme in DNA replication and chromosome segregation suppress genome instability. Cell Mol Life Sci 67: 2283–2296
33. van Attikum H, Gasser SM (2009) Crosstalk between histone modifications during the DNA damage response. Trends Cell Biol 19:207–217
34. Park EJ, Hur SK, Kwon J (2010) Human INO80 chromatin-remodelling complex contributes to DNA double-strand break repair via the expression of Rad54B and XRCC3 genes. Biochem J 431:179–187
35. Kashiwaba S et al (2010) The mammalian INO80 complex is recruited to DNA damage sites in an ARP8 dependent manner. Biochem Biophys Res Commun 402:619–625
36. Ruhl DD et al (2006) Purification of a human SRCAP complex that remodels chromatin by incorporating the histone variant H2A.Z into nucleosomes. Biochemistry 45:5671–5677
37. Wong MM, Cox LK, Chrivia JC (2007) The chromatin remodeling protein, SRCAP, is critical for deposition of the histone variant H2A.Z at promoters. J Biol Chem 282:26132–26139
38. Yang X et al (2012) Gene reactivation by 5-Aza-2'-deoxycytidine requires H2A.z insertion to establish but not to maintain nucleosome depleted regions. PLoS Genet 8:e1002604
39. Xu Y et al (2010) The p400 ATPase regulates nucleosome stability and chromatin ubiquitination during DNA repair. J Cell Biol 191:31–43
40. Gevry N et al (2009) Histone H2A.Z is essential for estrogen receptor signaling. Genes Dev 23:1522–1533
41. Slupianek A, Yerrum S, Safadi FF, Monroy MA (2010) The chromatin remodeling factor SRCAP modulates expression of prostate specific antigen and cellular proliferation in prostate cancer cells. J Cell Physiol 224:369–375
42. Svotelis A, Gevry N, Gaudreau L (2009) Regulation of gene expression and cellular proliferation by histone H2A.Z. Biochem Cell Biol 87:179–188
43. Ong CT, Corces VG (2011) Enhancer function: new insights into the regulation of tissue-specific gene expression. Nature reviews 12:283–293
44. Kelly TK et al (2010) H2A.Z maintenance during mitosis reveals nucleosome shifting on mitotically silenced genes. Mol Cell 39:901–911
45. Valdes-Mora F et al (2011) Acetylation of H2A.Z is a key epigenetic modification associated with gene deregulation and epigenetic remodeling in cancer. Genome Res 20(22):4299–4310
46. Zemach A, McDaniel IE, Silva P, Zilberman D (2010) Genome-wide evolutionary analysis of eukaryotic DNA methylation. Science 328:916–919
47. Zilberman D, Coleman-Derr D, Ballinger T, Henikoff S (2008) Histone H2A.Z and DNA methylation are mutually antagonistic chromatin marks. Nature 456:125–129
48. Li X et al (2011) Chromatin boundaries require functional collaboration between the hSET1 and NURF complexes. Blood 118:1386–1394
49. Sims JK, Wade PA (2011) Mi-2/NuRD complex function is required for normal S phase progression and assembly of pericentric heterochromatin. Mol Biol Cell 22:3094–3102
50. Lai AY, Wade PA (2011) Cancer biology and NuRD: a multifaceted chromatin remodelling complex. Nat Rev Cancer 11:588–596
51. Andreu-Vieyra C et al (2011) Dynamic nucleosome-depleted regions at androgen receptor enhancers in the absence of ligand in prostate cancer cells. Mol Cell Biol 31:4648–4662
52. Lidor Nili E et al (2010) p53 binds preferentially to genomic regions with high DNA-encoded nucleosome occupancy. Genome Res 20:1361–1368
53. Jones PA, Baylin SB (2002) The fundamental role of epigenetic events in cancer. Nature reviews 3:415–428
54. Jones PA, Baylin SB (2007) The epigenomics of cancer. Cell 128:683–692

55. Baylin SB, Jones PA (2011) A decade of exploring the cancer epigenome—biological and translational implications. Nat Rev Cancer 11:726–734
56. Egger G, Liang G, Aparicio A, Jones PA (2004) Epigenetics in human disease and prospects for epigenetic therapy. Nature 429:457–463
57. Jones PA, Laird PW (1999) Cancer epigenetics comes of age. Nat Genet 21:163–167
58. Xu W et al (2004) A methylation-mediator complex in hormone signaling. Genes Dev 18:144–156
59. Geiman TM et al (2004) DNMT3B interacts with hSNF2H chromatin remodeling enzyme, HDACs 1 and 2, and components of the histone methylation system. Biochem Biophys Res Commun 318:544–555
60. Geiman TM et al (2004) Isolation and characterization of a novel DNA methyltransferase complex linking DNMT3B with components of the mitotic chromosome condensation machinery. Nucleic Acids Res 32:2716–2729
61. Sunami E, de Maat M, Vu A, Turner RR, Hoon DS (2011) LINE-1 hypomethylation during primary colon cancer progression. PLoS One 6:e18884
62. Hatziapostolou M, Iliopoulos D (2011) Epigenetic aberrations during oncogenesis. Cell Mol Life Sci 68:1681–1702
63. Xu GL et al (1999) Chromosome instability and immunodeficiency syndrome caused by mutations in a DNA methyltransferase gene. Nature 402:187–191
64. Jelinic P, Shaw P (2007) Loss of imprinting and cancer. J Pathol 211:261–268
65. Lin JC et al (2007) Role of nucleosomal occupancy in the epigenetic silencing of the MLH1 CpG island. Cancer Cell 12:432–444
66. Tsuruta T et al (2011) miR-152 is a tumor suppressor microRNA that is silenced by DNA hypermethylation in endometrial cancer. Cancer Res 71(20):6450–6462
67. Agirre X et al (2009) Epigenetic silencing of the tumor suppressor microRNA Hsa-miR-124a regulates CDK6 expression and confers a poor prognosis in acute lymphoblastic leukemia. Cancer Res 69:4443–4453
68. Balaguer F et al (2010) Epigenetic silencing of miR-137 is an early event in colorectal carcinogenesis. Cancer Res 70:6609–6618
69. Zhang S et al (2011) Downregulation of miR-132 by promoter methylation contributes to pancreatic cancer development. Carcinogenesis 32:1183–1189
70. Huang YW et al (2009) Epigenetic repression of microRNA-129-2 leads to overexpression of SOX4 oncogene in endometrial cancer. Cancer Res 69:9038–9046
71. Wong KY et al (2011) Epigenetic inactivation of the miR-124-1 in haematological malignancies. PLoS One 6:e19027
72. Saito Y et al (2006) Specific activation of microRNA-127 with downregulation of the proto-oncogene BCL6 by chromatin-modifying drugs in human cancer cells. Cancer Cell 9:435–443
73. Dudziec E et al (2011) Hypermethylation of CpG islands and shores around specific microRNAs and mirtrons is associated with the phenotype and presence of bladder cancer. Clin Cancer Res 17:1287–1296
74. Kozaki K, Imoto I, Mogi S, Omura K, Inazawa J (2008) Exploration of tumor-suppressive microRNAs silenced by DNA hypermethylation in oral cancer. Cancer Res 68:2094–2105
75. Suzuki H et al (2010) Methylation-associated silencing of microRNA-34b/c in gastric cancer and its involvement in an epigenetic field defect. Carcinogenesis 31:2066–2073
76. Yan XJ et al (2011) Exome sequencing identifies somatic mutations of DNA methyltransferase gene DNMT3A in acute monocytic leukemia. Nat Genet 43:309–315
77. Tiu RV, Visconte V, Traina F, Schwandt A, Maciejewski JP (2011) Updates in cytogenetics and molecular markers in MDS. Curr Hematol Malig Rep 6:126–135
78. Tan PT, Wei AH (2011) The epigenomics revolution in myelodysplasia: a clinico-pathological perspective. Pathology 43:536–546
79. Bianco-Miotto T et al (2010) Global levels of specific histone modifications and an epigenetic gene signature predict prostate cancer progression and development. Cancer Epidemiol Biomarkers Prev 19:2611–2622

80. Kanai Y, Ushijima S, Nakanishi Y, Sakamoto M, Hirohashi S (2003) Mutation of the DNA methyltransferase (DNMT) 1 gene in human colorectal cancers. Cancer Lett 192:75–82
81. Miremadi A, Oestergaard MZ, Pharoah PD, Caldas C (2007) Cancer genetics of epigenetic genes. Hum Mol Genet 16(Spec No 1):R28–R49
82. Bhutani N et al (2011) Reprogramming towards pluripotency requires AID-dependent DNA demethylation. Nature 463:1042–1047
83. Guo JU, Su Y, Zhong C, Ming GL, Song H (2011) Emerging roles of TET proteins and 5-hydroxymethylcytosines in active DNA demethylation and beyond. Cell Cycle 10:2662–2668
84. Mullighan CG (2009) TET2 mutations in myelodysplasia and myeloid malignancies. Nat Genet 41:766–767
85. Jardin F et al (2011) TET2 and TP53 mutations are frequently observed in blastic plasmacytoid dendritic cell neoplasm. Br J Haematol 153:413–416
86. Quivoron C et al (2011) TET2 inactivation results in pleiotropic hematopoietic abnormalities in mouse and is a recurrent event during human lymphomagenesis. Cancer Cell 20:25–38
87. Bacher U et al (2010) Mutations of the TET2 and CBL genes: novel molecular markers in myeloid malignancies. Ann Hematol 89:643–652
88. Tefferi A et al (2009) Frequent TET2 mutations in systemic mastocytosis: clinical, KITD816V and FIP1L1-PDGFRA correlates. Leukemia 23:900–904
89. Tefferi A et al (2009) Detection of mutant TET2 in myeloid malignancies other than myeloproliferative neoplasms: CMML, MDS, MDS/MPN and AML. Leukemia 23:1343–1345
90. Tefferi A et al (2009) TET2 mutations and their clinical correlates in polycythemia vera, essential thrombocythemia and myelofibrosis. Leukemia 23:905–911
91. Kim YH et al (2011) TET2 promoter methylation in low-grade diffuse gliomas lacking IDH1/2 mutations. J Clin Pathol 64(10):850–852
92. Klemm L et al (2009) The B cell mutator AID promotes B lymphoid blast crisis and drug resistance in chronic myeloid leukemia. Cancer Cell 16:232–245
93. Tan AY, Manley JL (2009) The TET family of proteins: functions and roles in disease. J Mol Cell Biol 1:82–92
94. Esteller M (2007) Cancer epigenomics: DNA methylomes and histone-modification maps. Nature reviews 8:286–298
95. Kondo Y et al (2008) Gene silencing in cancer by histone H3 lysine 27 trimethylation independent of promoter DNA methylation. Nat Genet 40:741–750
96. Choi HN et al (2011) Expression and role of SIRT1 in hepatocellular carcinoma. Oncol Rep 26:503–510
97. Stunkel W et al (2007) Function of the SIRT1 protein deacetylase in cancer. Biotechnol J 2:1360–1368
98. Kim YR, Kim SS, Yoo NJ, Lee SH (2010) Frameshift mutation of SIRT1 gene in gastric and colorectal carcinomas with microsatellite instability. APMIS 118:81–82
99. Ansari KI, Mandal SS (2010) Mixed lineage leukemia: roles in gene expression, hormone signaling and mRNA processing. FEBS J 277:1790–1804
100. Gui Y et al (2011) Frequent mutations of chromatin remodeling genes in transitional cell carcinoma of the bladder. Nat Genet 43:875–878
101. Marschalek R (2010) Mixed lineage leukemia: roles in human malignancies and potential therapy. FEBS J 277:1822–1831
102. Morin RD et al (2011) Frequent mutation of histone-modifying genes in non-Hodgkin lymphoma. Nature 476:298–303
103. Shaknovich R, Melnick A (2011) Epigenetics and B-cell lymphoma. Curr Opin Hematol 18:293–299
104. Ashktorab H et al (2010) Distinct genetic alterations in colorectal cancer. PLoS One 5:e8879
105. Balakrishnan A et al (2007) Novel somatic and germline mutations in cancer candidate genes in glioblastoma, melanoma, and pancreatic carcinoma. Cancer Res 67:3545–3550

106. Wang XX et al (2011) Somatic mutations of the mixed-lineage leukemia 3 (MLL3) gene in primary breast cancers. Pathol Oncol Res 17:429–433
107. Liu H, Westergard TD, Hsieh JJ (2009) MLL5 governs hematopoiesis: a step closer. Blood 113:1395–1396
108. Hodge JC et al (2009) Identifying the molecular signature of the interstitial deletion 7q subgroup of uterine leiomyomata using a paired analysis. Genes Chromosomes Cancer 48:865–885
109. Damm F et al (2011) Prognostic importance of histone methyltransferase MLL5 expression in acute myeloid leukemia. J Clin Oncol 29:682–689
110. Dalgliesh GL et al (2010) Systematic sequencing of renal carcinoma reveals inactivation of histone modifying genes. Nature 463:360–363
111. Portela A, Esteller M (2010) Epigenetic modifications and human disease. Nat Biotechnol 28:1057–1068
112. Tsang DP, Cheng AS (2010) Epigenetic regulation of signaling pathways in cancer: role of the histone methyltransferase EZH2. J Gastroenterol Hepatol 26:19–27
113. Kondo Y et al (2007) Alterations of DNA methylation and histone modifications contribute to gene silencing in hepatocellular carcinomas. Hepatol Res 37:974–983
114. Vire E et al (2006) The Polycomb group protein EZH2 directly controls DNA methylation. Nature 439:871–874
115. Yang GF et al (2010) Intensive expression of Bmi-1 is a new independent predictor of poor outcome in patients with ovarian carcinoma. BMC Cancer 10:133
116. Bea S et al (2001) BMI-1 gene amplification and overexpression in hematological malignancies occur mainly in mantle cell lymphomas. Cancer Res 61:2409–2412
117. Brunner M et al (2008) Expression of VEGF-A/C, VEGF-R2, PDGF-alpha/beta, c-kit, EGFR, Her-2/Neu, Mcl-1 and Bmi-1 in Merkel cell carcinoma. Mod Pathol 21:876–884
118. Jaju RJ et al (2001) A novel gene, NSD1, is fused to NUP98 in the t(5;11)(q35;p15.5) in de novo childhood acute myeloid leukemia. Blood 98:1264–1267
119. Job B et al (2010) Genomic aberrations in lung adenocarcinoma in never smokers. PLoS One 5:e15145
120. Berdasco M et al (2009) Epigenetic inactivation of the Sotos overgrowth syndrome gene histone methyltransferase NSD1 in human neuroblastoma and glioma. Proc Natl Acad Sci USA 106:21830–21835
121. Kahl P et al (2006) Androgen receptor coactivators lysine-specific histone demethylase 1 and four and a half LIM domain protein 2 predict risk of prostate cancer recurrence. Cancer Res 66:11341–11347
122. van Haaften G et al (2009) Somatic mutations of the histone H3K27 demethylase gene UTX in human cancer. Nat Genet 41:521–523
123. Shi Y (2007) Histone lysine demethylases: emerging roles in development, physiology and disease. Nature reviews 8:829–833
124. Liu G et al (2009) Genomic amplification and oncogenic properties of the GASC1 histone demethylase gene in breast cancer. Oncogene 28:4491–4500
125. Xu Y, Yan W, Chen X (2010) SNF5, a core component of the SWI/SNF complex, is necessary for p53 expression and cell survival, in part through eIF4E. Oncogene 29:4090–4100
126. Oruetxebarria I et al (2004) P16INK4a is required for hSNF5 chromatin remodeler-induced cellular senescence in malignant rhabdoid tumor cells. J Biol Chem 279:3807–3816
127. Sansam CG, Roberts CW (2006) Epigenetics and cancer: altered chromatin remodeling via Snf5 loss leads to aberrant cell cycle regulation. Cell Cycle 5:621–624
128. Caramel J, Medjkane S, Quignon F, Delattre O (2008) The requirement for SNF5/INI1 in adipocyte differentiation highlights new features of malignant rhabdoid tumors. Oncogene 27:2035–2044
129. Caramel J, Quignon F, Delattre O (2008) RhoA-dependent regulation of cell migration by the tumor suppressor hSNF5/INI1. Cancer Res 68:6154–6161
130. McKenna ES et al (2008) Loss of the epigenetic tumor suppressor SNF5 leads to cancer without genomic instability. Mol Cell Biol 28:6223–6233

131. Wang X et al (2009) Oncogenesis caused by loss of the SNF5 tumor suppressor is dependent on activity of BRG1, the ATPase of the SWI/SNF chromatin remodeling complex. Cancer Res 69:8094–8101
132. Gadd S, Sredni ST, Huang CC, Perlman EJ (2010) Rhabdoid tumor: gene expression clues to pathogenesis and potential therapeutic targets. Lab Invest 90:724–738
133. Modena P et al (2005) SMARCB1/INI1 tumor suppressor gene is frequently inactivated in epithelioid sarcomas. Cancer Res 65:4012–4019
134. Kreiger PA et al (2009) Loss of INI1 expression defines a unique subset of pediatric undifferentiated soft tissue sarcomas. Mod Pathol 22:142–150
135. Trobaugh-Lotrario AD, Tomlinson GE, Finegold MJ, Gore L, Feusner JH (2009) Small cell undifferentiated variant of hepatoblastoma: adverse clinical and molecular features similar to rhabdoid tumors. Pediatr Blood Cancer 52:328–334
136. Cheng JX et al (2008) Renal medullary carcinoma: rhabdoid features and the absence of INI1 expression as markers of aggressive behavior. Mod Pathol 21:647–652
137. Lin H, Wong RP, Martinka M, Li G (2009) Loss of SNF5 expression correlates with poor patient survival in melanoma. Clin Cancer Res 15:6404–6411
138. Medina PP et al (2005) Transcriptional targets of the chromatin-remodelling factor SMARCA4/BRG1 in lung cancer cells. Hum Mol Genet 14:973–982
139. Wong AK et al (2000) BRG1, a component of the SWI-SNF complex, is mutated in multiple human tumor cell lines. Cancer Res 60:6171–6177
140. Rodriguez-Nieto S, Sanchez-Cespedes M (2009) BRG1 and LKB1: tales of two tumor suppressor genes on chromosome 19p and lung cancer. Carcinogenesis 30:547–554
141. Medina PP et al (2004) Genetic and epigenetic screening for gene alterations of the chromatin-remodeling factor, SMARCA4/BRG1, in lung tumors. Genes Chromosomes Cancer 41:170–177
142. Parsons DW et al (2010) The genetic landscape of the childhood cancer medulloblastoma. Science 331:435–439
143. Schneppenheim R et al (2010) Germline nonsense mutation and somatic inactivation of SMARCA4/BRG1 in a family with rhabdoid tumor predisposition syndrome. Am J Hum Genet 86:279–284
144. Sun A et al (2007) Aberrant expression of SWI/SNF catalytic subunits BRG1/BRM is associated with tumor development and increased invasiveness in prostate cancers. Prostate 67:203–213
145. de Zwaan SE, Haass NK (2010) Genetics of basal cell carcinoma. Australas J Dermatol 51:81–92; quiz 93–94
146. Moloney FJ et al (2009) Hotspot mutation of Brahma in non-melanoma skin cancer. J Invest Dermatol 129:1012–1015
147. Patsialou A, Wilsker D, Moran E (2005) DNA-binding properties of ARID family proteins. Nucleic Acids Res 33:66–80
148. Dallas PB et al (2000) The human SWI-SNF complex protein p270 is an ARID family member with non-sequence-specific DNA binding activity. Mol Cell Biol 20:3137–3146
149. Nagl NG Jr, Zweitzig DR, Thimmapaya B, Beck GR Jr, Moran E (2006) The c-myc gene is a direct target of mammalian SWI/SNF-related complexes during differentiation-associated cell cycle arrest. Cancer Res 66:1289–1293
150. Nagl NG Jr, Wang X, Patsialou A, Van Scoy M, Moran E (2007) Distinct mammalian SWI/SNF chromatin remodeling complexes with opposing roles in cell-cycle control. EMBO J 26:752–763
151. Nagl NG Jr et al (2005) The p270 (ARID1A/SMARCF1) subunit of mammalian SWI/SNF-related complexes is essential for normal cell cycle arrest. Cancer Res 65:9236–9244
152. Jones S et al (2010) Frequent mutations of chromatin remodeling gene ARID1A in ovarian clear cell carcinoma. Science 330:228–231
153. Wiegand KC et al (2010) ARID1A mutations in endometriosis-associated ovarian carcinomas. N Engl J Med 363:1532–1543
154. Maeda D et al (2010) Clinicopathological significance of loss of ARID1A immunoreactivity in ovarian clear cell carcinoma. Int J Mol Sci 11:5120–5128

155. Wiegand KC et al (2011) Loss of BAF250a (ARID1A) is frequent in high-grade endometrial carcinomas. J Pathol 224:328–333
156. Guan B et al (2011) Mutation and loss of expression of ARID1A in uterine low-grade endometrioid carcinoma. Am J Surg Pathol 35:625–632
157. Birnbaum DJ et al (2011) Genome profiling of pancreatic adenocarcinoma. Genes Chromosomes Cancer 50:456–465
158. Zhang X et al (2012) Frequent low expression of chromatin remodeling gene ARID1A in breast cancer and its clinical significance. Cancer Epidemiol 36(3):288–293
159. Wang X et al (2004) Expression of p270 (ARID1A), a component of human SWI/SNF complexes, in human tumors. Int J Cancer 112:636
160. Li XS, Trojer P, Matsumura T, Treisman JE, Tanese N (2010) Mammalian SWI/SNF–a subunit BAF250/ARID1 is an E3 ubiquitin ligase that targets histone H2B. Mol Cell Biol 30:1673–1688
161. Li M et al (2011) Inactivating mutations of the chromatin remodeling gene ARID2 in hepatocellular carcinoma. Nat Genet 43:828–829
162. Burrows AE, Smogorzewska A, Elledge SJ (2010) Polybromo-associated BRG1-associated factor components BRD7 and BAF180 are critical regulators of p53 required for induction of replicative senescence. Proc Natl Acad Sci USA 107:14280–14285
163. Drost J et al (2010) BRD7 is a candidate tumour suppressor gene required for p53 function. Nat Cell Biol 12:380–389
164. Kim S, Lee J, Park J, Chung J (2003) BP75, bromodomain-containing M(r) 75,000 protein, binds dishevelled-1 and enhances Wnt signaling by inactivating glycogen synthase kinase-3 beta. Cancer Res 63:4792–4795
165. Kikuchi M et al (2009) TRIM24 mediates ligand-dependent activation of androgen receptor and is repressed by a bromodomain-containing protein, BRD7, in prostate cancer cells. Biochim Biophys Acta 1793:1828–1836
166. Mantovani F, Drost J, Voorhoeve PM, Del Sal G, Agami R (2010) Gene regulation and tumor suppression by the bromodomain-containing protein BRD7. Cell Cycle 9:2777–2781
167. Liu H et al (2008) Promoter methylation inhibits BRD7 expression in human nasopharyngeal carcinoma cells. BMC Cancer 8:253
168. Varela I et al (2011) Exome sequencing identifies frequent mutation of the SWI/SNF complex gene PBRM1 in renal carcinoma. Nature 469:539–542
169. Xia W et al (2008) BAF180 is a critical regulator of p21 induction and a tumor suppressor mutated in breast cancer. Cancer Res 68:1667–1674
170. Hah N et al (2010) A role for BAF57 in cell cycle-dependent transcriptional regulation by the SWI/SNF chromatin remodeling complex. Cancer Res 70:4402–4411
171. Decristofaro MF et al (2001) Characterization of SWI/SNF protein expression in human breast cancer cell lines and other malignancies. J Cell Physiol 186:136–145
172. Gorrini C et al (2007) Tip60 is a haplo-insufficient tumour suppressor required for an oncogene-induced DNA damage response. Nature 448:1063–1067
173. Mattera L et al (2009) The p400/Tip60 ratio is critical for colorectal cancer cell proliferation through DNA damage response pathways. Oncogene 28:1506–1517
174. Notaridou M et al (2011) Common alleles in candidate susceptibility genes associated with risk and development of epithelial ovarian cancer. Int J Cancer 128:2063–2074
175. Wang CL et al (2009) Discovery of retinoblastoma-associated binding protein 46 as a novel prognostic marker for distant metastasis in nonsmall cell lung cancer by combined analysis of cancer cell secretome and pleural effusion proteome. J Proteome Res 8:4428–4440
176. Buganim Y et al (2010) A novel translocation breakpoint within the BPTF gene is associated with a pre-malignant phenotype. PLoS One 5:e9657
177. Thakur A et al (2007) Aberrant expression of X-linked genes RbAp46, Rsk4, and Cldn2 in breast cancer. Mol Cancer Res 5:171–181
178. Sakhinia E et al (2005) Routine expression profiling of microarray gene signatures in acute leukaemia by real-time PCR of human bone marrow. Br J Haematol 130:233–248
179. Kim MS, Chung NG, Kang MR, Yoo NJ, Lee SH (2011) Genetic and expressional alterations of CHD genes in gastric and colorectal cancers. Histopathology 58:660–668

180. Gorringe KL et al (2008) Mutation and methylation analysis of the chromodomain-helicase-DNA binding 5 gene in ovarian cancer. Neoplasia 10:1253–1258
181. Robbins CM et al (2011) Copy number and targeted mutational analysis reveals novel somatic events in metastatic prostate tumors. Genome Res 21:47–55
182. Fujita T et al (2008) CHD5, a tumor suppressor gene deleted from 1p36.31 in neuroblastomas. J Natl Cancer Inst 100:940–949
183. Bagchi A et al (2007) CHD5 is a tumor suppressor at human 1p36. Cell 128:459–475
184. Mulero-Navarro S, Esteller M (2008) Chromatin remodeling factor CHD5 is silenced by promoter CpG island hypermethylation in human cancer. Epigenetics 3:210–215
185. Wang J et al (2011) The involvement of CHD5 hypermethylation in laryngeal squamous cell carcinoma. Oral Oncol 47:601–608
186. Mokarram P et al (2009) Distinct high-profile methylated genes in colorectal cancer. PLoS One 4:e7012
187. Wang X, Lau KK, So LK, Lam YW (2009) CHD5 is down-regulated through promoter hypermethylation in gastric cancer. J Biomed Sci 16:95
188. Vissers LE et al (2004) Mutations in a new member of the chromodomain gene family cause CHARGE syndrome. Nat Genet 36:955–957
189. Wessels K et al (2010) Novel CHD7 mutations contributing to the mutation spectrum in patients with CHARGE syndrome. Eur J Med Genet 53:280–285
190. Schlesinger Y et al (2007) Polycomb-mediated methylation on Lys27 of histone H3 premarks genes for de novo methylation in cancer. Nat Genet 39:232–236
191. Widschwendter M et al (2007) Epigenetic stem cell signature in cancer. Nat Genet 39:157–158
192. Ohm JE et al (2007) A stem cell-like chromatin pattern may predispose tumor suppressor genes to DNA hypermethylation and heritable silencing. Nat Genet 39:237–242
193. Constantinides PG, Jones PA, Gevers W (1977) Functional striated muscle cells from non-myoblast precursors following 5-azacytidine treatment. Nature 267:364–366
194. Plimack ER, Kantarjian HM, Issa JP (2007) Decitabine and its role in the treatment of hematopoietic malignancies. Leuk Lymphoma 48:1472–1481
195. Matei DE, Nephew KP (2010) Epigenetic therapies for chemoresensitization of epithelial ovarian cancer. Gynecol Oncol 116:195–201
196. Cheng JC et al (2004) Preferential response of cancer cells to zebularine. Cancer Cell 6:151–158
197. Rodriguez-Paredes M, Esteller M (2011) Cancer epigenetics reaches mainstream oncology. Nat Med 17:330–339

Part II
The Impact of Epigenetic Alterations on Cancer Biology

Chapter 6
Epigenetic Regulation of miRNAs in Cancer

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Abstract MicroRNAs (miRNAs) are short noncoding RNAs with gene regulatory functions. It has been demonstrated that the genes encoding for miRNAs undergo the same regulatory epigenetic processes of protein coding genes. In turn, a specific subgroup of miRNAs, called epi-miRNAs, is able to directly target key enzymatic effectors of the epigenetic machinery (such as DNA methyltransferases, histone deacetylases, and polycomb genes), therefore indirectly affecting the expression of epigenetically regulated oncogenes and tumor suppressor genes. Also, several of the epigenetic drugs currently approved as anticancer agents affect the expression of miRNAs and this might explain part of their mechanism of action. This chapter focuses on the tight relationship between epigenetics and miRNAs and provides some insights on the translational implications of these findings, leading to the upcoming introduction of epigenetically related miRNAs in the treatment of cancer.

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6.1 Introduction

MicroRNAs (miRNAs) are small noncoding RNAs (ncRNAs), 19–25 nucleotides (nt) in length, which regulate gene expression. MiRNAs are involved in many biological processes ranging from development, differentiation, and cell cycle regulation to cell senescence and metabolism [1–5]. Mature miRNAs derive from much longer (hundreds nt long) primary transcripts, transcribed by RNA polymerase II as long, capped, polyadenylated precursor-miRNAs (pri-miRNAs) [1]. Then, the double-stranded RNA-specific ribonuclease Drosha, in conjunction with its binding partner DiGeorge syndrome critical region gene 8 (DGCR8, or Pasha), process pri-miRNAs into hairpin RNAs of 60–110 nt known as pre-miRNAs. Translocated from the cell nucleus to the cytoplasm by means of Exportin 5, the pre-miRNA is processed by a ribonuclease III (Dicer) and transactivating response RNA-binding protein (TRBP, which binds human immunodeficiency virus 1) into an 18- to 24-nt duplex. Finally, the duplex interacts with a large protein, RNA-induced silencing complex (RISC), which includes argonaute proteins (AGO1-4 in humans). One strand of the miRNA duplex remains stably associated with RISC and becomes the mature miRNA, which guides the RISC complex mainly (but not exclusively) to the 3′-untranslated region (UTR) of the target messenger RNAs (mRNAs) [1]. Consequently, the translation and/or stability of targeted mRNAs is impaired, causing a reduction in protein expression levels [6]. In addition to this "conventional" mechanism of action, miRNA regulatory effects on gene expression may be more varied than initially proposed. For example, miRNAs can also activate rather than suppress target mRNA expression in particular cell-cycle conditions [7], they can bind also to the coding and the 5′-UTR region of the target mRNAs [8, 9], and they can directly interact with proteins and function as gene promoter regulators [10]. Figure 6.1 summarizes the biogenesis and physiology of miRNAs.

Each miRNA has hundreds or thousands of target genes. We have demonstrated that a specific cluster of two miRNAs (namely, the miR-15a/16-1 cluster) is able to regulate, directly and indirectly, about 14% of the whole genome in a leukemic cell model [11]. Therefore, it is likely that the full coding genome is under the control of miRNAs. The full spectrum of miRNAs expressed in a specific cell type (the miRNome) is different between normal and pathologic tissues [12], and specific signatures of dys-regulated miRNAs harbor diagnostic and prognostic implications [13]. The first link between miRNAs and cancer came from the discovery that these ncRNAs are frequently located in cancer-associated genomic regions, which include minimal regions of amplification, loss of heterozygosity, and common breakpoints in or near oncogenes or tumor suppressor genes (TSGs) and fragile sites (preferential sites of chromatide exchange, deletion, translocation, amplification, or integration of plasmid DNA and tumor-associated viruses) [14]. Since then, myriad studies have investigated aberrations in the miRNome in most types of human cancer (for reviews, see [15–21]). In particular, while some miRNAs act mainly as TSGs, others are frequently overexpressed in human tumors and target TSGs, thereby exerting

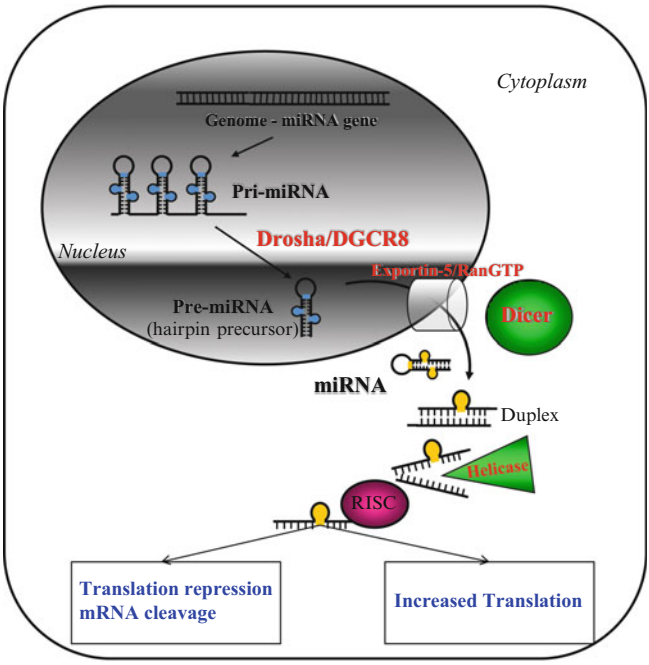

Fig. 6.1 Biogenesis and physiology of miRNAs. MiRNAs are transcribed as pri-miRNAs (in some cases as a cluster of multiple miRs, such as miR-15a and miR-16-1 on the long arm of chromosome 13) and then processed in a hairpin shaped pre-miRNA precursor in the nucleus of the cell. The precursor is then transported in the cytoplasm by means of Exportin 5 and processed until it becomes a single-stranded mature miRNA that eventually binds to a ribonucleoproteic complex (RISC) which directs the miRNA to its target mRNAs. As a result, both translational repression (or mRNA cleavage) and increased target translation can occur (see text for more explanation)

a tumorigenic function. MiRNAs with well-established roles as oncogenes, for instance, include the miR-17-92 cluster, which is transactivated by the *c-MYC* oncogene and dramatically accelerates lymphomagenesis in murine models [22, 23]; miR-155, which induces leukemia in transgenic murine models [24] and has an important function as a regulator of inflammation and the immune response [25–27], and miR-21, which targets important TSGs, such as *PTEN1* [28] and *PDCD4*, in several types of cancer [29–31]. Conversely, the miR-15a/16-1 cluster acts as a TSG in chronic lymphocytic leukemia (CLL) by targeting the antiapoptotic gene *BCL2* [32]. Interestingly, the same miR-15a-16-1 cluster also acts as an oncogene (OG), in CLL, by directly targeting the pro-apoptotic gene *p53* [33], leading to the conclusion that each miRNA should not be labeled exclusively as an OG or as a TSG, since it may have a dual nature (both as OG and TSG) [34], in which the overall effect depends on the specific conditions (tumor type, species specificity, concentration, etc.) in which it operates.

It has been demonstrated that miRNAs, similar to protein coding genes, (PCG), can undergo epigenetic regulation. More recently, it has been shown that a specific

group of miRNAs, called epi-miRNAs, can affect the epigenetic regulation of a given gene by targeting key enzymatic effectors of the epigenetic machinery, such as DNA methyltransferases (DNMTs), histone deacetylases (HDACs), and polycomb genes.

This chapter focuses on the interactions between epigenetics and miRNAs and presents how this intertwined relationship harbors fundamental implications for human carcinogenesis.

6.2 MiRNAs Are Epigenetically Regulated in Cancer

The expression of miRNAs undergoes epigenetic regulation, similarly to PCG. This regulation involves both chromatin modifications and miRNA gene promoter methylation. By treating a breast cancer cell line with the HDAC inhibitor LAQ824, Scott et al. demonstrated that the expression levels of 27 miRNAs are rapidly modified [35], indicating that HDAC and chromatin conformation affects the miRNome in human cancer. Similarly, Saito et al. showed that by treating bladder cancer cells with both a DNA demethylating agent (5-aza-2′-deoxycytidine, 5-AZA) and an HDAC inhibitor (4-phenylbutyric acid, 4-PBA) the expression levels of about 5% of all human miRNAs increased [36]. Among the most strictly epigenetically regulated miRNAs, there is miR-127, an ncRNA embedded in a CpG island and kept epigenetically silenced by both promoter hypermethylation and histone modifications in cancer cells [36]. Interestingly, this miRNA (which belongs to a large cluster that includes miR-136, -431, -432, and -433) is the only member of the cluster whose re-expression was observed when cells were treated with two epigenetic drugs [36]. Moreover, when cells were treated with each drug alone, no variation in miR-127 expression was detected [36], suggesting that miR-127 epigenetic regulation occurs by combined promoter methylation and chromatin histone modifications. Since the *BCL6* oncogene is a direct target of this miRNA [36], miR-127 acts as a TSG, therefore the severe epigenetic control of its expression represents an important mechanism for bladder carcinogenesis.

Using an HCT-116 colorectal cancer cell line with a double knockout (DKO) of DNMT1 (maintenance DNMT) and DNMT3b (de novo DNMT), Lujambio et al. compared miRNA levels of the DKO and wild-type cells. About 6% of the 320 miRNAs analyzed were upregulated in the DKO cells [37]. Among the dys-regulated miRNAs, only miR-124a was embedded in a CpG island, which is densely methylated in this cancer cell line but not in normal tissue. This might suggest that DNMTs act both directly and indirectly in miRNA expression control. MiRNA-124a directly targets CDK6, and restoration of its expression reduces the levels of CDK6 and impacts the phosphorylation status of the CDK6-downstream effector Rb protein [37]. In a group of 353 acute lymphoblastic leukemia (ALL) patients, Roman-Gomez et al. identified a signature of 13 miRNAs, embedded in CpG islands, with high heterochromatic markers (such as high levels of K9H3me2 and/or low levels of K4H3me3) [38, 39]. Treatment with 5-AZA upregulated at least 1 of the 13 miRNAs in 65% of ALLs

[38]. Among these miRNAs, miR-124a was methylated in 59% of ALLs and hypermethylation of its promoter was associated with higher relapse and mortality rates than in the non-hypermethylated cases: multivariate analysis confirmed that miR-124a promoter methylation status is an independent prognostic factor for disease-free and overall survival [39]. Moreover, miR-124a directly silences *CDK6* in ALL patients [39], confirming the impact of miR-124a on the CDK6-Rb pathway. Recently, Ando et al. showed that hypermethylation of the miR-124a promoter is involved in the formation of an epigenetic field defect, a gastric cancer predisposition condition characterized by the accumulation of abnormal DNA methylation in normal-appearing gastric mucosa that is mostly induced by *Helicobacter pylori* infection [40]. These findings reveal that miR-124a promoter hypermethylation is also an early event in gastric carcinogenesis.

In addition to miR-124a, miR-107, another epigenetically controlled miRNA, targets *CDK6* and affects pancreatic carcinogenesis [41]. In HCT-116 cells deficient for *DNMT1* and *DNMT3B*, Bruckner et al. showed increased expression of let-7a-3, an miRNA normally silenced by promoter hypermethylation in the wild-type cell line [42]. In lung adenocarcinoma primary tumors the let-7a-3 promoter was found to be hypomethylated [42], whereas it was found to be hypermethylated in epithelial ovarian cancer. This hypermethylation was associated with low expression of IGF2 (insulin-like growth factor 2) and with a good prognosis [43]. Therefore, DNA methylation could act as a protective mechanism by silencing miRNAs with oncogenic functions. Also miR-1 is epigenetically regulated and frequently silenced by promoter hypermethylation in hepatocellular carcinoma [44]. However, hypomethylation and re-expression of miR-1 were observed in DNMT1-null HCT-116 cells (but not in DNMT3B-null cells) [44], revealing that the maintenance DNMT is specifically and mainly responsible for miR-1 epigenetic regulation. Overall, these studies demonstrate that epigenetic factors can control human carcinogenesis, not only by directly affecting the expression of OGs and TSGs, but also by affecting the expression of miRNAs involved in oncogenic pathways. In addition, epigenetic control of miRNAs may be tissue-specific (since no variation in miRNA expression was observed in lung cancer cells treated with demethylating agents, HDAC inhibitors, or their combination [45]), miRNA-specific (e.g., miR-127 within the cluster it belongs to [36]), and epigenetic-effector-specific (e.g., miR-1 mainly regulated by DNMT1 [44]).

Epigenetically regulated miRNAs are also affecting one of the main aspects of malignancy: the ability to metastasize. Lujambio et al. treated three lymph node–metastatic cell lines with 5-AZA and checked miRNA levels by high-throughput microarray analysis [46]. They identified four miRNAs (namely, miR-148a, miR-34b/c, and miR-9) that showed cancer-specific CpG island hypermethylation. Epigenetic silencing of these miRNAs was also significantly associated with metastasis in human malignancies [46], while the reintroduction of miR-148a and miR-34b/c into cancer cells with epigenetic inactivation inhibited both motility and metastatic potential of the cells in xenograft models. The miR-34b/c cluster is also epigenetically regulated in colorectal cancer (promoter hypermethylation in 90% of primary colorectal cancer tumors vs. normal colon mucosa) [47], whereas

epigenetic silencing of miR-9 and miR-148a (together with miR-152, -124a, and -663) has also been described in breast cancer. In breast cancer cell lines treated with 5-AZA miR-9 was reactivated, while the levels of other aberrantly methylated miRNAs were unchanged [48], further proving that different epigenetic processes can control miRNA levels in different types of cancer.

MiR-342 is located in an intron of the Ena/Vasp-like (*EVL*) gene and represents a good model to study the relationship between miRNAs and the epigenetic regulation of cognate host genes. *EVL* promoter hypermethylation occurs in 86% of colorectal cancers and is present in 67% of adenomas at diagnosis, suggesting that it is an early event in colon carcinogenesis [49]. Treatment with 5-AZA and the HDAC inhibitor trichostatin A restores the synchronized expression of EVL and miR-342 [49]. Another gene, the *EGFL7* gene, which is frequently downregulated in several cancer cell lines and in primary bladder and prostate tumors, hosts miR-126 in one intron. The mature miR-126 can be encoded by three different transcripts of the cognate host gene, each of them with its own promoter. However, miR-126 is concomitantly upregulated with one of the EGFL7 transcripts that has a CpG-island promoter when cancer cell lines are treated with DNA methylation and histone deacetylation inhibitors, indicating that the silencing of intronic miRNAs in cancer may occur by means of epigenetic changes in cognate host genes [50].

Fazi et al. showed that transcription factors can recruit epigenetic effectors to miRNA promoter regions to regulate their expression. The AML1/ETO fusion oncoprotein, the aberrant product of the t(8;21) translocation in acute myeloid leukemia, can bind to the pre-miR-223 region. The oncoprotein recruits epigenetic effectors (i.e., DNMTs, HDAC1, and MeCP2), leading to aberrant hypermethylation of the CpG site near the AML1/ETO binding site and H3-H4 deacetylation of the same chromatin region [51].

In summary, several studies have addressed how epigenetics regulates miRNA expression in human cancer. It has emerged that epigenetic factors account for several of the miRNome aberrancies observed in human cancer, ultimately implicated in both carcinogenesis and in metastasis formation. Therefore, a better understanding of miRNA epigenetic regulation will lead to a better comprehension of the mechanisms responsible for abnormal miRNA levels in cancer and to the development of strategies able to revert these anomalies. Interestingly, miRNAs can also affect the expression of epigenetically regulated PCGs, revealing a further layer of complexity between miRNome and epigenome.

6.3 Epi-miRNAs Affect the Expression of Epigenetically Regulated Genes in Cancer

In addition to being epigenetically regulated, like PCG, miRNAs can also affect the expression of epigenetically regulated genes by targeting key enzymes responsible for epigenetic reactions. We call this group of miRNAs, epi-miRNAs. Some epi-miRNAs are also epigenetically regulated themselves. Our group provided the first

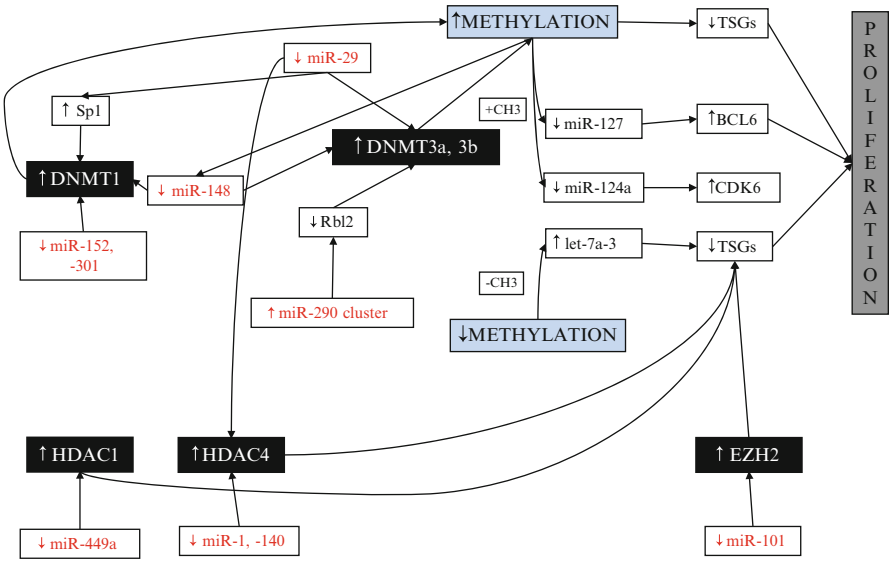

Fig. 6.2 Epi-miRNAs and cancer. Epi-miRNAs (in *red*) directly target effectors of the epigenetic machinery (in *black boxes*) and indirectly affect the expression of epigenetically regulated miRNAs and protein coding genes, ultimately affecting carcinogenesis. *TSGs* tumor suppressor genes; *DNMT* DNA methyltransferase; *HDAC* histone deacetylase; *EZH2* enhancer of zeste homolog 2; *BCL6* B-cell CLL/lymphoma 6; *CDK6* cyclin-dependent kinase 6; *SP1* Sp1 transcription factor; *RBL2* retinoblastoma-like 2 (p130); CH_3 methyl group

evidence that miRNAs can regulate the expression of members of the epigenetic machinery in humans [52]. We demonstrated in both lung cancer cell lines and primary tumors that a family of miRNAs (namely the miR-29 family, composed of miR-29a, -29b, and -29c) directly targets both DNMT3a and DNMT3b, the two key de novo DNMTs. We observed that miR-29 restoration reduces global DNA methylation, induces re-expression of TSGs (such as *WWOX* and *FHIT*, whose expression is silenced by promoter hypermethylation in lung cancer), and exerts an overall antitumoral effect both in vitro and in vivo [52]. The global hypomethylating effect observed in tumor cells upon miR-29 re-expression is the result of a direct targeting effect of these miRNAs on DNMT3a and DNMT3b, and of an indirect silencing effect on DNMT1, occurring through the direct targeting of the DNMT1 transactivating factor SP1 [53]. Figure 6.2 summarizes the relationship between epi-miRNAs and cancer. Duursma et al. [54] have shown that miR-148 also directly targets DNMT3b by binding to a conserved target sequence located in the coding region of the mRNA. Intriguingly, the authors concluded that the targeting of the coding region may play a role in determining the relative abundance of different splice variants of DNMT3b. Furthermore, miRNAs can affect the expression of DNMTs also through an indirect mechanism. Sinkkonen et al. showed that in mouse embryonic stem (ES) cells, members of the miR-290 cluster directly target Rbl2, a factor contributing to the suppression of *DNMT3* genes [55]. By restoring the expression of

the miR-290 cluster, de novo methylation, which had been disrupted in ES Dicer$^{-/-}$ cells, was reestablished, suggesting that DNMTs are indirectly regulated by the miR-290 cluster. These results were confirmed by Benetti et al. [56], who also observed that the aberrant DNA methylation occurring after miR-290 cluster silencing in ES Dicer$^{-/-}$ cells is responsible for increased telomere recombination and aberrant telomere elongation. Notably, the miR-290 Rbl2-mediated regulation of *DNMT3a* and *DNMT3b* was not observed in HEK293 cells with knockdown of Dicer [55], revealing that the described regulatory mechanism might be restricted to ES cells. Moreover, neither of the above-mentioned studies identified the miR-29 family as direct regulators of de novo DNMTs, suggesting that this interaction could also be species-, tumor-, or even histotype-specific.

Epi-miRNAs can also target *DNMT1*. In a study by Braconi et al., it was shown that miR-148a, miR-152, and miR-301 directly target *DNMT1* in cholangiocarcinoma cells [57], resulting in the re-expression of the *RASSF1A* and *p16INK4a* genes, two well-known TSGs that are epigenetically silenced in several malignancies. As previously reported, miR-29b indirectly targets *DNMT1*, by directly silencing its activator SP1 in hematological malignancies [53]. These studies suggest that miR-29b plays a key role in the epigenetic control of human genome.

Epi-miRNAs also regulate the expression of HDACs and PRC genes. *HDAC4* is a direct target of both miR-1, miR-140, and miR-29b [58–60], whereas miR-449a binds to the 3′-UTR region of *HDAC1* [61]. *HDAC1* is upregulated in several types of cancer, and miR-449a re-expression in prostate cancer cells induces cell-cycle arrest, apoptosis, and a senescent-like phenotype by reducing the levels of HDAC1 [61]. EZH2 is the catalytic subunit of PRC2 and is responsible for heterochromatin formation by trimethylating histone H3 on lysine 27 (H3K27me3), leading to the silencing of several TSGs. Varambally et al. showed in prostate cancer cell lines and primary tumors that the level of EZH2 is inversely correlated with the expression of miR-101, which decreases during cancer progression. These findings suggest a role for miR-101 as an epi-miRNA, a hypothesis that was tested and confirmed by showing that miR-101 directly targets EZH2 both in prostate and in bladder cancer models [62, 63]. Moreover, the miR-101-mediated suppression of EZH2 inhibits cancer cell proliferation and colony formation, revealing a role for miR-101 as a TSG that is mediated by its modulatory effects on the cancer epigenome [63].

In summary, an increasing number of epi-miRNAs is being identified and will clarify which epigenetic effectors are involved in the regulation of OGs and TSGs. This knowledge will lead to the development of new strategies to prevent and cure human carcinogenesis by selective modulation of the epi-miRNome.

6.4 Epigenetics and miRNAs: Clinical Implications and Final Remarks

The epigenetics–miRNA relationship harbors several clinical implications. First, some of the demethylating agents (such as 5-AZA or Vidaza) used to show that miRNAs are re-expressed upon demethylation and therefore undergo epigenetic

regulations are drugs, currently approved for the treatment of myelodysplastic syndromes (MDS) [64]. Therefore, part of the observed therapeutic effects of 5-AZA or decitabine might be mediated by their effect on the miRNome. Also, currently available anticancer drugs (such as Bortezomib) induce the expression of miR-29b [65], a key epi-miRNA targeting both DNMTs and HDACs. Moreover, SAHA (suberoylanilide hydroxamic acid), also known as Vorinostat is an HDAC inhibitor currently approved in the treatment of cutaneous T cell lyphomas, may exert an anticancer effect by re-expressing epigenetically regulated miRNAs [66, 67]. Valproic acid (VPA) is also an HDAC inhibitor currently in phase III studies for the treatment of cervical and ovarian cancer, which is able to modulate the expression of miRNAs in human cord blood-derived multipotent stem cells [68].

Overall, while basic research scientists are trying to improve their understanding of the relationship existing between epigenetics and miRNAs, clinicians have started interpreting some of the effects of epigenetic drugs in terms of their effects on the miRNome. This interaction represents an ideal translational setting, capable of bringing novel insights deriving from basic science to the patients. In addition to better understanding the implications and function of currently available epigenetic drugs on the miRNome, it is likely that in the near future this knowledge will assist in the development of miRNA- and epi-miRNA-based therapies. These therapies will be tailored to the specific set of genes that need to be reverted to a physiological expression, in order to achieve an anticancer effect. Therefore, their effect will specifically affect tumor cells, without introducing any major epigenetic perturbation in noncancerous cells, therefore leading to less side effects. These days are not far to come and will provide a new powerful therapeutic tool in the war against cancer.

References

1. Bartel DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116:281–297
2. Pasquinelli AE, Hunter S, Bracht J (2005) MicroRNAs: a developing story. Curr Opin Genet Dev 15:200–205
3. Harfe BD (2005) MicroRNAs: in vertebrate development. Curr Opin Genet Dev 15:410–415
4. Carleton M, Cleary MA, Linsley PS (2007) MicroRNAs and cell cycle regulation. Cell Cycle 6:2127–2132
5. Boehm M, Slack FJ (2006) MicroRNA control of lifespan and metabolism. Cell Cycle 5:837–840
6. Filipowicz W, Bhattacharyya SN, Sonenberg N (2008) Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? Nature Reviews 9:102–114
7. Vasudevan S, Tong Y, Steitz JA (2007) Switching from repression to activation: microRNAs can up-regulate translation. Science 318:1931–1934
8. Tay Y, Zhang J, Thomson AM, Lim B, Rigoutsos I (2008) MicroRNAs to Nanog, Oct4 and Sox2 coding regions modulate embryonic stem cell differentiation. Nature 455:1124–1128
9. Vatolin S, Navaratne K, Weil RJ (2006) A novel method to detect functional microRNA targets. J Mol Biol 358:983–996
10. Eiring AM et al (2010) MiR-328 functions as an RNA decoy to modulate hnRNP E2 regulation of mRNA translation in leukemic blasts. Cell 140:652–665

11. Calin GA et al (2008) MiR-15a and miR-16-1 cluster functions in human leukemia. Proc Natl Acad Sci USA 105:5166–5171
12. Sevignani C, Calin GA, Siracusa LD, Croce CM (2006) Mammalian microRNAs: a small world for fine-tuning gene expression. Mamm Genome 17:189–202
13. Calin GA et al (2005) A microRNA signature associated with prognosis and progression in chronic lymphocytic leukemia. N Engl J Med 353:1793–1801
14. Calin GA et al (2004) Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. Proc Natl Acad Sci USA 101:2999–3004
15. Nelson KM, Weiss GJ (2008) MicroRNAs and cancer: past, present, and potential future. Mol Cancer Ther 7:3655–3660
16. Fabbri M, Croce CM, Calin GA (2009) MicroRNAs in the ontogeny of leukemias and lymphomas. Leuk Lymphoma 50:160–170
17. Fabbri M, Croce CM, Calin GA (2008) MicroRNAs. Cancer J 14:1–6
18. Fabbri M et al (2008) MicroRNAs and noncoding RNAs in hematological malignancies: molecular, clinical and therapeutic implications. Leukemia 22:1095–1105
19. Deng S, Calin GA, Croce CM, Coukos G, Zhang L (2008) Mechanisms of microRNA deregulation in human cancer. Cell Cycle 7:2643–2646
20. Garzon R, Croce CM (2008) MicroRNAs in normal and malignant hematopoiesis. Curr Opin Hematol 15:352–358
21. Croce CM (2009) Causes, and consequences of microRNA dysregulation in cancer. Nat Rev Genet 10:704–714
22. He L et al (2005) A microRNA polycistron as a potential human oncogene. Nature 435: 828–833
23. Mendell JT (2008) miRiad roles for the miR-17-92 cluster in development and disease. Cell 133:217–222
24. Costinean S et al (2006) Pre-B cell proliferation and lymphoblastic leukemia/high-grade lymphoma in E(mu)-miR155 transgenic mice. Proc Natl Acad Sci USA 103:7024–7029
25. Thai TH et al (2007) Regulation of the germinal center response by microRNA-155. Science 316:604–608
26. Rodriguez A et al (2007) Requirement of bic/microRNA-155 for normal immune function. Science 316:608–611
27. Tili E et al (2007) Modulation of miR-155 and miR-125b levels following lipopolysaccharide/TNF-alpha stimulation and their possible roles in regulating the response to endotoxin shock. J Immunol 179:5082–5089
28. Cully M, You H, Levine AJ, Mak TW (2006) Beyond PTEN mutations: the PI3K pathway as an integrator of multiple inputs during tumorigenesis. Nat Rev Cancer 6:184–192
29. Meng F et al (2007) MicroRNA-21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer. Gastroenterology 133:647–658
30. Asangani IA et al (2008) MicroRNA-21 (miR-21) post-transcriptionally downregulates tumor suppressor Pdcd4 and stimulates invasion, intravasation and metastasis in colorectal cancer. Oncogene 27:2128–2136
31. Frankel LB et al (2008) Programmed cell death 4 (PDCD4) is an important functional target of the microRNA miR-21 in breast cancer cells. J Biol Chem 283:1026–1033
32. Cimmino A et al (2005) miR-15 and miR-16 induce apoptosis by targeting BCL2. Proc Natl Acad Sci USA 102:13944–13949
33. Fabbri M et al (2011) Association of a microRNA/TP53 feedback circuitry with pathogenesis and outcome of B-cell chronic lymphocytic leukemia. JAMA 305:59–67
34. Fabbri M, Ivan M, Cimmino A, Negrini M, Calin GA (2007) Regulatory mechanisms of microRNAs involvement in cancer. Expert Opin Biol Ther 7:1009–1019
35. Scott GK, Mattie MD, Berger CE, Benz SC, Benz CC (2006) Rapid alteration of microRNA levels by histone deacetylase inhibition. Cancer Res 66:1277–1281
36. Saito Y et al (2006) Specific activation of microRNA-127 with downregulation of the proto-oncogene BCL6 by chromatin-modifying drugs in human cancer cells. Cancer Cell 9: 435–443

37. Lujambio A et al (2007) Genetic unmasking of an epigenetically silenced microRNA in human cancer cells. Cancer Res 67:1424–1429
38. Roman-Gomez J et al (2009) Epigenetic regulation of microRNAs in acute lymphoblastic leukemia. J Clin Oncol 27:1316–1322
39. Agirre X et al (2009) Epigenetic silencing of the tumor suppressor microRNA Hsa-miR-124a regulates CDK6 expression and confers a poor prognosis in acute lymphoblastic leukemia. Cancer Res 69(10):4443–4453
40. Ando T et al (2009) DNA methylation of microRNA genes in gastric mucosae of gastric cancer patients: its possible involvement in the formation of epigenetic field defect. Int J Cancer 124:2367–2374
41. Lee KH et al (2009) Epigenetic silencing of microRNA miR-107 regulates cyclin-dependent kinase 6 expression in pancreatic cancer. Pancreatology 9:293–301
42. Brueckner B et al (2007) The human let-7a-3 locus contains an epigenetically regulated microRNA gene with oncogenic function. Cancer Res 67:1419–1423
43. Lu L, Katsaros D, de la Longrais IA, Sochirca O, Yu H (2007) Hypermethylation of let-7a-3 in epithelial ovarian cancer is associated with low insulin-like growth factor-II expression and favorable prognosis. Cancer Res 67:10117–10122
44. Datta J et al (2008) Methylation mediated silencing of MicroRNA-1 gene and its role in hepatocellular carcinogenesis. Cancer Res 68:5049–5058
45. Yanaihara N et al (2006) Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. Cancer Cell 9:189–198
46. Lujambio A et al (2008) A microRNA DNA methylation signature for human cancer metastasis. Proc Natl Acad Sci USA 105:13556–13561
47. Toyota M et al (2008) Epigenetic silencing of microRNA-34b/c and B-cell translocation gene 4 is associated with CpG island methylation in colorectal cancer. Cancer Res 68:4123–4132
48. Lehmann U et al (2008) Epigenetic inactivation of microRNA gene hsa-mir-9-1 in human breast cancer. J Pathol 214:17–24
49. Grady WM et al (2008) Epigenetic silencing of the intronic microRNA hsa-miR-342 and its host gene EVL in colorectal cancer. Oncogene 27:3880–3888
50. Saito Y et al (2009) Epigenetic therapy upregulates the tumor suppressor microRNA-126 and its host gene EGFL7 in human cancer cells. Biochem Biophys Res Commun 379:726–731
51. Fazi F et al (2007) Epigenetic silencing of the myelopoiesis regulator microRNA-223 by the AML1/ETO oncoprotein. Cancer Cell 12:457–466
52. Fabbri M et al (2007) MicroRNA-29 family reverts aberrant methylation in lung cancer by targeting DNA methyltransferases 3A and 3B. Proc Natl Acad Sci USA 104:15805–15810
53. Garzon R et al (2009) MicroRNA -29b induces global DNA hypomethylation and tumor suppressor gene re-expression in acute myeloid leukemia by targeting directly DNMT3A and 3B and indirectly DNMT1. Blood 113:6411–6418
54. Duursma AM, Kedde M, Schrier M, le Sage C, Agami R (2008) miR-148 targets human DNMT3b protein coding region. RNA 14:872–877
55. Sinkkonen L et al (2008) MicroRNAs control de novo DNA methylation through regulation of transcriptional repressors in mouse embryonic stem cells. Nat Struct Mol Biol 15:259–267
56. Benetti R et al (2008) A mammalian microRNA cluster controls DNA methylation and telomere recombination via Rbl2-dependent regulation of DNA methyltransferases. Nat Struct Mol Biol 15:268–279
57. Braconi C, Huang N, Patel T (2010) MicroRNA-dependent regulation of DNA methyltransferase-1 and tumor suppressor gene expression by interleukin-6 in human malignant cholangiocytes. Hepatology 51:881–890
58. Chen JF et al (2006) The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation. Nat Genet 38:228–233
59. Tuddenham L et al (2006) The cartilage specific microRNA-140 targets histone deacetylase 4 in mouse cells. FEBS Lett 580:4214–4217
60. Li Z et al (2009) Biological functions of miR-29b contribute to positive regulation of osteoblast differentiation. J Biol Chem 282:15676–15684

61. Noonan EJ et al (2009) miR-449a targets HDAC-1 and induces growth arrest in prostate cancer. Oncogene 28:1714–1724
62. Varambally S et al (2008) Genomic loss of microRNA-101 leads to overexpression of histone methyltransferase EZH2 in cancer. Science 322:1695–1699
63. Friedman JM et al (2009) The putative tumor suppressor microRNA-101 modulates the cancer epigenome by repressing the polycomb group protein EZH2. Cancer Res 69:2623–2629
64. Kaminskas E, Farrell AT, Wang YC, Sridhara R, Pazdur R (2005) FDA drug approval summary: azacitidine (5-azacytidine, Vidaza) for injectable suspension. Oncologist 10:176–182
65. Liu S et al (2010) Sp1/NFkappaB/HDAC/miR-29b regulatory network in KIT-driven myeloid leukemia. Cancer Cell 17:333–347
66. Shin S et al (2009) MicroRNAs that respond to histone deacetylase inhibitor SAHA and p53 in HCT116 human colon carcinoma cells. Int J Oncol 35:1343–1352
67. Kretzner L et al (2011) Combining histone deacetylase inhibitor vorinostat with aurora kinase inhibitors enhances lymphoma cell killing with repression of c-Myc, hTERT, and microRNA levels. Cancer Res 71:3912–3920
68. Lee S et al (2011) Histone deacetylase regulates high mobility group A2-targeting microRNAs in human cord blood-derived multipotent stem cell aging. Cell Mol Life Sci 68:325–336

Chapter 7
DNA Hypomethylation and Activation of Germline-Specific Genes in Cancer

Charles De Smet and Axelle Loriot

Abstract DNA methylation, occurring at cytosines in CpG dinucleotides, is a potent mechanism of transcriptional repression. Proper genomic methylation patterns become profoundly altered in cancer cells: both gains (hypermethylation) and losses (hypomethylation) of methylated sites are observed. Although DNA hypomethylation is detected in a vast majority of human tumors and affects many genomic regions, its role in tumor biology remains elusive. Surprisingly, DNA hypomethylation in cancer was found to cause the aberrant activation of only a limited group of genes. Most of these are normally expressed exclusively in germline cells and were grouped under the term "cancer-germline" (CG) genes. CG genes represent unique examples of genes that rely primarily on DNA methylation for their tissue-specific expression. They are also being exploited to uncover the mechanisms that lead to DNA hypomethylation in tumors. Moreover, as CG genes encode tumor-specific antigens, their activation in cancer highlights a direct link between epigenetic alterations and tumor immunity. As a result, clinical trials combining epigenetic drugs with anti-CG antigen vaccines are being considered.

7.1 Introduction

Although DNA hypomethylation was the first epigenetic alteration to be described in human cancers, its effect on gene expression programs and tumor biology has remained enigmatic. Initial examination of cancer genomes identified most losses of DNA methylation in repeated elements [29]. This is not surprising, since these DNA elements are highly abundant and comprise most of the CpG sites that are normally methylated in healthy somatic tissues. A crucial question was whether

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DNA hypomethylation also affected protein-encoding genes, leading to their aberrant expression in tumor cells. It appeared, however, that genome hypomethylation in tumors is not generally associated with the ectopic activation of a multitude of genes [5]. A plausible explanation for this is that most tissue-specific genes use other regulatory mechanisms, including histone modifications, and that DNA methylation, if present, serves merely as secondary layer of repression. Losses of DNA methylation within such genes would therefore not be sufficient to trigger transcriptional activation.

Later work, aiming at isolating genes that code for tumor-specific antigens, led to the identification of a particular group of genes, which are normally expressed exclusively in germline cells but become aberrantly activated in a wide variety of tumors [86]. Given this expression profile, these genes were termed "cancer-germline" (CG) genes. Interestingly, CG genes were found to rely primarily on DNA methylation for repression in normal somatic tissues, and their activation in tumors was shown to be a direct consequence of genome hypomethylation [22]. These observations highlighted an unexpected link between epigenetic alterations in tumors and cancer immunity. They also provided clear examples of genes that owe their tissue-specific expression to DNA methylation. Moreover, CG genes are being exploited to try to uncover the molecular mechanisms underlying genome hypomethylation in tumors, as this epigenetic process remains largely unexplained.

7.2 Characterization of CG Genes

Human tumors express specific antigens, as evidenced by the existence in the blood of cancer patients of cytolytic T lymphocytes (CTL) that recognize antigens present on their tumor cells but not on normal cells [10]. Using a gene library transfection approach and a CTL clone isolated from a melanoma patient, Boon and colleagues identified the first human tumor antigen-encoding gene [85]. The gene was named melanoma antigen 1 or *MAGE-1* (later renamed *MAGEA1*). *MAGEA1* expression was not found in normal tissues except for testis, but was instead detected in a significant fraction of melanoma samples, as well as in various other tumor types [20, 23]. The same genetic approach led to the identification of other melanoma antigen genes, namely *BAGE*, *GAGE*, and *MAGEA3*, a gene closely related to *MAGEA1* [9, 34, 84]. For these genes too, expression among normal tissues was restricted to testis, and activation in tumors was detected among various cancer types. Additional tumor antigen genes were subsequently identified, using an alternative cloning approach, called SEREX (serological analysis of recombinant tumor cDNA expression libraries), and based on the presence of high titers of antitumor IgGs in the blood of tumor-bearing patients [73]. Again, several of the identified genes, including *SSX2* and *NY-ESO-1*, had their normal expression restricted to testis and were activated in a percentage of different tumor types. Later studies indicated that the normal expression of most isolated genes was confined to the germ cells in both testis and fetal ovary [44, 52, 82].

Together, these findings led to the important notion that specific antigens in tumors arise from the aberrant activation of genes that are normally transcribed exclusively in the germline. From an immunological point of view, this dual expression pattern is understandable. Unlike most somatic cells, germ cells lack MHC class I molecules, which are required to present antigenic peptides at the cell surface [37]. Activation of germline-specific genes in tumor cells therefore leads to the expression of truly tumor-specific antigens, which can be recognized as nonself by the immune system.

Further studies using cDNA subtraction procedures or database mining have permitted the identification of additional genes expressed in germ cells and cancer but not in normal somatic tissues [56, 60, 63, 75]. Some genes identified in this way were subsequently shown to encode tumor-specific antigens recognized by CTLs [86]. Altogether about 50 human genes or gene families were identified, which displayed specific expression in the germline and activation in a significant proportion of cancers [2]. These genes appear to exert a variety of cellular functions, but on the basis of their common expression pattern they were grouped under the term cancer-germline (CG) genes. CG genes are dispersed on several chromosomes, with a marked preference for the X chromosome. In human cancers, CG genes are expressed more frequently in specific tumor types, like for instance lung cancer, head and neck cancer, bladder cancer, and melanoma [76]. Other tumor types like colon cancer, renal cancer, and leukemia only rarely show activation of CG genes. An important feature of CG genes is their frequent co-activation in tumors [74]. It was observed indeed that positive tumors often express several CG genes. Clearly, the widespread and concerted expression of CG genes in tumors indicates that their activation in cancer results from a global gene activation process, rather than stochastic individual events.

7.3 DNA Demethylation in the Activation of CG Genes in Tumors

The marked tendency of CG genes to become co-expressed in tumors suggested that these genes share, at least in part, a common mechanism of transcriptional activation. Initial studies were performed with the *MAGEA1* gene in order to identify essential promoter elements and corresponding transcription factors that may contribute to the cell-type-specific expression of the gene. Surprisingly, however, transfection experiments revealed that all cells, including those that do not express *MAGEA1*, contain transcription factors capable of inducing significant *MAGEA1* promoter activity [24]. Transfection experiments with other CG gene promoter constructs led to similar results [17, 89]. This implied that nonexpressing cells have a repression mechanism, probably operating at the chromatin level that protects CG gene promoters against spurious activation.

The initial observation by Weber and colleagues that *MAGEA1* could be induced in nonexpressing melanoma cell lines following treatment with the DNA

methylation inhibitor 5-aza-2′-deoxycytidine provided a first hint that DNA methylation may contribute to the transcriptional regulation of this gene [91]. This was confirmed by studies showing that the promoter of *MAGEA1* is invariably methylated in all normal somatic tissues and instead unmethylated in germ cells [26]. Likewise, activation of the *MAGEA1* gene in tumors was strictly correlated with demethylation of its promoter [26]. Further studies showed that DNA methylation was similarly involved in the regulation of other CG genes [17, 26, 52, 56, 89]. Altogether, these observations indicated that CG genes rely on DNA methylation for repression in somatic tissues, and that aberrant activation of these genes in tumors results from demethylation of their promoter.

Interestingly, demethylation and activation of CG genes in tumors was found to correlate with global genome hypomethylation [14, 25, 45]. This association was further confirmed by a study on microdissected tumor samples, revealing that intratumor heterogeneity of CG gene expression also correlates with global genome hypomethylation levels [96]. These observations provided therefore the first clear evidence that the process of genome-wide demethylation, common to many cancers, not only affects repeated sequences but also single copy genes, and can lead to aberrant gene activation. The frequent co-activation of CG genes in tumors likely reflects the global process of DNA demethylation, which can simultaneously affect many loci across the cancer genome.

7.4 DNA Methylation in the Regulation of Germline Genes

Considering the potent effect of DNA methylation on transcriptional repression, it was originally proposed that this DNA modification might serve as a general mechanism to control the programmed expression of tissue-specific genes [39, 72]. Evidence, however, indicates that most tissue-specific genes rely on mechanisms other than DNA methylation for repression in nonexpressing cells [8, 88]. This may be ascribed to the distribution of CpG sequences, where cytosine methylation can occur. Vertebrate genomes show a general depletion of CpG dinucleotides, which was attributed to the high mutability of methylated cytosines, and hence the progressive disappearance of this sequence during evolution [7]. Discrete genomic regions however, which appear generally free of CpG methylation, maintained a high density of CpG sites. These so-called CpG islands often overlap gene promoters [19]. Many tissue-specific genes contain a methylation-free CpG island within their promoter and can therefore not rely on DNA methylation for repression in nonexpressing tissues. On the other hand, genes with few CpG sites within their promoter are only little affected by DNA methylation, and often show an inconstant relationship between promoter methylation and transcriptional silencing [12]. It was therefore proposed that DNA methylation in vertebrates is solely involved in the control of retrotransposable elements, monoallelically expressed imprinted genes, and X chromosome inactivation, the only cases where consistent methylation of CpG-rich regions appeared to exist [101].

This view was challenged by the discovery of CG genes, which were found to be characterized by the presence of a high density of CpG sites within their promoter [26]. Yet, unlike classical CpG islands, CpG-rich promoters of CG genes are methylated in all normal somatic tissues. CG gene promoters appear therefore favorably disposed to DNA methylation-mediated regulation. Consistently, transfection experiments with in vitro methylated CG gene constructs indicated that DNA methylation was sufficient to repress transcription, even in cells that express the corresponding endogenous CG gene, and therefore obviously contain appropriate transcriptional activators [17, 26, 27, 78, 89]. This and the above-mentioned observation that unmethylated CG gene promoters are transcriptionally active in nonexpressing cells provided strong evidence that DNA methylation is an essential component of the repression of this group of germline-specific genes in somatic cells.

More recently, genome-wide studies were conducted in order to identify the distribution of differentially methylated CpG sites across the genome of distinct types of human cells [77, 93]. These studies revealed the existence of novel sets of genes with a CpG-rich promoter that was densely methylated in somatic tissues (in addition to the previously characterized CG genes). Remarkably, most of these genes were specifically demethylated and expressed in testis. It appears therefore that DNA methylation has a particular role in the regulation of germline-specific genes.

Why would DNA methylation be particularly suitable for the regulation of genes with specific expression in germline cells rather than in other cell types? A plausible explanation may be that methylation-dependent germline genes have the advantage of being little exposed to the evolutionary loss of methylated CpGs, because they are unmethylated precisely in the cells that transmit their genome to the offspring. As a result, such genes maintain a high density of CpG sites within their promoter and remain therefore fully responsive to DNA methylation.

7.5 Mechanisms Leading to Hypomethylation of CG Genes in Cancer

CG genes have served as model sequences to investigate the distribution and dynamics of methylation losses in tumor genomes. Detailed analysis of the *MAGEA1* locus revealed preferential hypomethylation of a restricted region surrounding the transcription start site of the gene in expressing tumor cells, suggesting that hypomethylated CpG sites are unevenly distributed across cancer genomes [27]. Consistently, recent genome-wide DNA methylation studies confirmed that DNA hypomethylation in tumors adopts mosaic patterns, with defined hypomethylated domains (between one kilobase and several megabases in size) surrounded by normally methylated regions [66, 71, 92]. These observations indicate that certain genomic regions, including CG promoters, are particularly susceptible to DNA hypomethylation in tumors.

The possibility that *MAGEA1*-expressing tumor cells possess a DNA demethylation activity targeted towards the 5′-region of the gene was investigated [27, 58].

Thus, a large genomic fragment comprising the *MAGEA1* gene was methylated in vitro and then stably transfected into several human tumor cell lines, where the endogenous *MAGEA1* gene is hypomethylated and active. The newly integrated *MAGEA1* transgenes did not undergo demethylation, indicating that the process that once led to demethylation of the endogenous *MAGEA1* gene was not preserved in these cells. Remarkably, when unmethylated *MAGEA1* constructs were introduced into such cells, de novo methylation of the transgenes occurred except in a region overlapping the *MAGEA1* promoter [27]. This mechanism of protection against de novo DNA methylation was lost when mutations that impair the *MAGEA1* promoter activity were introduced into the transgene, or when the transgene was transfected into tumor cells that induce only little *MAGEA1* promoter activity. Altogether, these data suggest that site-specific hypomethylation of *MAGEA1* in tumors results from a past event of transient DNA demethylation and is maintained locally by the presence of potent transcriptional activators that prevent remethylation.

In vivo studies, evaluating global genome methylation levels in colon and breast cancers, demonstrated that DNA hypomethylation is present in the early stages of the disease, and does not progress towards later stages, adding support the transient nature of the DNA demethylation process [30, 41]. Other studies, however, reported a higher prevalence of genome hypomethylation and an increased frequency of CG gene activation in more advanced tumor stages [53, 100]. This was interpreted as an indication that DNA demethylation might instead be a continuous process leading to progressive methylation losses with tumor development. Other interpretations for the increased hypomethylation in advanced tumor genomes, which implicate a transient DNA demethylation process, are however possible: (1) transient demethylation would initially produce a mixed population of precancerous cells with varying levels of DNA hypomethylation, and cells with the most hypomethylated genome would later be selected to contribute to the more advanced stages of the disease; or (2) the transient demethylation process could occur at varying time points during tumor progression and would therefore be more likely to have already occurred in late stage tumor samples [22]. Additional support for a transient DNA demethylation process comes from the observation that tumor cell lines with a hypomethylated genome do not show further CpG methylation losses during culturing [32, 55, 94]. Of note, many tumor cells display instead de novo methylation activities [3, 43].

Considering the suggested dynamics of DNA demethylation in tumors, it is reasonable to propose that hypomethylation of CG genes in tumors is mediated by two groups of factors: those that contribute to the transient DNA demethylation process and those that are required to protect the CG gene promoter region against subsequent remethylation.

7.5.1 Process of DNA Demethylation

Factors contributing to the DNA demethylation process during cancer development remain unknown. The apparent transient nature of this process suggests that activation of such demethylation-inducing factors might occur in association with

one (or several) of the multiple steps through which precancerous cells are progressing before acquiring full malignancy. Interestingly, a recent study evaluating genome methylation levels in an isogenic series of human mammary epithelial cell cultures transitioning from normal to malignantly transformed revealed that most losses of DNA methylation occurred at the stage of acquisition of indefinite lifespan [67]. Another study reported that genome hypomethylation and CG gene activation is more prevalent in tumors displaying the alternative telomere (ALT) maintenance phenotype rather than telomerase activation, the two possible mechanisms by which cancer cells stabilize their telomeres and acquire immortality [83]. These observations establish therefore a possible link between DNA demethylation and cellular immortalization. Underlying molecular mechanisms remain, however, to be identified.

Theoretically, DNA demethylation in tumor cells could possibly occur through two distinct processes commonly referred to as active demethylation and passive demethylation [16]. Active demethylation would involve the activation of demethylating enzymes, which can remove methylation marks from the DNA in a replication-independent manner. Enzymes contributing to active DNA demethylation in animal cells are beginning to be characterized [16], but their potential involvement in cancer genome demethylation has not yet been reported. Passive demethylation on the other hand, would rely on the inhibition of DNA methyltransferases, which normally preserve the DNA methylation marks through the successive replication cycles. Three DNA methyltransferases exist in mammals: DNMT1, DNMT3A, and DNMT3B [6]. DNMT1 is primarily involved in DNA methylation maintenance, as it appears to be specialized in copying preexisting methylation sites onto the newly synthesized strand during replication. DNMT3A and DNMT3B instead have de novo DNA methylation activity and are responsible for the establishment of new DNA methylation marks in the developing embryo. For CG genes in particular, studies based on targeted depletion of the distinct DNMTs indicate that DNMT1 is the principal enzyme for methylation maintenance [42, 57]. It is therefore likely that passive DNA demethylation of CG genes in tumors would necessarily involve factors that decrease the amount or impair proper functioning of DNMT1. In certain tumor cells, however, combined depletion of DNMT1 and DNMT3 enzymes was required to obtain efficient demethylation and activation of CG genes [42, 95]. This indicates that de novo methyltransferases can be targeted to these genes, where they might restore lost methylation sites, and underscores the importance of acquiring mechanisms of protection against remethylation for long-term activation.

7.5.2 Factors that Protect Against Remethylation

Studies with the *MAGEA1* promoter suggest that protection of the promoter against DNA remethylation is dependent on the level of transcriptional activation [27]. It is therefore likely that maintenance of CG gene promoter hypomethylation in tumor cells relies on the presence of appropriate transcription factors, as well as on the activation of such factors by upstream signaling pathways.

Several DNA-binding factors have been identified, which appear to induce activation of CG gene promoters. Transcriptional activation of several genes of the *MAGEA* family has been shown to depend on the binding of ETS transcription factors within their promoter [21, 24]. Interestingly, ETS-binding sequences in *MAGEA* promoters contain a CpG site, and it was shown that methylation of this site inhibits binding of the corresponding factor [25]. In the promoter of *MAGEA1*, two ETS-binding sites were shown to be essential to maintain hypomethylation of the promoter in expressing tumor cells, as evidenced by remethylation of transfected *MAGEA1* constructs containing mutations within these two essential promoter elements [27]. The ETS family of transcription factors comprises about 30 members in humans, which all bind a similar DNA motif with a central GGAA/T sequence [68]. The precise member(s) involved in the regulation of *MAGEA* genes remain(s) to be characterized.

SP1 is another transcription factor, which was shown to contribute to the activation of several *MAGEA* genes, as well as the *CTAG1* gene (also termed *NY-ESO-1*) [24, 46]. The ubiquitously expressed SP1 factor acts as a transcriptional activator and recognizes a consensus DNA sequence (GC box element), which includes a CpG site [80]. SP1-binding elements are therefore often present in CG-rich promoter sequences. Binding of SP1 to the *CTAG1* gene was shown to occur only in cells where the promoter is unmethylated [46]. Interestingly, SP1-binding elements were previously shown to be involved in preserving the methylation-free status of classical CpG-island promoters [13, 62]. It is therefore likely that, once bound to the demethylated promoter of CG genes, SP1 proteins contribute to protect the region against remethylation.

BORIS (also known as CTCFL) is a testis-specific paralog of the ubiquitously expressed DNA-binding protein CTCF, which is involved in various aspects of epigenetic regulation, including gene imprinting and X chromosome inactivation [59]. Both proteins share a highly similar central DNA-binding domain, and recognize therefore overlapping DNA sequences, but contain divergent amino- and carboxy-terminal domains. The gene-encoding BORIS belongs to the CG group of genes, as its expression is regulated by DNA methylation and becomes activated in a wide variety of tumors [38, 49, 87, 95]. Remarkably, it has been demonstrated that in expressing tumors cells, BORIS is targeted to the promoters of other CG genes, namely *MAGEA1* and *CTAG1*, where its recruitment coincides with loss of CTCF binding [40, 87]. BORIS exerts transcriptional activation of CG genes, possibly in cooperation with SP1 transcription factors [46, 87]. In one study, forced overexpression of BORIS led to demethylation (albeit only partially) and activation of various CG genes in normal human fibroblasts, suggesting that BORIS activation in tumors might represent a primary triggering event for the epigenetic de-repression of other CG genes [87]. However, similar experiments from other groups did not confirm CG gene demethylation and activation resulting from BORIS overexpression [49, 97]. Moreover, it was found that many tumors display activation of various CG genes in the absence of BORIS expression. It is therefore unlikely that BORIS is a necessary factor for the derepression of other CG genes in tumors. Its presence in certain tumor cells may, however, facilitate maintenance of the hypomethylated and active state of CG gene promoters.

Many more transcription factors involved in CG gene regulation remain to be identified, and it is likely that each particular CG gene is controlled by a distinct combination of transcription factors. Tissue-specific differences in the content of transcription factors probably account for the fact that, while CG genes tend to be co-activated in hypomethylated tumors, some of them nevertheless show preferential activation in specific tumor types [36, 56].

Cell signaling through tyrosine kinase receptors appears to represent an additional level of control of CG gene regulation. A study in mast cell lines reported that signaling through KIT, an oncogenic receptor hyper-activated in several types of cancers, increases transcription of *MAGE* genes [99]. Other studies revealed that signaling through FGFR2, an FGF receptor often down-regulated in thyroid and pituitary cancers, exerts a negative effect on *MAGEA3* and *MAGEA6* transcription [51, 102]. It is therefore possible that particular dysregulations in cancers, such as those affecting cell signaling pathways, increase the activity of transcription factors that target CG genes, and thereby facilitate long-term activation of these genes in hypomethylated tumor cells. This may partially explain the observation that experimental DNA demethylation, by the use of DNMT inhibitors, often induces CG gene activation more efficiently in tumor cells than in normal cells [47].

7.5.3 Histone Modifications

Active CG gene promoters in tumors usually display a hypomethylated region that comprises one to several kilobases [27]. It is therefore likely that the protective influence of transcription factors against DNA remethylation extends beyond their narrow-binding site. Consistently, impaired binding of ETS transcription factors to *MAGEA1* transgenes, as caused by mutations in their recognition sites, resulted in de novo methylation of CpG sites within the entire promoter region, not just those located nearby the mutated ETS-binding sites [27]. This regional, rather than site-specific effect, might be related to the presence of modifications on the chromatin, such as histone modifications, which after being initiated by specific transcription factors often propagate themselves over larger domains [31]. Histone modifications can indeed influence DNA methylation states [15]. Repressive histone marks, such as methylation of lysine 9 and 27 of histone H3 (H3K9 and H3K27), favor local DNA methylation, whereas active marks, such as histone acetylation or methylation of lysine 4 of histone H3 (H3K4), appear to exclude the DNA methylation machinery. Studies from several groups have shown that demethylation and activation of CG genes in tumor cells is always associated with gains in histone acetylation and H3K4 methylation [42, 70]. The repressed state of human CG genes instead has been associated to a certain extent with the presence of H3K27 and H3K9 methylation marks [42, 70]. The exact relationship between histone modifications changes and DNA demethylation in CG gene promoters remains unclear. A crucial question is whether the varying histone modifications in CG gene promoters are a cause or a consequence of DNA methylation alterations. Studies using inhibitors of histone-modifying enzymes showed that these were on their own unable to induce significant

demethylation and activation of CG genes. Only in combination with inhibitors of DNA methylation, did they significantly modulate the level of activation of CG genes [35, 54, 70]. These observations support the notion that DNA methylation exerts a dominant role in the epigenetic repression of CG genes. But it remains possible that histone modifications assume the responsibility of maintaining the active status of the promoter following its demethylation.

7.5.4 Multiple Factors Determining CG Gene Activation in Tumors

Considering the above, it appears that activation of a particular CG gene in a tumor cell will depend on several factors: (1) the extent of CpG methylation losses resulting from the transient DNA demethylation process; (2) the level of de novo DNA methylation activities in the cell, which might induce remethylation of the promoter; (3) the presence of transcriptional activators and histone-modifying enzymes capable of counteracting remethylation activities. The likelihood that a CG gene becomes activated in a tumor cell probably depends on a complex balance between these different factors (Fig. 7.1).

7.6 Oncogenic Function of CG Genes

Activation of CG genes in tumor cells raises the possibility that their proteins might have oncogenic activities. The biological function of most of these genes, which encode very diverse proteins, remains however poorly understood. One extreme possibility is that the main contribution of DNA hypomethylation to tumor progression resides in its repercussions on genomic instability [33], and that the accompanying activation of CG genes is merely a side effect with no impact on malignancy (other than inducing the expression of tumor antigens). Another possibility has been proposed, in which the concerted expression of CG genes in cancer would correspond to the activation of a gametogenic program, thereby bestowing tumor cells with germ cell properties, including the capacity to self-renew (a feature of spermatogonial stem cells) and increased motility (a feature of sperm cells) [79]. Activation of CG genes in tumors is however only partial, making it very unlikely that all genes necessary for inducing a gametogenic program become expressed at the same time. Nevertheless, it remains possible that some CG genes contribute to tumor progression. Several MAGE proteins were found to inhibit p53 transactivation function, thereby exerting antiapoptotic properties [28, 64, 98]. GAGE proteins were also shown to render cells resistant to apoptosis [18]. Other studies reported that MAGEA11 serves as a co-stimulator for the androgen receptor and might therefore contribute to the development of prostate tumors that have become independent of the presence of androgen for their growth [4, 48]. Moreover, it was noted that

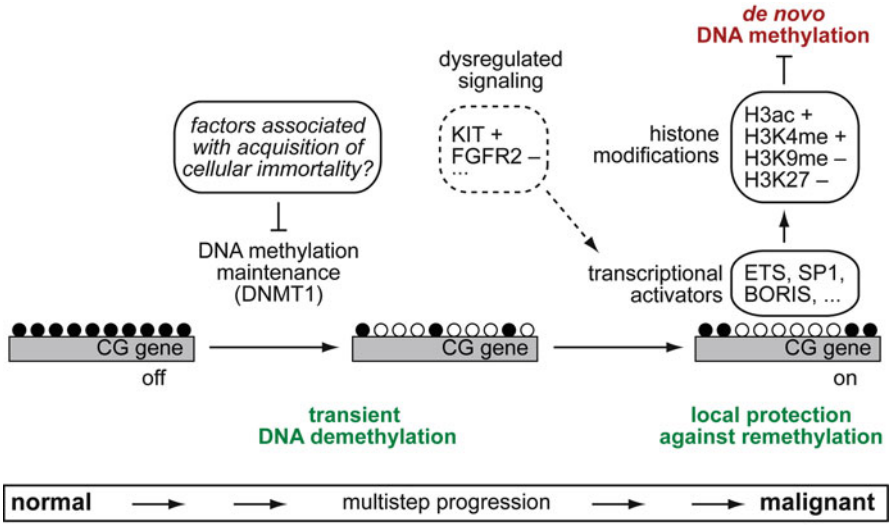

Fig. 7.1 Proposed model of demethylation and activation of CG genes during tumor development. The activation of CG genes in tumors depends on several factors: the extent of the transient DNA demethylation process, occurring at some step of tumor development; the level of counteracting de novo methylation activities in the cell; and the presence of transcriptional activators that protect the CG gene promoter against remethylation, for instance by increasing (+) or decreasing (–) distinct histone marks locally. *Filled circles* represent methylated CpG, *empty circles* unmethylated cytosines

several CG genes, including *BORIS*, *BRDT,* and *ATAD2*, encode nuclear proteins that have a potential impact on chromatin structures and might therefore be involved in the epigenetic alterations commonly affecting cancer genomes [90]. Altogether, these observations support the notion that the activation of several CG genes in tumors, resulting from DNA demethylation, might be associated with the acquisition of oncogenic properties.

Surprisingly, however, two independent studies indicate that *MAGEA4* displays instead tumor-suppressor functions. In one study, MAGEA4 was shown to interact with gankyrin and to inhibit anchorage-independent growth in vitro and tumor formation in mice [65]. In the other study, MAGEA4 was found to promote tumor cell death and to increase their sensitivity to apoptotic stimuli [69]. Clearly, more studies will be required before we can evaluate the full spectrum of consequences of CG gene activation in tumors.

7.7 DNA Hypomethylation in Cancer: An Immunological Paradox

There is now compelling evidence that the immune system is able to identify and destroy tumor cells [81]. This immune surveillance of cancer is believed to provide a barrier to cancer development, even though progressing tumors eventually escape

this obstacle by activating a variety of immune evasion strategies. Evidence for the existence of such surveillance of cancer by the immune system is provided for instance by the observation that solid tumors are often infiltrated by lymphocytes. Not surprisingly, several of these tumor-infiltrating lymphocytes were shown to be directed against antigens encoded by CG genes [50]. This suggests therefore that DNA hypomethylation and the consequent activation of CG genes has, at least at some stage of oncogenesis, a detrimental effect on tumor development. Yet, DNA hypomethylation is observed in most tumors, suggesting that it must otherwise have a strong tumor-promoting effect that outweighs this negative immunogenic effect.

7.8 Epigenetically Assisted Cancer Immunotherapy

Clinical trials of therapeutic vaccination of cancer patients using antigens encoded by CG genes are underway. Noticeable clinical responses were observed, albeit in only a fraction of the treated patients [11]. An interesting possibility to increase vaccination efficiencies would be the use of epigenetic drugs, such as the DNA methylation inhibitor decitabine, which should increase the number of expressed CG genes in the tumors, thereby rendering them more visible to the immune system. Importantly, decitabine is expected to induce reactivation of epigenetically silenced tumor-suppressor genes as well, and hence to reduce the growth rate of the tumors at the same time. Clinical trials combining decitabine and vaccination against antigens encoded by CG gene have been initiated [1].

There are, however, several points concerning the efficiency and safety of such approaches, which remain to be addressed. The first point concerns the specificity of decitabine-induced expression of CG genes in tumor cells rather than normal cells. Although studies have found that tumor cells are more sensitive to decitabine [47], it is obvious that the drug also induces CG genes in normal cell cultures, including fibroblasts and blood lymphocytes [25, 56, 61]. It will therefore be crucial to monitor decitabine/vaccine-treated patients for potential autoimmune reactions directed against their healthy tissues. Another concern relates to the duration of CG gene expression following decitabine treatment. Several studies have shown that CG gene expression in tumor cells was only transient following exposure to decitabine [26, 91]. This may be related to the absence of appropriate transcription factors, and hence lack of protection of the promoters against remethylation. The duration of CG gene expression in tumor cells may be critical to allow complete rejection by the immune cells. In this particular immune context, tumor cells that lose CG gene expression might be strongly selected. Prolonged decitabine treatment or combination with another epigenetic drug favoring protection of CG promoters against remethylation (e.g., drugs affecting histone marks) might be a solution to the problem. Finally, as genome hypomethylation is obviously associated with tumor development, there is a concern that decitabine treatment may generate strongly hypomethylated tumor cells with increased malignancy [33]. This is particularly problematic if it is confirmed that CG genes themselves exert oncogenic functions.

Clearly, a better understanding of the mechanisms of activation and of the biological functions of CG genes should help to resolve these questions, and may help to design the most efficient and safest ways to epigenetically augment tumor immunogenicity, thereby rendering cancer cells more vulnerable to vaccination.

References

1. Akers SN, Odunsi K, Karpf AR (2010) Regulation of cancer germline antigen gene expression: implications for cancer immunotherapy. Future Oncol 6(5):717–732
2. Almeida LG, Sakabe NJ, deOliveira AR, Silva MC, Mundstein AS, Cohen T, Chen YT, Chua R, Gurung S, Gnjatic S, Jungbluth AA, Caballero OL, Bairoch A, Kiesler E, White SL, Simpson AJ, Old LJ, Camargo AA, Vasconcelos AT (2009) CTdatabase: a knowledge-base of high-throughput and curated data on cancer-testis antigens. Nucleic Acids Res 37(Database issue):D816–819
3. Antequera F, Boyes J, Bird A (1990) High levels of de novo methylation and altered chromatin structure at CpG islands in cell lines. Cell 62(3):503–514
4. Bai S, He B, Wilson EM (2005) Melanoma antigen gene protein MAGE-11 regulates androgen receptor function by modulating the interdomain interaction. Mol Cell Biol 25(4):1238–1257
5. Baylin SB, Herman JG, Graff JR, Vertino PM, Issa JP (1998) Alterations in DNA methylation: a fundamental aspect of neoplasia. Adv Cancer Res 72:141–196
6. Bestor TH (2000) The DNA methyltransferases of mammals. Hum Mol Genet 9(16):2395–2402
7. Bird AP (1980) DNA methylation and the frequency of CpG in animal DNA. Nucleic Acids Res 8:1499–1504
8. Bird A (2002) DNA methylation patterns and epigenetic memory. Genes Dev 16(1):6–21
9. Boël P, Wildmann C, Sensi M-L, Brasseur R, Renauld J-C, Coulie P, Boon T, van der Bruggen P (1995) BAGE, a new gene encoding an antigen recognized on human melanomas by cytolytic T lymphocytes. Immunity 2:167–175
10. Boon T, Cerottini J-C, Van den Eynde B, van der Bruggen P, Van Pel A (1994) Tumor antigens recognized by T lymphocytes. Annu Rev Immunol 12:337–365
11. Boon T, Coulie PG, Van den Eynde BJ, van der Bruggen P (2006) Human T cell responses against melanoma. Annu Rev Immunol 24:175–208
12. Boyes J, Bird A (1992) Repression of genes by DNA methylation depends on CpG density and promoter strength: evidence for involvement of a methyl-CpG binding protein. EMBO J 11(1):327–333
13. Brandeis M, Frank D, Keshet I, Siegfried Z, Mendelsohn M, Nemes A, Temper V, Razin A, Cedar H (1994) Sp1 elements protect a CpG island from de novo methylation. Nature 371(6496):435–438
14. Cadieux B, Ching TT, VandenBerg SR, Costello JF (2006) Genome-wide hypomethylation in human glioblastomas associated with specific copy number alteration, methylenetetrahydrofolate reductase allele status, and increased proliferation. Cancer Res 66(17):8469–8476
15. Cedar H, Bergman Y (2009) Linking DNA methylation and histone modification: patterns and paradigms. Nat Rev Genet 10(5):295–304
16. Chen ZX, Riggs AD (2011) DNA methylation and demethylation in mammals. J Biol Chem 286(21):18347–18353
17. Cho B, Lee H, Jeong S, Bang YJ, Lee HJ, Hwang KS, Kim HY, Lee YS, Kang GH, Jeoung DI (2003) Promoter hypomethylation of a novel cancer/testis antigen gene CAGE is correlated with its aberrant expression and is seen in premalignant stage of gastric carcinoma. Biochem Biophys Res Commun 307(1):52–63

18. Cilensek ZM, Yehiely F, Kular RK, Deiss LP (2002) A member of the GAGE family of tumor antigens is an anti-apoptotic gene that confers resistance to Fas/CD95/APO-1, Interferon-gamma, taxol and gamma-irradiation. Cancer Biol Ther 1(4):380–387
19. Cross SH, Bird AP (1995) CpG islands and genes. Curr Opin Genet Dev 5:309–314
20. De Plaen E, Arden K, Traversari C, Gaforio JJ, Szikora J-P, De Smet C, Brasseur F, van der Bruggen P, Lethé B, Lurquin C, Brasseur R, Chomez P, De Backer O, Cavenee W, Boon T (1994) Structure, chromosomal localization and expression of twelve genes of the MAGE family. Immunogenetics 40:360–369
21. De Plaen E, Naerhuyzen B, De Smet C, Szikora J-P, Boon T (1997) Alternative promoters of gene MAGE4a. Genomics 40:305–313
22. De Smet C, Loriot A (2010) DNA hypomethylation in cancer: epigenetic scars of a neoplastic journey. Epigenetics 5(3):206–213
23. De Smet C, Lurquin C, van der Bruggen P, De Plaen E, Brasseur F, Boon T (1994) Sequence and expression pattern of the human MAGE2 gene. Immunogenetics 39:121–129
24. De Smet C, Courtois SJ, Faraoni I, Lurquin C, Szikora JP, De Backer O, Boon T (1995) Involvement of two Ets binding sites in the transcriptional activation of the MAGE1 gene. Immunogenetics 42(4):282–290
25. De Smet C, De Backer O, Faraoni I, Lurquin C, Brasseur F, Boon T (1996) The activation of human gene MAGE-1 in tumor cells is correlated with genome-wide demethylation. Proc Natl Acad Sci USA 93(14):7149–7153
26. De Smet C, Lurquin C, Lethé B, Martelange V, Boon T (1999) DNA methylation is the primary silencing mechanism for a set of germ line- and tumor-specific genes with a CpG-rich promoter. Mol Cell Biol 19:7327–7335
27. De Smet C, Loriot A, Boon T (2004) Promoter-dependent mechanism leading to selective hypomethylation within the 5′ region of gene MAGE-A1 in tumor cells. Mol Cell Biol 24(11):4781–4790
28. Doyle JM, Gao J, Wang J, Yang M, Potts PR (2010) MAGE-RING protein complexes comprise a family of E3 ubiquitin ligases. Mol Cell 39(6):963–974
29. Ehrlich M (2002) DNA methylation in cancer: too much, but also too little. Oncogene 21(35):5400–5413
30. Feinberg AP, Gehrke CW, Kuo KC, Ehrlich M (1988) Reduced genomic 5-methylcytosine content in human colonic neoplasia. Cancer Res 48(5):1159–1161
31. Felsenfeld G, Groudine M (2003) Controlling the double helix. Nature 421(6921):448–453
32. Flatau E, Gonzales FA, Michalowsky LA, Jones PA (1984) DNA methylation in 5-aza-2′-deoxycytidine-resistant variants of C3H 10T1/2C18 cells. Mol Cell Biol 4(10):2098–2102
33. Gaudet F, Hodgson JG, Eden A, Jackson-Grusby L, Dausman J, Gray JW, Leonhardt H, Jaenisch R (2003) Induction of tumors in mice by genomic hypomethylation. Science 300(5618):489–492
34. Gaugler B, Van den Eynde B, van der Bruggen P, Romero P, Gaforio JJ, De Plaen E, Lethé B, Brasseur F, Boon T (1994) Human gene MAGE-3 codes for an antigen recognized on a melanoma by autologous cytolytic T lymphocytes. J Exp Med 179:921–930
35. Goodyear O, Agathanggelou A, Novitzky-Basso I, Siddique S, McSkeane T, Ryan G, Vyas P, Cavenagh J, Stankovic T, Moss P, Craddock C (2010) Induction of a CD8+ T-cell response to the MAGE cancer testis antigen by combined treatment with azacitidine and sodium valproate in patients with acute myeloid leukemia and myelodysplasia. Blood 116(11):1908–1918
36. Grunwald C, Koslowski M, Arsiray T, Dhaene K, Praet M, Victor A, Morresi-Hauf A, Lindner M, Passlick B, Lehr HA, Schafer SC, Seitz G, Huber C, Sahin U, Tureci O (2006) Expression of multiple epigenetically regulated cancer/germline genes in nonsmall cell lung cancer. Int J Cancer 118(10):2522–2528
37. Haas GG Jr, D'Cruz OJ, De Bault LE (1988) Distribution of human leukocyte antigen-ABC and -D/DR antigens in the unfixed human testis. Am J Reprod Immunol Microbiol 18(2):47–51
38. Hoffmann MJ, Muller M, Engers R, Schulz WA (2006) Epigenetic control of CTCFL/BORIS and OCT4 expression in urogenital malignancies. Biochem Pharmacol 72(11):1577–1588

39. Holliday R, Pugh JE (1975) DNA modification mechanisms and gene activity during development. Science 186:226–232
40. Hong JA, Kang Y, Abdullaev Z, Flanagan PT, Pack SD, Fischette MR, Adnani MT, Loukinov DI, Vatolin S, Risinger JI, Custer M, Chen GA, Zhao M, Nguyen DM, Barrett JC, Lobanenkov VV, Schrump DS (2005) Reciprocal binding of CTCF and BORIS to the NY-ESO-1 promoter coincides with derepression of this cancer-testis gene in lung cancer cells. Cancer Res 65(17):7763–7774
41. Jackson K, Yu MC, Arakawa K, Fiala E, Youn B, Fiegl H, Muller-Holzner E, Widschwendter M, Ehrlich M (2004) DNA hypomethylation is prevalent even in low-grade breast cancers. Cancer Biol Ther 3(12):1225–1231
42. James SR, Link PA, Karpf AR (2006) Epigenetic regulation of X-linked cancer/germline antigen genes by DNMT1 and DNMT3b. Oncogene 25(52):6975–6985
43. Jones PA, Wolkowicz MJ, Rideout WM III, Gonzales FA, Marziasz CM, Coetzee GA, Tapscott SJ (1990) De novo methylation of the MyoD1 CpG island during the establishment of immortal cell lines. Proc Natl Acad Sci USA 87(16):6117–6121
44. Jungbluth AA, Chen YT, Stockert E, Busam KJ, Kolb D, Iversen K, Coplan K, Williamson B, Altorki N, Old LJ (2001) Immunohistochemical analysis of NY-ESO-1 antigen expression in normal and malignant human tissues. Int J Cancer 92(6):856–860
45. Kaneda A, Tsukamoto T, Takamura-Enya T, Watanabe N, Kaminishi M, Sugimura T, Tatematsu M, Ushijima T (2004) Frequent hypomethylation in multiple promoter CpG islands is associated with global hypomethylation, but not with frequent promoter hypermethylation. Cancer Sci 95(1):58–64
46. Kang Y, Hong JA, Chen GA, Nguyen DM, Schrump DS (2007) Dynamic transcriptional regulatory complexes including BORIS, CTCF and Sp1 modulate NY-ESO-1 expression in lung cancer cells. Oncogene 26(30):4394–4403
47. Karpf AR, Lasek AW, Ririe TO, Hanks AN, Grossman D, Jones DA (2004) Limited gene activation in tumor and normal epithelial cells treated with the DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine. Mol Pharmacol 65(1):18–27
48. Karpf AR, Bai S, James SR, Mohler JL, Wilson EM (2009) Increased expression of androgen receptor coregulator MAGE-11 in prostate cancer by DNA hypomethylation and cyclic AMP. Mol Cancer Res 7(4):523–535
49. Kholmanskikh O, Loriot A, Brasseur F, De Plaen E, De Smet C (2008) Expression of BORIS in melanoma: lack of association with MAGE-A1 activation. Int J Cancer 122(4):777–784
50. Khong HT, Wang QJ, Rosenberg SA (2004) Identification of multiple antigens recognized by tumor-infiltrating lymphocytes from a single patient: tumor escape by antigen loss and loss of MHC expression. J Immunother 27(3):184–190
51. Kondo T, Zhu X, Asa SL, Ezzat S (2007) The cancer/testis antigen melanoma-associated antigen-A3/A6 is a novel target of fibroblast growth factor receptor 2-IIIb through histone H3 modifications in thyroid cancer. Clin Cancer Res 13(16):4713–4720
52. Koslowski M, Bell C, Seitz G, Lehr HA, Roemer K, Muntefering H, Huber C, Sahin U, Tureci O (2004) Frequent nonrandom activation of germ-line genes in human cancer. Cancer Res 64(17):5988–5993
53. Lin CH, Hsieh SY, Sheen IS, Lee WC, Chen TC, Shyu WC, Liaw YF (2001) Genome-wide hypomethylation in hepatocellular carcinogenesis. Cancer Res 61(10):4238–4243
54. Link PA, Gangisetty O, James SR, Woloszynska-Read A, Tachibana M, Shinkai Y, Karpf AR (2009) Distinct roles for histone methyltransferases G9a and GLP in cancer germ-line antigen gene regulation in human cancer cells and murine embryonic stem cells. Mol Cancer Res 7(6):851–862
55. Lorincz MC, Schubeler D, Goeke SC, Walters M, Groudine M, Martin DI (2000) Dynamic analysis of proviral induction and de novo methylation: implications for a histone deacetylase-independent, methylation density-dependent mechanism of transcriptional repression. Mol Cell Biol 20(3):842–850
56. Loriot A, Boon T, De Smet C (2003) Five new human cancer-germline genes identified among 12 genes expressed in spermatogonia. Int J Cancer 105(3):371–376

57. Loriot A, De Plaen E, Boon T, De Smet C (2006) Transient down-regulation of DNMT1 methyltransferase leads to activation and stable hypomethylation of MAGE-A1 in melanoma cells. J Biol Chem 281(15):10118–10126
58. Loriot A, Sterpin C, De Backer O, De Smet C (2008) Mouse embryonic stem cells induce targeted DNA demethylation within human MAGE-A1 transgenes. Epigenetics 3(1):38–42
59. Loukinov DI, Pugacheva E, Vatolin S, Pack SD, Moon H, Chernukhin I, Mannan P, Larsson E, Kanduri C, Vostrov AA, Cui H, Niemitz EL, Rasko JE, Docquier FM, Kistler M, Breen JJ, Zhuang Z, Quitschke WW, Renkawitz R, Klenova EM, Feinberg AP, Ohlsson R, Morse HC III, Lobanenkov VV (2002) BORIS, a novel male germ-line-specific protein associated with epigenetic reprogramming events, shares the same 11-zinc-finger domain with CTCF, the insulator protein involved in reading imprinting marks in the soma. Proc Natl Acad Sci USA 99(10):6806–6811
60. Lucas S, De Smet C, Arden KC, Viars CS, Lethe B, Lurquin C, Boon T (1998) Identification of a new MAGE gene with tumor-specific expression by representational difference analysis. Cancer Res 58(4):743–752
61. Lurquin C, De Smet C, Brasseur F, Muscatelli F, Martelange V, De Plaen E, Brasseur R, Monaco AP, Boon T (1997) Two members of the human MAGEB gene family located in Xp21.3 are expressed in tumors of various histological origins. Genomics 46(3):397–408
62. Macleod D, Charlton J, Mullins J, Bird AP (1994) Sp1 sites in the mouse aprt gene promoter are required to prevent methylation of the CpG island. Genes Dev 8(19):2282–2292
63. Martelange V, De Smet C, De Plaen E, Lurquin C, Boon T (2000) Identification on a human sarcoma of two new genes with tumor-specific expression. Cancer Res 60(14):3848–3855
64. Monte M, Simonatto M, Peche LY, Bublik DR, Gobessi S, Pierotti MA, Rodolfo M, Schneider C (2006) MAGE-A tumor antigens target p53 transactivation function through histone deacetylase recruitment and confer resistance to chemotherapeutic agents. Proc Natl Acad Sci USA 103(30):11160–11165
65. Nagao T, Higashitsuji H, Nonoguchi K, Sakurai T, Dawson S, Mayer RJ, Itoh K, Fujita J (2003) MAGE-A4 interacts with the liver oncoprotein gankyrin and suppresses its tumorigenic activity. J Biol Chem 278(12):10668–10674
66. Novak P, Jensen T, Oshiro MM, Watts GS, Kim CJ, Futscher BW (2008) Agglomerative epigenetic aberrations are a common event in human breast cancer. Cancer Res 68(20):8616–8625
67. Novak P, Jensen TJ, Garbe JC, Stampfer MR, Futscher BW (2009) Stepwise DNA methylation changes are linked to escape from defined proliferation barriers and mammary epithelial cell immortalization. Cancer Res 69(12):5251–5258
68. Oikawa T, Yamada T (2003) Molecular biology of the Ets family of transcription factors. Gene 303:11–34
69. Peikert T, Specks U, Farver C, Erzurum SC, Comhair SA (2006) Melanoma antigen A4 is expressed in non-small cell lung cancers and promotes apoptosis. Cancer Res 66(9): 4693–4700
70. Rao M, Chinnasamy N, Hong JA, Zhang Y, Zhang M, Xi S, Liu F, Marquez VE, Morgan RA, Schrump DS (2011) Inhibition of histone lysine methylation enhances cancer-testis antigen expression in lung cancer cells: implications for adoptive immunotherapy of cancer. Cancer Res 71(12):4192–4204
71. Rauch TA, Zhong X, Wu X, Wang M, Kernstine KH, Wang Z, Riggs AD, Pfeifer GP (2008) High-resolution mapping of DNA hypermethylation and hypomethylation in lung cancer. Proc Natl Acad Sci USA 105(1):252–257
72. Riggs AD (1989) DNA methylation and cell memory. Cell Biophys 15(1–2):1–13
73. Sahin U, Tureci O, Schmitt H, Cochlovius B, Johannes T, Schmits R, Stenner F, Luo G, Schobert I, Pfreundschuh M (1995) Human neoplasms elicit multiple specific immune responses in the autologous host. Proc Natl Acad Sci USA 92:11810–11813
74. Sahin U, Tureci O, Chen YT, Seitz G, Villena-Heinsen C, Old LJ, Pfreundschuh M (1998) Expression of multiple cancer/testis (CT) antigens in breast cancer and melanoma: basis for polyvalent CT vaccine strategies. Int J Cancer 78(3):387–389

75. Scanlan MJ, Gordon CM, Williamson B, Lee SY, Chen YT, Stockert E, Jungbluth A, Ritter G, Jager D, Jager E, Knuth A, Old LJ (2002) Identification of cancer/testis genes by database mining and mRNA expression analysis. Int J Cancer 98(4):485–492
76. Scanlan MJ, Simpson AJ, Old LJ (2004) The cancer/testis genes: review, standardization, and commentary. Cancer Immun 4:1
77. Shen L, Kondo Y, Guo Y, Zhang J, Zhang L, Ahmed S, Shu J, Chen X, Waterland RA, Issa JP (2007) Genome-wide profiling of DNA methylation reveals a class of normally methylated CpG island promoters. PLoS Genet 3(10):2023–2036
78. Sigalotti L, Coral S, Nardi G, Spessotto A, Cortini E, Cattarossi I, Colizzi F, Altomonte M, Maio M (2002) Promoter methylation controls the expression of MAGE2, 3 and 4 genes in human cutaneous melanoma. J Immunother 25(1):16–26
79. Simpson AJ, Caballero OL, Jungbluth A, Chen YT, Old LJ (2005) Cancer/testis antigens, gametogenesis and cancer. Nat Rev Cancer 5(8):615–625
80. Suske G (1999) The Sp-family of transcription factors. Gene 238(2):291–300
81. Swann JB, Smyth MJ (2007) Immune surveillance of tumors. J Clin Invest 117(5):1137–1146
82. Takahashi K, Shichijo S, Noguchi M, Hirohata M, Itoh K (1995) Identification of MAGE-1 and MAGE-4 proteins in spermatogonia and primary spermatocytes of testis. Cancer Res 55(16):3478–3482
83. Tilman G, Loriot A, Van Beneden A, Arnoult N, Londono-Vallejo JA, De Smet C, Decottignies A (2009) Subtelomeric DNA hypomethylation is not required for telomeric sister chromatid exchanges in ALT cells. Oncogene 28(14):1682–1693
84. Van den Eynde B, Peeters O, De Backer O, Gaugler B, Lucas S, Boon T (1995) A new family of genes coding for an antigen recognized by autologous cytolytic T lymphocytes on a human melanoma. J Exp Med 182:689–698
85. van der Bruggen P, Traversari C, Chomez P, Lurquin C, De Plaen E, Van den Eynde B, Knuth A, Boon T (1991) A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. Science 254(5038):1643–1647
86. Van Der Bruggen P, Zhang Y, Chaux P, Stroobant V, Panichelli C, Schultz ES, Chapiro J, Van Den Eynde BJ, Brasseur F, Boon T (2002) Tumor-specific shared antigenic peptides recognized by human T cells. Immunol Rev 188(1):51–64
87. Vatolin S, Abdullaev Z, Pack SD, Flanagan PT, Custer M, Loukinov DI, Pugacheva E, Hong JA, Morse H III, Schrump DS, Risinger JI, Barrett JC, Lobanenkov VV (2005) Conditional expression of the CTCF-paralogous transcriptional factor BORIS in normal cells results in demethylation and derepression of MAGE-A1 and reactivation of other cancer-testis genes. Cancer Res 65(17):7751–7762
88. Walsh CP, Bestor TH (1999) Cytosine methylation and mammalian development. Genes Dev 13(1):26–34
89. Wang Z, Zhang J, Zhang Y, Lim SH (2006) SPAN-Xb expression in myeloma cells is dependent on promoter hypomethylation and can be upregulated pharmacologically. Int J Cancer 118(6):1436–1444
90. Wang J, Emadali A, Le Bescont A, Callanan M, Rousseaux S, Khochbin S (2011) Induced malignant genome reprogramming in somatic cells by testis-specific factors. Biochim Biophys Acta 1809(4–6):221–225
91. Weber J, Salgaller M, Samid D, Johnson B, Herlyn M, Lassam N, Treisman J, Rosenberg SA (1994) Expression of the MAGE-1 tumor antigen is up-regulated by the demethylating agent 5-aza-2′-deoxycytidine. Cancer Res 54(7):1766–1771
92. Weber M, Davies JJ, Wittig D, Oakeley EJ, Haase M, Lam WL, Schubeler D (2005) Chromosome-wide and promoter-specific analyses identify sites of differential DNA methylation in normal and transformed human cells. Nat Genet 37(8):853–862
93. Weber M, Hellmann I, Stadler MB, Ramos L, Paabo S, Rebhan M, Schubeler D (2007) Distribution, silencing potential and evolutionary impact of promoter DNA methylation in the human genome. Nat Genet 39(4):457–466
94. Wilson VL, Jones PA (1983) DNA methylation decreases in aging but not in immortal cells. Science 220(4601):1055–1057

95. Woloszynska-Read A, James SR, Link PA, Yu J, Odunsi K, Karpf AR (2007) DNA methylation-dependent regulation of BORIS/CTCFL expression in ovarian cancer. Cancer Immun 7:21
96. Woloszynska-Read A, Mhawech-Fauceglia P, Yu J, Odunsi K, Karpf AR (2008) Intertumor and intratumor NY-ESO-1 expression heterogeneity is associated with promoter-specific and global DNA methylation status in ovarian cancer. Clin Cancer Res 14(11):3283–3290
97. Woloszynska-Read A, James SR, Song C, Jin B, Odunsi K, Karpf AR (2010) BORIS/CTCFL expression is insufficient for cancer-germline antigen gene expression and DNA hypomethylation in ovarian cell lines. Cancer Immun 10:6
98. Yang B, O'Herrin SM, Wu J, Reagan-Shaw S, Ma Y, Bhat KM, Gravekamp C, Setaluri V, Peters N, Hoffmann FM, Peng H, Ivanov AV, Simpson AJ, Longley BJ (2007) MAGE-A, mMage-b, and MAGE-C proteins form complexes with KAP1 and suppress p53-dependent apoptosis in MAGE-positive cell lines. Cancer Res 67(20):9954–9962
99. Yang B, Wu J, Maddodi N, Ma Y, Setaluri V, Longley BJ (2007) Epigenetic control of MAGE gene expression by the KIT tyrosine kinase. J Invest Dermatol 127(9):2123–2128
100. Yegnasubramanian S, Haffner MC, Zhang Y, Gurel B, Cornish TC, Wu Z, Irizarry RA, Morgan J, Hicks J, DeWeese TL, Isaacs WB, Bova GS, De Marzo AM, Nelson WG (2008) DNA hypomethylation arises later in prostate cancer progression than CpG island hypermethylation and contributes to metastatic tumor heterogeneity. Cancer Res 68(21):8954–8967
101. Yoder JA, Walsh CP, Bestor TH (1997) Cytosine methylation and the ecology of intragenomic parasites. Trends Genet 13(8):335–340
102. Zhu X, Asa SL, Ezzat S (2008) Fibroblast growth factor 2 and estrogen control the balance of histone 3 modifications targeting MAGE-A3 in pituitary neoplasia. Clin Cancer Res 14(7):1984–1996

Chapter 8
APC and DNA Demethylation in Cell Fate Specification and Intestinal Cancer

Angela Andersen and David A. Jones

Abstract Most cases of colon cancer are initiated by mutation or loss of the tumor suppressor gene adenomatous polyposis coli (*APC*). APC controls many cellular functions including intestinal cell proliferation, differentiation, migration, and polarity. This chapter focuses on the role of APC in regulating a recently identified DNA demethylase system, consisting of a cytidine deaminase and a DNA glycosylase. A global decrease in DNA methylation is known to occur soon after loss of APC; however, how this occurs and its contribution to tumorigenesis has been unclear. In the absence of wild-type *APC*, ectopic expression of the DNA demethylase system leads to the hypomethylation of specific loci, including intestinal cell fating genes, and stabilizes intestinal cells in an undifferentiated state. Further, misregulation of this system may influence the acquisition of subsequent genetic mutations that drive tumorigenesis.

Colon cancer is the second leading cause of cancer-related death in the western world [1]. Truncating mutations in the tumor suppressor gene (TSG) adenomatous polyposis coli (*APC*) underlie 70–80% of sporadic colon cancers, and germ line mutations in *APC* cause familial adenomatous polyposis (FAP) syndrome, which inevitably leads to colon cancer unless the colon is removed [2, 3]. Mutations in *APC* are observed in early intestinal lesions including aberrant crypt foci, and their frequency is similar in benign adenomas and advanced stage carcinomas, suggesting that the loss of *APC* function initiates tumorigenesis [4]. Additional genetic and epigenetic events affect the rate of tumor progression. Changes in DNA methylation are detected in early stage adenomas, and can be classified as drivers or passengers of tumor progression, analogous to genetic mutations [5–8]. Mutations that activate

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the oncogene *KRAS* are infrequent in small polyps but are common in larger, less differentiated adenomas [9]. Loss of p53 function appears to arise even later in tumor progression and is observed mostly in carcinomas [10]. Technological advances in genome and epigenome analyses should facilitate extensive characterization of the spectrum, sequence, and interdependence of the molecular events that promote colon cancer and should also enable the development of more precise, personalized diagnoses and treatments.

8.1 Tumor Suppressor Functions of APC

A well-appreciated role for *APC* in tumor suppression is as a negative regulator of Wnt signaling [11]. In the absence of Wnt signaling, *APC* forms a destruction complex with Axin and two kinases, casein kinase 1 and glycogen syntase kinase 3β, that phosphorylate the transcriptional co-activator β-catenin. Phosphorylated β-catenin is then ubiquitinated and targeted for proteasomal degradation. Wnt signaling inhibits the formation of the destruction complex, thereby stabilizing β-catenin, which subsequently translocates to the nucleus, binds to the transcription factor TCF4, and activates target genes such as *c-myc* and *cyclin D1*. Deleterious mutations in *APC* stabilize β-catenin and are thus thought to trigger ectopic Wnt signaling. This, in turn, affects multiple cellular functions including adhesion, migration, apoptosis, and proliferation. Consistent with this model, stabilizing mutations within the gene encoding β-catenin are sufficient to initiate adenoma formation in transgenic mice and are associated with about 7% of sporadic colon cancers [12–14].

At the same time, a number of studies have suggested that loss of APC function is not sufficient to induce Wnt signaling. For example, tissues lacking functional APC do not always exhibit the predicted nuclear localization of β-catenin associated with activated Wnt signaling [15]. Blaker et al. showed that early adenomas with mild dysplasia displayed elevated levels of β-catenin in the cytoplasm but not the nucleus, whereas β-catenin was nuclear only in late stage adenomas. In addition, Anderson et al. examined grossly uninvolved and adenoma tissues taken from FAP patients and were unable to identify unambiguous staining for nuclear β-catenin in over 90% of the adenomas [16]. Recent advances suggest that Wnt signaling induces posttranslational modifications of β-catenin that regulate its subcellular localization and function as a transcriptional co-activator with TCF4. For instance, β-catenin is upregulated but confined to the cytoplasm in the intestines of homozygous *apc* mutant zebrafish (*apc*^{mcr}) embryos [17]. These mutant zebrafish display a decrease in the number of intestinal epithelial cells, consistent with reduced Wnt signaling and cell proliferation. This study showed that activation of EGF signaling was required to cooperate with loss of APC in order to stimulate nuclear translocation of β-catenin, activate Wnt signaling, and induce proliferation in *apc*^{mcr} mutant fish. The nuclear accumulation of β-catenin depended on Rac1 and Jnk2 activity, extend-

ing previous observations that these kinases are required for canonical Wnt signaling during mouse development [18]. Similarly, the detection of nuclear β-catenin in advanced human colon adenomas is coincident with increased levels of phospho-cJun, an indicator of JNK activity. Thus, loss of APC appears to stabilize β-catenin without necessarily inducing nuclear translocation and activation of target genes. In this model, aberrant Wnt/β-catenin signaling is a distinct event that contributes to tumor progression after loss of APC.

Indeed, the mechanism of tumor initiation following loss of APC activity may involve functions that are independent of β-catenin. For instance, APC binds to microtubules and regulates mitotic spindle dynamics, which in turn may influence many cellular functions, including chromosome segregation, genomic stability, and cell polarity [19–21]. APC was recently shown to promote asymmetric division of intestinal stem cells, possibly by affecting cell shape [22]. In addition, APC also acts as a positive regulator of retinoic acid (RA) biosynthesis, and, as a result, intestinal cell fate specification [23–26]. Retinoic acid is known to play important roles in controlling cell patterning, fate, and differentiation through the binding and activation of specific RA receptors, retinoid A receptors (RARα, RARβ, and RARγ), or retinoid X receptors (RXRα, RXRβ, and RXRγ) [27]. These receptors belong to the nuclear hormone receptor superfamily and are thought to act, following ligand binding, as direct activators or repressors of gene transcription [28]. A number of studies have implicated retinoids in normal colonocyte function and in the development of colon neoplasms. Compelling evidence for retinoic acid in intestinal development comes from previous studies demonstrating that retinol dehydrogenases Rdh1 and Rdh1l were essential for normal development and in intestinal differentiation in zebrafish [25, 26]. Specifically, knockdown of either Rdh1 or Rdh1l function resulted in well-known RA-deficient phenotypes including loss of pectoral fin formation, lack of jaw development, small eyes, absence of differentiated exocrine pancreas, and aberrant intestinal development. Further studies demonstrated a clear genetic connection between loss of APC and impaired retinoic acid biosynthesis. *apc*^{mcr} zebrafish lack rdhs expression and share a number of developmental phenotypes present in rdh-deficient fish. In addition, exogenous retinoic acid can improve developmental abnormalities in APC-deficient zebrafish, including failed intestinal cell differentiation. Despite the data implicating retinoic acid in intestinal cell functions, the direct functions of retinoic acid in this context remained unexplained.

8.2 Aberrant DNA Methylation Is Associated with Colon Cancer Progression

Retinoic acid induces cell differentiation of different cell types in vitro and in vivo and is thus associated with changes in DNA methylation [28–30]. About 4% of cytosines in a vertebrate genome are methylated by the action of DNA methyltransferases (Dnmt) [31]. Methylcytosine can further be converted to hydroxymethylcytosine,

formylmethylcytosine, and carboxymethylcytosine [32–34]. Methylated cytosine usually occurs at CpG dinucleotides, although significant cytosine methylation outside the CpG context is observed in embryonic stem cells and induced pluripotent cells [35]. Methylated CpG sites are enriched within repetitive sequences such as long interspersed nuclear elements (LINEs) and satellites. Dense methylation of these regions contributes to genomic stability by silencing retrotransposons and suppressing recombination. In contrast, CpG islands, which are short CpG-rich regions frequently found within promoters, tend to be unmethylated in normal tissue [36]. CpG island shores, which are regions located outside of gene promoters but within 2 kb of CpG islands, are differentially methylated in pluripotent cells, different tissues and tumors [37, 38]. Methylation of CpG islands and CpG island shores is associated with gene silencing; however, DNA methylation within gene bodies and intergenic regions has been shown to promote transcription [39]. In addition, DNA methylation was recently shown to influence alternative splicing [40]. Thus, specific patterns of DNA methylation throughout the genome regulate genomic stability and cell-type-specific gene expression.

Aberrant DNA methylation occurs soon after loss of APC, and evidence suggests that it promotes cancer progression. Widespread DNA hypomethylation, inferred from a decrease in LINE-1 methylation, is observed in small adenomas as well as late-stage carcinomas. It was recently shown that most of this hypomethylation corresponds to large, discrete blocks encompassing half the genome and consisting of repetitive sequences as well as genes [41]. Genes within these hypomethylated blocks displayed increased expression variability in different cancer samples, but were not expressed in normal samples, and it was postulated that this stochastic gene expression may contribute to tumor heterogeneity and facilitate the survival of cancer cells in different environments. Demethylation is thought to induce genomic instability by activating retrotransposons and by increasing the frequency of recombination events within repetitive heterochromatin. In addition, hypomethylation could contribute to the chromatin restructuring and nuclear disorganization associated with cancer cells. Smaller regions outside of these blocks were also differentially methylated relative to normal tissue. Hypomethylation was typically observed at CpG island shores and correlated with increased gene expression. In contrast, hypermethylation was associated with CpG islands and gene silencing. The genes that were identified as differentially methylated in colon cancer are enriched for those that are normally differentially methylated between tissues and appear to function in pluripotency, differentiation, and cell fate specification.

8.3 APC Regulates DNA Demethylation and Cell Fate Through Retinoic Acid

DNA methylation may be lost passively or actively removed. Passive demethylation occurs when unmethylated cytosine is incorporated into DNA during replication in the absence of maintenance Dnmt activity. In contrast, during active demethylation methylated cytosines are replaced with unmethylated ones by an enzymatic process

independent of DNA replication. Both mechanisms of demethylation likely contribute to the DNA hypomethylation observed in tumors. An age-dependent decrease of methylation has been observed both in normal tissues and in tumors, consistent with errors in methylated cytosine replication fidelity [42]. This passive, gradual loss of DNA methylation could facilitate tumor initiation or progression by triggering genomic instability and changes in gene expression.

Genetic mutations may also lead to aberrant DNA demethylation. Recently, it was shown that homozygous *apc*^{mcr} zebrafish embryos have reduced DNA methylation at the promoters of genes implicated in intestinal cell fate specification and colorectal cancer, such as *hoxd13a* and *pitx2* [43]. Moreover, these APC-deficient embryos had upregulated the components of a DNA demethylase system, including the cytidine deaminases Aid and Apobec2a, the thymine glycosylase Mbd4, and the DNA repair protein Gadd45α [44, 45]. Knockdown of Mbd4 or of the cytosine deaminases in *apc*^{mcr} zebrafish embryos restored methylation levels. In addition, human colon adenoma samples harboring germ line *APC* mutations also showed reduced DNA methylation at the corresponding loci and upregulation of Aid, Mbd4, and Gadd45α. Thus, APC prevents hypomethylation of key intestinal fating and colorectal cancer genes by repressing the demethylase system.

The upregulation of the demethylase system upon loss of APC was shown to be a consequence of loss of RA production, not misregulated Wnt signaling. Treatment of mutant zebrafish embryos with all-trans retinoic acid, which restores RA levels, but not a pharmacological inhibitor of Cox2, which reduces β-catenin levels downstream of activated Wnt signaling, precluded the upregulation of Aid, Mbd4, and Gadd45α. Further, pharmacological inhibition of RA production in wild-type, adult zebrafish also increased the expression of the demethylase genes and reduced cytosine methylation. Together these observations indicated that DNA demethylation and the expression of the demethylase system are regulated by RA production downstream of APC [43].

Genetic or epigenetic deregulation of genes controlling cell fate decisions can lead to tumorigenesis by precluding the differentiation of progenitor cells [43]. Indeed, DNA hypomethylation of *apc*^{mcr} zebrafish embryos is associated with an expansion of intestinal progenitor cells, revealed by the promoter demethylation and increased expression of intestinal cell fating genes and of *aldh1a2*, a marker of colon crypt progenitor cells, and by the decreased expression of a marker for intestinal differentiation, *fabp2*. Knockdown of the demethylase system components induced intestinal differentiation, indicating that hypomethylation is required to stabilize intestinal cells in a progenitor-like state. In addition, increased cell proliferation was observed in the brain of *apc*^{mcr} zebrafish embryos, and this also depended on the demethylase system. Patterning defects were excluded since the mutant embryos expressed primordial brain and intestinal markers. These data support a role for APC in cell fate specification and differentiation through the regulation of RA production and, in turn, DNA methylation. Thus, loss of APC may initiate tumorigenesis in part by hypomethylating and deregulating cell fate genes, resulting in the expansion of proliferative, progenitor-like cells.

The proposed mechanism of demethylation by this system couples enzyme-mediated deamination of methylated cytosine (me-dC), to produce thymine (dT), with

glycosylase-mediated base excision repair to replace the dG:dT mismatch with a dG:dC base pair [45]. Aid, Mbd4, and Gadd45α were shown promote demethylation of a methylated, double-stranded DNA fragment injected into wild-type zebrafish embryos, and also of bulk genomic DNA. The injected DNA fragment is not replicated, excluding a passive mechanism of demethylation arising from rounds of DNA replication without subsequent cytosine methylation of the newly synthesized strand. Further, co-expression of Aid with a catalytic mutant of Mbd4 in zebrafish embryos stabilized the dG:dT mismatches that would be generated by deamination. Indeed, Aid and a related cytosine deaminase Apobec1 have been shown to deaminate me-dC to dT within single-stranded DNA in vitro [46]. Nevertheless, the field awaits biochemical support for the proposed mechanism and insight into how Aid accesses me-dC within duplex DNA. Given that Mbd4 can recognize and extrude me-dC from duplex DNA, this component of the demethylase system could both target the deaminase to me-dC and promote substrate accessibility [47, 48]. Consistent with this model, Mbd4 was required not only for repair of the dG:dT mismatch, but also for Aid-mediated deamination of me-dC in zebrafish embryos. Moreover, Gadd45α appears to stabilize the physical interaction of Mbd4 with Aid [45]. The stable association of a deaminase with a glycosylase may be important not only for targeting demethylation but also for mediating the repair of the dG:dT intermediate.

That APC may suppress tumor formation partly through negative regulation of DNA demethylase components is consistent with previous observations. Mice carrying the APC multiple intestinal neoplasia (Apc^{min}) mutant allele, which produces truncated APC, develop intestinal lesions similar to human FAP and are frequently employed as a mouse model for colon carcinogenesis. Interestingly, genetic deletion of the cytidine deaminase Apobec1 reduced adenoma formation in $Apc^{min/+}$ mice [49]. Apobec1 is highly expressed in the small intestine and targets a number of mRNAs for C to U editing [50]. It had previously been shown that Apobec1 binds and stabilizes cyclooxygenase 2 (Cox2) mRNA in vitro [51]. Adenomas from $Apc^{min/+}$ *Apobec-1*$^{-/-}$ mice displayed decreased expression of Cox2 and it was suggested that this could account for the reduced tumor burden. This model is consistent with previous reports that Cox2 expression is increased in adenomas, and that genetic or pharmacological inhibition of Cox2 also decreases polyp formation in APC mutant mice [52]. However, Apobec1 can also deaminate DNA, and this activity may also promote tumor progression. Deamination of dC or me-dC results in transitions to dT, and Apobec-1 knockout mice would be predicted to have a reduced frequency of these mutations. This in turn could decrease polyp initiation by preventing second-hit mutations. In addition, given that components of the DNA demethylase system are ectopically expressed in the absence of APC, Apobec1 may also cooperate with a thymine glycosylase to promote DNA demethylation, altered gene expression, and the expansion of intestinal progenitor cells in $Apc^{min/+}$ mice. Thus, $Apc^{min/+}$ *Apobec-1*$^{-/-}$ mice may display reduced adenoma formation in part due to reduced transition mutations and to restored DNA methylation patterns and differentiation of intestinal progenitor cells.

In considering the development of APC loss-dependent colorectal cancer, it is plausible to envision a role for DNA demethylation given its role in reprogramming

in other systems. Genome-wide demethylation of the paternal genome in the mammalian zygote occurs within hours after fertilization [53–55]. Later in embryogenesis, during specification of mouse primordial germ cells, the cytosine methylation that underlies parental imprints is erased and pluripotency is reestablished [56, 57]. Interestingly, genome-wide bisulphite sequencing analysis revealed an increase in global DNA methylation levels in PGCs derived from Aid-null embryos relative to wild-type embryos [58]. However, significant demethylation occurred even in the absence of Aid, suggesting that this process may involve other deaminases like Apobec1 [46] or another mechanism. Similarly, reduced levels of DNA demethylation in zebrafish required simultaneous knockdown of Aid and Apobec2 [45], suggesting redundancy among members of the Aid/Apobec family. DNA demethylation is also a rate-limiting step for reprogramming somatic cells to a pluripotent state [59–61]. Indeed, Aid was required for the demethylation and induction of pluripotency genes in heterokaryons generated by fusing mouse embryonic stem (ES) cells with human fibroblasts. Importantly, Aid-mediated DNA demethylation did not require cell proliferation or DNA replication, providing further support for a role for Aid in active DNA demethylation. Prior to cell fusion, Aid is bound to distinct, methylated promoters in each cell type. For instance, Aid associates with the methylated promoters of Oct4 and Nanog in fibroblasts, but not with their unmethylated promoters in ES cells [61]. These observations suggest that cell-type-specific factors stimulate Aid's deaminase activity at methylated target loci. Thus, active DNA demethylation mechanisms employing deaminases stabilize a pluripotent state in different biological contexts.

The misregulation of the demethlyase system in APC-deficient animals may also reconcile some apparent contradictions arising from previous studies. Adenoma formation in $Apc^{min/+}$ mice is suppressed either by pharmacologic inhibition of Dnmt activity with 5-aza-deoxycytidine or by genetic loss of the DNA methyltransferase *Dnmt1* or *Dnmt3b* [62–65]. However, 5-aza-deoxycytidine did not preclude microadenoma formation, nor did it preclude adenoma progression once a polyp had formed, suggesting an irreversible event occurs prior to, and is required for, the transition to a macroadenoma. Microadenomas have lost the wild-type allele of *APC*, indicating that this step is not rate limiting for macroadenoma formation. One explanation for these findings could be that hypermethylation and silencing of TSGs is required for tumor growth, and that reducing Dnmt activity inhibits this step [66, 67]. It has been shown that the CpG islands upstream of some TSGs are methylated in some cells within the normal intestinal mucosa of $Apc^{min/+}$ mice, and that their methylation increases in polyps [62]. Genetic loss of *Dnmt1* reduced the extent of methylation at these sites in both normal mucosa and polyps, and reduced polyp formation, extending the correlation between localized methylation and tumor growth. Although these observations are consistent with a reduction in TSG expression promoting tumor progression, DNA methylation could also contribute to tumorigenesis by affecting the rate and spectrum of genetic mutations [68, 69]. Spontaneous or enzymatic deamination of me-dC yields dT, resulting in a dC to dT transition mutation if it is not repaired prior to replication. Transition mutations at CpG dinucleotides, the target for DNA methylation, contribute significantly to tumorigenesis despite the under-representation of CpG in the genome [70, 71]. Loss of *APC* could increase

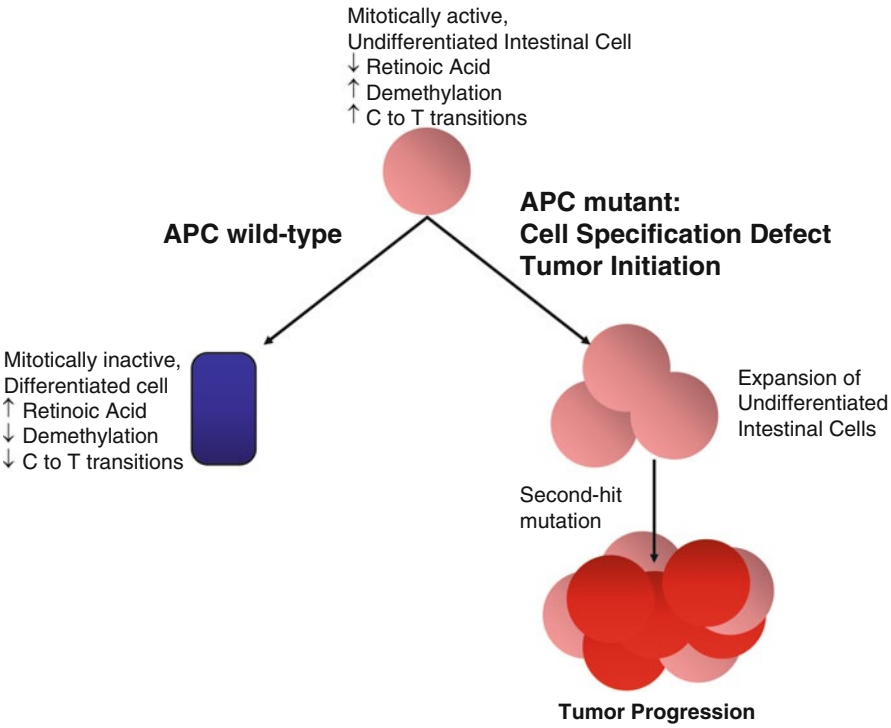

Fig. 8.1 In the intestine, APC promotes differentiation through the production of retinoic acid and the negative regulation of DNA demethylase components. In APC mutants, there is decreased retinoic acid production, maintaining cells in an undifferentiated state due to the continued expression of the demethylase system and of genes controlling cell fate and proliferation. In addition, expression of the demethylase system may promote C to T transition mutations. Both the cell specification defect and accumulation of second-hit mutations upon loss of APC may contribute to tumorigenesis

the rate of dC to dT transitions due to the upregulation of deaminases such as Aid and Apobec2 [43]. Thus, in addition to stabilizing a progenitor-like state, loss of *APC* and deregulation of the DNA demethylase system may separately contribute to tumorigenesis by increasing the likelihood of second-hit transition mutations. In this model, inhibition of Dnmt activity would suppress adenoma formation upon loss of *APC* by reducing the levels of me-dC, a substrate for deamination, which ultimately decreases the frequency of tumor-promoting dC to dT transitions. Similarly, genetic loss of Mbd4, which can repair the dT generated by deamination of me-dC, increased the rate of dC to dT transitions at CpG dinucleotides and accelerated intestinal tumorigenesis in APC mutant mice [72, 73].

The above findings support a new model linking loss of APC, impaired intestinal differentiation, and tumor initiation to RA-mediated control of DNA methylation dynamics. APC serves a critical role in cell fate specification by positive regulation of RA production and, in turn, inhibition of the DNA demethylase system (Fig. 8.1).

In the absence of APC function, there is an expansion of intestinal progenitor cells. Further, the misregulation of deaminases downstream of loss of APC may lead to an increased frequency of second-hit mutations. In this way, loss of APC may both directly and indirectly affect tumor initiation and progression.

References

1. Markowitz SD (2007) Aspirin and colon cancer–targeting prevention? N Engl J Med 356(21):2195–2198
2. Bienz M, Clevers H (2000) Linking colorectal cancer to Wnt signaling. Cell 103(2): 311–320
3. Fearon ER (2011) Molecular genetics of colorectal cancer. Annu Rev Pathol 6:479–507
4. Kinzler KW, Vogelstein B (1996) Lessons from hereditary colorectal cancer. Cell 87(2):159–170
5. Sunami E et al (2011) LINE-1 hypomethylation during primary colon cancer progression. PLoS One 6(4):e18884
6. Feinberg AP et al (1988) Reduced genomic 5-methylcytosine content in human colonic neoplasia. Cancer Res 48(5):1159–1161
7. Cravo M et al (1996) Global DNA hypomethylation occurs in the early stages of intestinal type gastric carcinoma. Gut 39(3):434–438
8. Goelz SE et al (1985) Hypomethylation of DNA from benign and malignant human colon neoplasms. Science 228(4696):187–190
9. Fearon ER, Vogelstein B (1990) A genetic model for colorectal tumorigenesis. Cell 61(5):759–767
10. Baker SJ et al (1990) p53 gene mutations occur in combination with 17p allelic deletions as late events in colorectal tumorigenesis. Cancer Res 50(23):7717–7722
11. Clevers H (2006) Wnt/beta-catenin signaling in development and disease. Cell 127(3):469–480
12. Sparks AB et al (1998) Mutational analysis of the APC/beta-catenin/Tcf pathway in colorectal cancer. Cancer Res 58(6):1130–1134
13. Morin PJ et al (1997) Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC. Science 275(5307):1787–1790
14. Romagnolo B et al (1999) Intestinal dysplasia and adenoma in transgenic mice after overexpression of an activated beta-catenin. Cancer Res 59(16):3875–3879
15. Blaker H et al (2003) Somatic mutations in familial adenomatous polyps. Nuclear translocation of beta-catenin requires more than biallelic APC inactivation. Am J Clin Pathol 120(3):418–423
16. Anderson CB, Neufeld KL, White RL (2002) Subcellular distribution of Wnt pathway proteins in normal and neoplastic colon. Proc Natl Acad Sci USA 99(13):8683–8688
17. Phelps RA et al (2009) A two-step model for colon adenoma initiation and progression caused by APC loss. Cell 137(4):623–634
18. Wu X et al (2008) Rac1 activation controls nuclear localization of beta-catenin during canonical Wnt signaling. Cell 133(2):340–353
19. Caldwell CM, Green RA, Kaplan KB (2007) APC mutations lead to cytokinetic failures in vitro and tetraploid genotypes in Min mice. J Cell Biol 178(7):1109–1120
20. Caldwell CM, Kaplan KB (2009) The role of APC in mitosis and in chromosome instability. Adv Exp Med Biol 656:51–64
21. Green RA, Wollman R, Kaplan KB (2005) APC and EB1 function together in mitosis to regulate spindle dynamics and chromosome alignment. Mol Biol Cell 16(10):4609–4622
22. Quyn AJ et al (2010) Spindle orientation bias in gut epithelial stem cell compartments is lost in precancerous tissue. Cell Stem Cell 6(2):175–181
23. Jette C et al (2004) The tumor suppressor adenomatous polyposis coli and caudal related homeodomain protein regulate expression of retinol dehydrogenase L. J Biol Chem 279(33):34397–34405

24. Nadauld LD et al (2006) Adenomatous polyposis coli control of C-terminal binding protein-1 stability regulates expression of intestinal retinol dehydrogenases. J Biol Chem 281(49): 37828–37835
25. Nadauld LD et al (2004) Adenomatous polyposis coli control of retinoic acid biosynthesis is critical for zebrafish intestinal development and differentiation. J Biol Chem 279(49): 51581–51589
26. Nadauld LD et al (2005) The zebrafish retinol dehydrogenase, rdh1l, is essential for intestinal development and is regulated by the tumor suppressor adenomatous polyposis coli. J Biol Chem 280(34):30490–30495
27. Mark M, Ghyselinck NB, Chambon P (2009) Function of retinoic acid receptors during embryonic development. Nucl Recept Signal 7:e002
28. Duester G (2008) Retinoic acid synthesis and signaling during early organogenesis. Cell 134(6):921–931
29. Deb-Rinker P et al (2005) Sequential DNA methylation of the Nanog and Oct-4 upstream regions in human NT2 cells during neuronal differentiation. J Biol Chem 280(8):6257–6260
30. Fisher CL, Fisher AG (2011) Chromatin states in pluripotent, differentiated, and reprogrammed cells. Curr Opin Genet Dev 21(2):140–146
31. Wild L, Flanagan JM (2010) Genome-wide hypomethylation in cancer may be a passive consequence of transformation. Biochim Biophys Acta 1806(1):50–57
32. Ito S et al (2011) Tet proteins can convert 5-methylcytosine to 5-formylcytosine and 5-carboxylcytosine. Science 333(6047):1300–1303
33. Kriaucionis S, Heintz N (2009) The nuclear DNA base 5-hydroxymethylcytosine is present in Purkinje neurons and the brain. Science 324(5929):929–930
34. Tahiliani M et al (2009) Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. Science 324(5929):930–935
35. Lister R et al (2009) Human DNA methylomes at base resolution show widespread epigenomic differences. Nature 462(7271):315–322
36. Deaton AM, Bird A (2011) CpG islands and the regulation of transcription. Genes Dev 25(10):1010–1022
37. Doi A et al (2009) Differential methylation of tissue- and cancer-specific CpG island shores distinguishes human induced pluripotent stem cells, embryonic stem cells and fibroblasts. Nat Genet 41(12):1350–1353
38. Irizarry RA et al (2009) The human colon cancer methylome shows similar hypo- and hypermethylation at conserved tissue-specific CpG island shores. Nat Genet 41(2):178–186
39. Wu H et al (2010) Dnmt3a-dependent nonpromoter DNA methylation facilitates transcription of neurogenic genes. Science 329(5990):444–448
40. Shukla S et al (2011) CTCF-promoted RNA polymerase II pausing links DNA methylation to splicing. Nature 479(7371):74–79
41. Hansen KD et al (2011) Increased methylation variation in epigenetic domains across cancer types. Nat Genet 43(8):768–775
42. Suzuki K et al (2006) Global DNA demethylation in gastrointestinal cancer is age dependent and precedes genomic damage. Cancer Cell 9(3):199–207
43. Rai K et al (2010) DNA demethylase activity maintains intestinal cells in an undifferentiated state following loss of APC. Cell 142(6):930–942
44. Barreto G et al (2007) Gadd45a promotes epigenetic gene activation by repair-mediated DNA demethylation. Nature 445(7128):671–675
45. Rai K et al (2008) DNA demethylation in zebrafish involves the coupling of a deaminase, a glycosylase, and gadd45. Cell 135(7):1201–1212
46. Morgan HD et al (2004) Activation-induced cytidine deaminase deaminates 5-methylcytosine in DNA and is expressed in pluripotent tissues: implications for epigenetic reprogramming. J Biol Chem 279(50):52353–52360
47. Hendrich B et al (1999) Genomic structure and chromosomal mapping of the murine and human Mbd1, Mbd2, Mbd3, and Mbd4 genes. Mamm Genome 10(9):906–912

48. Wu P et al (2003) Mismatch repair in methylated DNA. Structure and activity of the mismatch-specific thymine glycosylase domain of methyl-CpG-binding protein MBD4. J Biol Chem 278(7):5285–5291
49. Blanc V et al (2007) Deletion of the AU-rich RNA binding protein Apobec-1 reduces intestinal tumor burden in Apc(min) mice. Cancer Res 67(18):8565–8573
50. Rosenberg BR et al (2011) Transcriptome-wide sequencing reveals numerous APOBEC1 mRNA-editing targets in transcript 3′ UTRs. Nat Struct Mol Biol 18(2):230–236
51. Anant S et al (2004) Apobec-1 protects intestine from radiation injury through posttranscriptional regulation of cyclooxygenase-2 expression. Gastroenterology 127(4):1139–1149
52. Oshima M et al (1996) Suppression of intestinal polyposis in Apc delta716 knockout mice by inhibition of cyclooxygenase 2 (COX-2). Cell 87(5):803–809
53. Mayer W et al (2000) Demethylation of the zygotic paternal genome. Nature 403(6769): 501–502
54. Oswald J et al (2000) Active demethylation of the paternal genome in the mouse zygote. Curr Biol 10(8):475–478
55. Santos F et al (2002) Dynamic reprogramming of DNA methylation in the early mouse embryo. Dev Biol 241(1):172–182
56. Hajkova P et al (2002) Epigenetic reprogramming in mouse primordial germ cells. Mech Dev 117(1–2):15–23
57. Feng S, Jacobsen SE, Reik W (2010) Epigenetic reprogramming in plant and animal development. Science 330(6004):622–627
58. Popp C et al (2010) Genome-wide erasure of DNA methylation in mouse primordial germ cells is affected by AID deficiency. Nature 463(7284):1101–1105
59. Simonsson S, Gurdon J (2004) DNA demethylation is necessary for the epigenetic reprogramming of somatic cell nuclei. Nat Cell Biol 6(10):984–990
60. Mikkelsen TS et al (2008) Dissecting direct reprogramming through integrative genomic analysis. Nature 454(7200):49–55
61. Bhutani N et al (2010) Reprogramming towards pluripotency requires AID-dependent DNA demethylation. Nature 463(7284):1042–1047
62. Eads CA, Nickel AE, Laird PW (2002) Complete genetic suppression of polyp formation and reduction of CpG-island hypermethylation in Apc(Min/+) Dnmt1-hypomorphic Mice. Cancer Res 62(5):1296–1299
63. Yamada Y et al (2005) Opposing effects of DNA hypomethylation on intestinal and liver carcinogenesis. Proc Natl Acad Sci USA 102(38):13580–13585
64. Lin H et al (2006) Suppression of intestinal neoplasia by deletion of Dnmt3b. Mol Cell Biol 26(8):2976–2983
65. Laird PW et al (1995) Suppression of intestinal neoplasia by DNA hypomethylation. Cell 81(2):197–205
66. Linhart HG et al (2007) Dnmt3b promotes tumorigenesis in vivo by gene-specific de novo methylation and transcriptional silencing. Genes Dev 21(23):3110–3122
67. Eads CA et al (2000) Fields of aberrant CpG island hypermethylation in Barrett's esophagus and associated adenocarcinoma. Cancer Res 60(18):5021–5026
68. Jones PA et al (1992) Methylation, mutation and cancer. Bioessays 14(1):33–36
69. Laird PW, Jaenisch R (1994) DNA methylation and cancer. Hum Mol Genet 3 Spec No:1487–1495
70. Greenblatt MS et al (1994) Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. Cancer Res 54(18):4855–4878
71. Holliday R, Grigg GW (1993) DNA methylation and mutation. Mutat Res 285(1):61–67
72. Wong E et al (2002) Mbd4 inactivation increases Cright-arrowT transition mutations and promotes gastrointestinal tumor formation. Proc Natl Acad Sci USA 99(23):14937–14942
73. Millar CB et al (2002) Enhanced CpG mutability and tumorigenesis in MBD4-deficient mice. Science 297(5580):403–405

Chapter 9
Epigenetic Changes During Cell Transformation

Bernard W. Futscher

Abstract Malignant cancer emerges from normal healthy cells in a multistep process that involves both genetic and epigenetic lesions. Both genetic and environmental inputs participate in driving the epigenetic changes that occur during human carcinogenesis. The pathologic changes seen in DNA methylation and histone posttranslational modifications are complex, deeply intertwined, and act in concert to produce malignant transformation. To better understand the causes and consequences of the pathoepigenetic changes in cancer formation, a variety of experimentally tractable human cell line model systems that accurately reflect the molecular alterations seen in the clinical disease have been developed. Results from studies using these cell line model systems suggest that early critical epigenetic events occur in a stepwise fashion prior to cell immortalization. These epigenetic steps coincide with the cell's transition through well-defined cell proliferation barriers of stasis and telomere dysfunction. Following cell immortalization, stressors, such as environmental toxicants, can induce malignant transformation in a process in which the epigenetic changes occur in a smoother progressive fashion, in contrast to the stark stepwise epigenetic changes seen prior to cell immortalization. It is hoped that developing a clearer understanding of the identity, timing, and consequences of these epigenetic lesions will prove useful in future clinical applications that range from early disease detection to therapeutic intervention in malignant cancer.

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9.1 Introduction

Malignant cancer cells arise from normal cells via a multistep process that involves both genetic and epigenetic change. Similar to genetic lesions, epigenetic lesions can be diverse in nature, serving to alter the structure and function of the genome thereby participating in a cell's acquisition of limitless uncontrolled growth and the phenotypic hallmarks of the malignant cancer cell. In general, the degree of epigenetic difference between cancer cells and normal cells greatly exceeds the epigenetic differences that are seen between normal cells of different phenotypes and even different germ layers (e.g., fibroblasts and epithelial cells). Since epigenetic mechanisms are a primary determinant governing normal cell identity, this comparison underscores how epigenetically different cancer cells are from normal cells. Mutation and altered expression of proteins involved in the writing or reading of the epigenetic code are two mechanisms that help produce aberrant epigenetic changes seen in not only cancer, but other human diseases as well. The complexity and the frequency of the epigenetic changes seen in cancer cells, however, seem to defy explanations that rely on a single event. Instead, it appears that pathologic epigenetic change during carcinogenesis results from myriad genetic mutations and environmental inputs which perturb the manifold nodes of epigenetic regulation.

Environmental inputs acting on the epigenetic nodes are highly variable and can include contributions from both physiologic and xenobiotic sources such as hormonal status; microenvironmental milieu; nutritional, metabolic, or oxidative state; and toxicant and therapeutic drug exposures. Since the epigenetic state is important in governing cell identity, cellular nodes of epigenetic control acted upon by stimuli will show some variation between different cell types, suggesting that environmental inputs may show cell type selectivity, as well as display activity towards a broad array of cell types. Once these epigenetic changes are "fixed" into the chromatin, they can be vertically transmitted through cell generations. The inherent plasticity of the epigenetic control systems coupled to the cancer cell's limitless replicative potential provides the ability to generate extraordinary phenotypic diversity and rapidly respond to changing environmental stimuli and stresses.

Chromatin is rich in epigenetic marks, and these marks participate in the regulation and control of likely most or all genomic functions. The primary epigenetic mark found on DNA, 5-methylcytosine, is produced via the enzymatic methylation of the C5 position of cytosine through the action of multiple specialized DNA methyltransferases. The patterns and levels of DNA methylation across the genome have been mapped for a variety of normal and cancer cells, with cancer cells showing complex and extensive patterns of DNA methylation derangements. These DNA methylation derangements either participate in or reflect a number of different genomic processes, with its role in the regulation of gene expression being the best understood. Other C5 cytosine modifications have been identified recently, such as 5-hydroxymethylcytosine. It appears that these newly identified modifications are a result of an active DNA demethylation process and it is likely that these DNA epigenetic marks will prove biologically important; however, it has not yet been elucidated how these marks change and participate in the process of malignant transformation.

Posttranslational histone modifications are an additional layer of epigenetic control altered during human carcinogenesis. These posttranslational modifications include acetylation, methylation, phosphorylation, ubiquitylation, and sumoylation, and over 40 different amino acid residues in histones are currently known to undergo one or more of these modifications, especially in histones H3 and H4. Similar to DNA methylation, the histone posttranslational marks participate in a number of different genomic processes. Some histone marks are highly predictive of gene promoter location and transcriptional activity, such as histone H3K4 trimethylation and histone H3 and H4 lysine acetylation, and these modifications show strong negative correlations with DNA methylation levels in a typical genomic region. Other posttranslational histone modifications are linked to a transcriptionally repressed state and display positive correlations with DNA methylation levels, such as H3K9 methylation repressive marks. Still other histone marks, such as H3K27 trimethylation, are closely linked to transcriptional repression, preferentially target developmentally regulated genes and largely appears to be a repressive epigenetic control system that operates independently of the repressive DNA methylation system. Overall, a number of in vitro studies have provided clear mechanistic links between DNA methylation and histone modification state indicating that the control of the DNA methylation and histone modification patterns are deeply intertwined. As such, it is not surprising that, similar to DNA methylation, the normal levels and patterns of histone posttranslational modifications become compromised in human cancer cells.

In a clinical setting, the multistep nature of epithelial cell malignant transformation manifests as hyperplasia, dysplasia, benign tumor, carcinoma in situ, and finally frank malignancy and metastases; analogous pathologic progressions can be seen in some hematologic pathologies, as well, and may very well exist for most or all human cancers. Analysis of clinical specimens has shown that epigenetic aberrations are seen in the earliest stages of this multistep process, although obtaining quantitative information-rich epigenetic data from minute clinical specimens creates unique technical challenges that have slowed the ability to identify pathoepigenetic events that directly translate to clinical impact with respect to the detection, prognostication, treatment, and management of human cancer. For example, technical limitations such as specimen size and quality have hindered success in analyzing the posttranslational modification state of histones in clinical specimens. With respect to DNA methylation analysis, quantitative high resolution approaches for the analysis of the minute clinical cancer specimens typically available have been available for over 20 years in the form of bisulfite sequencing [1, 2], and today comprehensive DNA methylome sequencing approaches have emerged and should attain wide availability over the next few years [3, 4]. In the translational science arena, there are a few early applications where the results indicate DNA methylation analysis may be a useful tool in predicting response to cancer therapy [5, 6]. Results such as these should provide significant optimism and encouragement to investigators that epigenetic analysis will prove useful in the areas of prediction, detection, prognostication, as well as treatment of cancer. While significant progress has been made in understanding the causes, consequences, and temporal sequence of pathologic epigenetic events in cancer, their utility on the clinical management of cancer is largely a promissory note with their potential not yet fully realized.

9.2 Laboratory Model Systems of Cell Transformation

To better discover and understand the pathoepigenetic events that mechanistically participate in the conversion of a normal cell to a malignant cell, there is value in using experimentally tractable models systems that faithfully reflect the in vivo process. To this end, a variety of useful and complementary in vitro human cell line and animal model systems have been developed that recapitulate aspects of clinical multistep carcinogenesis and that allow for detailed analysis of epigenetic/epigenomic events as they unfold during the transformation from the normal to the malignant phenotype. These models have a number of advantages as laboratory tools—certainly the most important being that the genetic and epigenetic changes present in them accurately reflect the known (epi)genetic etiology of the clinical form of the disease, thereby providing a solid platform for the discovery and dissection of new epigenetic events relevant to clinical cancer. These cell line systems also allow for the production of pure and reproducible populations of cells that can be fairly easily generated in large number and at relatively low costs. In our experience, the epigenetic state of the cell line models we have employed does not vary to a significant extent when grown under appropriate and consistent conditions. We routinely verify cell line identity using STR profiling using 13 CODIS markers; reference DNA fingerprinting data for most of the widely used cell lines are available from cell line collections such as the ATCC or from the investigators who developed the models [7, 8].

A majority of the human cell culture model systems that have been developed perhaps best address the final step(s) of malignant human cancer, specifically the steps that follow cell immortalization. Since immortalization through telomerase activation may be a rate limiting step in human carcinogenesis, these models may not be best suited for the identification of the earliest epigenetic events in carcinogenesis. Cell model systems that adequately address the earliest steps in human carcinogenesis, prior to cell immortalization, are more limited. These are discussed later in the chapter. As is always the case, each model system used to evaluate the steps from normal finite life span cell to immortal malignant cancer cell has distinct qualities and limitations. Together, these laboratory models allow for the molecular dissection of epigenetic dysfunction during the pathologic process and help provide new insights that can be used to develop approaches to better detect, prognosticate, treat, and manage the myriad human cancers.

9.3 Immortalization to Malignant Transformation

Cell line systems that model the epigenetic events that occur following epithelial cell immortalization are widespread and provide useful tools to study malignant transformation (meant here as the in vitro assessments of anchorage independent growth and tumor forming ability in immunocompromised mice). These immortal-

ized cell line model systems have generally overcome normal cell proliferation barriers either by (1) direct immortalization of primary cell strains through overexpression of hTERT, (2) selective genetic strategies that inactivate the p16/Rb and p53 pathways, frequently via viral approaches, or (3) establishing cell lines from cultured pathologic specimens that are already immortal, but not fully malignant. A variety of immortalized variants of different epithelial cell models have been generated and examples include, but are not limited to, prostate epithelial cells immortalized by HPV18 (RWPE), bronchial epithelial cells immortalized with SV40 (HBE16, BEAS-2B), keratinocytes that arose spontaneously in culture from primary cells (HaCAT), breast epithelial cells derived from diseased tissue (MCF10A) or non-diseased healthy tissue (HMEC), and urinary bladder cells immortalized with hTERT or SV40 (UROtsa) [9–18]. Although some approaches used to immortalize cells are not themselves etiologic agents involved in clinical human carcinogenesis (e.g., viral inactivation of p53 or the genetic introduction of hTERT), they do provide reproducible approaches that target proteins and pathways known to be critical to the human tumor cell phenotype.

These immortalized cell line systems should not be considered normal cells; however, since they have had perhaps the most dramatic phenotypic shift possible—acquisition of limitless replicative potential. In addition, these cells have often also acquired genetic abnormalities (e.g., deletions, translocations, aneuploidy). It is highly likely that these immortalized cells have undergone changes in the epigenetic state, if compared to its normal finite life span counterpart, although detailed studies to this end are limited. Indeed, the p53 inactivation strategies used in immortalization strategies may instigate epigenetic change itself. Following a cellular stress, activated p53 binds to DNA in a sequence-specific manner while also recruiting coactivators or corepressors to participate in transcriptional regulation. Thus, loss of p53 binding and coactivator/corepressor recruitment may produce long-term epigenetic changes at p53 target loci disrupting their normal transcriptional regulation and altering attendant cellular phenotypes [19–21]. As such, these immortalized models likely provide more limited information regarding the nature of the epigenetic changes that may occur early in multistep carcinogenesis and prior to immortalization. Overall, these models have proven useful in identifying novel epigenetic changes, the molecular mechanisms responsible for these epigenetic changes, and the genetic and/or environmental events that provoke the epigenetic changes.

9.4 Epigenetic Remodeling by Environmental Arsenicals

Our laboratory has been interested in the effect that environmental arsenicals has on the epigenetic state. Arsenic is a widespread environmental toxicant that exists as a number of different molecular species and ranks as the 20th most common element in the earth's crust. Humans may be exposed to arsenicals to varying degrees through water, air, soil, and food. Arsenic may also be the world's most well recognized poison. Acute high dose exposure to arsenic has been used repeatedly throughout

history for murder by intentional poisoning and has earned the moniker, “Poison of Kings and King of Poisons [22].” In contrast, various forms of arsenic have also been used for centuries to treat a wide range of illnesses, including syphilis, malaria, asthma, chorea, eczema, psoriasis, and cancer [23]. Today, one molecular species of arsenic, arsenic trioxide (As_2O_3) is an FDA-approved therapy to treat acute promyelocytic leukemia and also shows promising anticancer activity in laboratory models of other human cancers [24–26]. In the most common setting, however, that of chronic low dose, environmental exposures, arsenicals are associated with a number of human maladies, among them cancer, neurologic disorders, cardiovascular disease, developmental abnormalities, and diabetes [27–30].

Of all the pathologic effects associated with long-term arsenic exposure, cancer is the most widely studied. A number of epidemiological studies have convincingly linked human arsenic exposure with various cancers, especially cancers of the lung, urinary tract, and skin [31]. Arsenicals are classified as a group 1 carcinogen by the International Agency for Research on Cancer (IARC); however, a precise mechanism of arsenical action remains wanting. A few observations suggest that epigenetic remodeling may be important in arsenical-associated cancers. Arsenicals do not appear to cause point mutations and on their own are unable to cause cancer in standard animal assays or immortalize primary human epithelial cells [32, 33]. However, earlier studies showed arsenicals can change DNA methylation levels [34], and long-term nontoxic exposure to arsenicals has been sufficient to reproducibly induce malignant transformation in a variety of immortalized nonmalignant human epithelial cells derived from tissues with known arsenical sensitivity. Examples of cell line models that have been malignantly transformed by arsenicals include HaCaT, BEAS-2B, RWPE, and UROtsa [35–39].

Human transitional carcinoma of the bladder arises from the transformation of urinary bladder epithelial cells, and those tumors that progress clinically to a malignant phenotype generally demonstrate genetic inactivation of the p16/Rb and p53 pathways [40]. In vitro, benign immortalized urothelial cell lines that resemble the earlier stages of clinical bladder cancer can be reproducibly generated from finite life span urothelial cell strains via genetic manipulations that target these pathways for inactivation. In our studies of epigenetic changes that occur during the transition from a benign immortal cell to a malignant cancer cell, we have used the immortalized, non-tumorigenic human urothelial cell line, UROtsa, generated from the urothelial cells of a young female donor and immortalized using a temperature sensitive SV40 large-T antigen construct [14]. Further evaluation of these cells has revealed hypodiploidy, genetic deletion of a small region of chromosome 9 that contains p16, and hTERT expression (unpublished observations).

Malignant transformation of UROtsa cells using long-term nontoxic exposures to environmental toxicants such as arsenic has been successfully performed by multiple independent laboratories [36, 39]. The phenotypic manifestations of the malignant conversion process can first be detected in these cells at approximately 12 weeks of exposure at a faster growth rate. With increased exposure time, the ability to form colonies in an anchorage independent fashion occurs, and finally arsenic-exposed

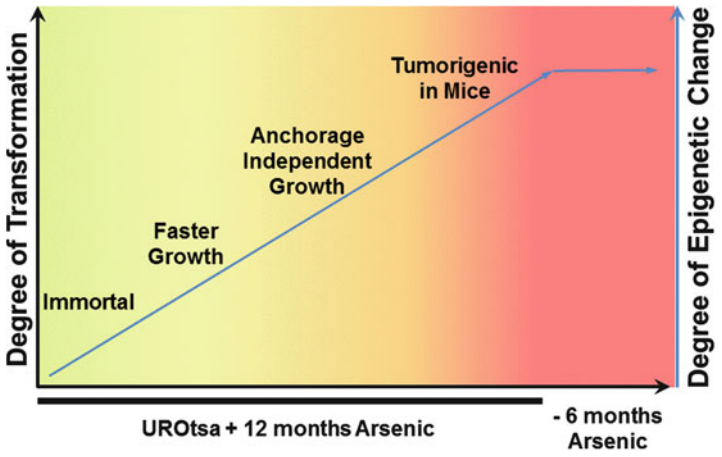

Fig. 9.1 UROtsa cell line model of malignant transformation. The immortalized urothelial cell line UROtsa was exposed to arsenicals for periods of up to a year. Arsenical exposed cells were probed at various time points for markers of malignant transformation. After 3 months there was a significant increase in proliferation rate, after 6 months a significant increase in anchorage independent growth, and after 12 months, arsenic exposed cells formed tumors in immune compromised mice [36, 39]. Progressive epigenetic changes occur during this transition from a benign immortal to malignant phenotype

UROtsa cells acquire the ability to form tumors in immunocompromised mice. Interestingly, the arsenical-induced malignant phenotype is stable, as removal of the toxicant for at least 6 months has not led to the reversion to a more benign phenotype (Fig. 9.1).

Broad epigenetic changes begin to rise in UROtsa cells during exposure to arsenic at concentrations seen in real-world situations, such as can be found in drinking water from wells (5–10 ppb). We examined epigenetic changes in a genome-wide and temporal manner using histone modification-specific chromatin and 5-methylcytosine-specific immunoprecipitations coupled to two-color DNA microarray analysis. We found global changes emerging around 12 weeks after initial exposure. These epigenetic changes appear progressive—the degree of epigenetic change increases at the individual targets with time. The epigenetic changes also are stable—after malignant transformation, the toxicant can be removed, but the malignant phenotype as well as the epigenetic changes remains. Some of the epigenetic changes identified were in genes overtly relevant to the malignant phenotype and have functional roles in cancer in general, and bladder cancer in particular [41], while the roles for most of the changes seen remain enigmatic. It appears unlikely that the observed epigenetic changes seen in UROtsa following arsenical exposure are simply due to the outgrowth or simple selection of a preexisting clone, since the arsenical-transformed cells grow significantly faster (~35%) than the non-malignant parental UROtsa cell line. Rather, it seems possible that (epi)genetic alterations may arise during and as a result of arsenic exposure, and given enough time (cell divisions), which is provided by the cell immortality, and optimal growth

conditions, a faster growing, more malignant population of cells emerges, which are then selected for based on their growth characteristics.

Probing the DNA methylation profile of the arsenical transformed UROtsa cells and comparing them to the non-transformed immortal parental cells revealed that ~3% of the assessed regions were hypermethylated, while ~1% were hypomethylated. The hypermethylation events occurred mostly within gene promoters, whereas the hypomethylation events were more prevalent in repetitive elements spread throughout the genome [42], consistent with what is well established for human cancers. We attempted to assess whether the DNA methylation changes acquired during malignant transformation were specifically or randomly distributed in the genome by analyzing two different arsenical-transformed UROtsa cell lines, created in two different laboratories using two different arsenicals (i.e., sodium arsenite and monomethyl arsenous acid). A statistical analysis of the numerical size of the overlap of aberrantly DNA methylated promoters between these two cell lines indicates that the DNA methylation changes seen are nonrandom and suggest that common epigenetic changes occur in association with arsenical malignant transformation.

The types of DNA methylation changes observed during the arsenical-mediated malignant transformation can be roughly divided into two groups, focal and long range. Focal DNA methylation events refer to DNA differentially methylated regions that cover a single gene promoter and are typically ≤1 kb in size. These types of aberrant DNA hypermethylation events seem to predominate and are closely linked to the silencing of a large number of tumor suppressor genes. In the UROtsa malignant transformation model, several potential tumor suppressor genes were found to be hypermethylated such as DBCCR1 (deleted in bladder cancer chromosome region candidate1); its relevance to bladder cancer having been previously ascertained [41]. Overall, the DNA hypermethylation changes were correlated to corresponding losses in the permissive histone modification marks of histone acetylation and H3K4 methylation and loss of gene expression, although as is often the case, apparent exceptions to the general rules could also be detected.

The DNA differentially methylated regions that cover much larger contiguous regions, along with corresponding changes in histone modifications, are linked to chromatin remodeling of more extended regions of the genome in a process termed long-range epigenetic silencing [43]. This type of epigenetic lesion has been found in a number of human cancer cell lines as well as clinical tumor specimens, suggesting that this type of coordinate epigenetic regulation over large regions may be a common and important event in cancer [43–46]. Interestingly, it appears that the gain of aberrant agglomerative DNA methylation changes and associated long-range epigenetic silencing can be observed over the time course of arsenical-mediated transformation of UROtsa from a benign to a malignant phenotype. Recent studies in the laboratory indicate that the PCDH and HOXC gene clusters undergo extensive aberrant DNA and that these epigenetic lesions are also found in malignant human bladder cancer specimens. Overall, these results suggest that the UROtsa malignant transformation model may be a laboratory tool to discern the molecular underpinnings responsible for long-range epigenetic silencing and identifies a

significant environmental toxicant as a possible etiologic agent of this pathologic epigenetic lesion.

In an initial measure evaluating the commonality of the epigenetic change in arsenical-induced malignant transformation, we sought other human epithelial cell line models of arsenical-mediated malignant transformation. The immortalized human prostate epithelial cell line RWPE-1 was shown to undergo genomic hypomethylation after chronic exposure to AsIII [47, 48], and we have made preliminary comparisons between this model and the UROtsa model. We have found a significant overlap in gene promoters targeted for aberrant DNA methylation in both the UROtsa and RWPE models of arsenical-mediated malignant transformation that is beyond what is expected by random chance. These results suggest that a common ground of epigenetic change occurs in these laboratory models of arsenical exposure and suggests that they may be useful to help identify new epigenetically targeted genes important to malignant transformation and the cellular processes responsible for these epigenetic changes.

Epigenetic regulation resides at a nexus of gene–environment interactions. Together these results suggest that environmental arsenicals may exert their carcinogenic activity by eliciting epigenetic change thereby acting as an epimutagen, an agent whose exposure induces stable and heritable changes to the epigenetic state. The epigenetic changes seen are linked to gene expression changes and coincide with the advent of an increasingly malignant phenotype. Furthermore, results from epigenome-wide analysis suggest that common regions are epigenetically targeted during arsenical-mediated malignant transformation. Importantly, the DNA methylation changes seen in the laboratory models are consistent with what is seen in the relevant in vivo correlates—clinical cancer specimens. These experimentally tractable systems provide a unique opportunity to better discern the causes and consequences of epigenetic change in arsenical-associated cancers.

9.5 Epigenetic Models of Finite Life span to Immortalization (and Beyond)

A cell model we have found particularly useful to study the epigenetics of cell transformation is the human mammary epithelial cell (HMEC) model system developed by Dr. Martha Stampfer during the past 30 years [9, 44, 49, 50]. The utility of this model system for the examination of the early molecular events in human breast carcinogenesis has been demonstrated in a number of studies, both with respect to genetic and epigenetic events [49–53]. In our estimation this isogenic cell model system offers a number of benefits and allows for the temporal analysis of molecular events that occur during the transitions from finite life span through immortalization and on to malignant transformation. This model also allows one to study the effects that directed genetic changes and environmental stressors can have on the epigenetic state.

In this model system, cultured finite life span HMEC must overcome two distinct proliferation barriers in order to achieve immortality and ultimately acquire a malignant phenotype. The first proliferation barrier is termed stasis or stress-induced senescence and is mediated by the Rb protein, characterized by elevated levels of p16INK4A. This first barrier, stasis, has been overcome or bypassed in cultured HMEC by various means, such as exposure to benzo(a)pyrene. The resultant post-stasis cells commonly show p16 inactivation by gene mutation or promoter hypermethylation [50, 54]. Loss of p16 expression due to silencing or mutation is also a common event during in vivo human breast cell transformation [55]. When grown in a serum-free medium, rare HMEC will "spontaneously" silence p16, generating a type of post-stasis HMEC population that has been called post-selection [9, 54]. HMEC that escape the stasis barrier can continue to proliferate for dozens of additional population doublings before encountering a second more stringent proliferation barrier resulting from critically shortened telomeres [49, 56]. When approaching the telomere dysfunction barrier, HMEC exhibit increased chromosomal instability and a DNA damage response. Rare cells that gain telomerase expression may escape this barrier and become immortal, whereby HMEC activates telomerase by as yet undefined, and potentially novel, epigenetic mechanisms. In addition, HMEC systems can acquire immortality through genetic perturbations. For example, under appropriate circumstances direct genetic introduction of constructs that express CMYC, or ZNF217, hTERT can promote HMEC immortalization [57, 58]. Nondirected mutagenesis can also promote HMEC immortalization, as evidenced by the effects of the complete carcinogen benzo(a)pyrene on HMEC. This limitless replicative potential allows for the acquisition and accumulation of additional epigenetic and genetic events that promote the development of additional malignant properties [50, 59–61].

We have used this HMEC model system to begin to develop a timeline of the DNA methylation changes that occurs over the course of multistep breast carcinogenesis, with a particular interest on the earliest stages of the process. Figure 9.2 shows a generalized view of cells we have analyzed, their temporal position in relation to the cellular proliferation barriers, the approximate clinical correlates, and the timing of DNA methylation changes. This figure is an example and not an exhaustive or detailed review of the HMEC strains and cell lines or the multiple treatments and exposures used to create them, and for a more detailed view one can see [62] or visit http://hmec.lbl.gov/mindex.html. In our initial studies using this model system, DNA methylation state was determined using 5-methylcytosine antibody immunopreciptations (MeDIP) coupled to two-color hybridization on a custom 13,500 element human gene promoter microarray and verified using the orthogonal technology of mass spectrometric analysis using Sequenom MassArray [63].

Overall, in this model we observed a stepwise progression of DNA methylation changes with each step coinciding with overcoming a cellular proliferation barrier [62]. In HMEC that overcame stasis produced by stress-inducing serum-free medium, we found, in addition to p16 methylation, hundreds of other differentially methylated regions in the post-stasis cells when compared to pre-stasis cells, representing approximately 2% of all gene promoters on the microarray. These DNA

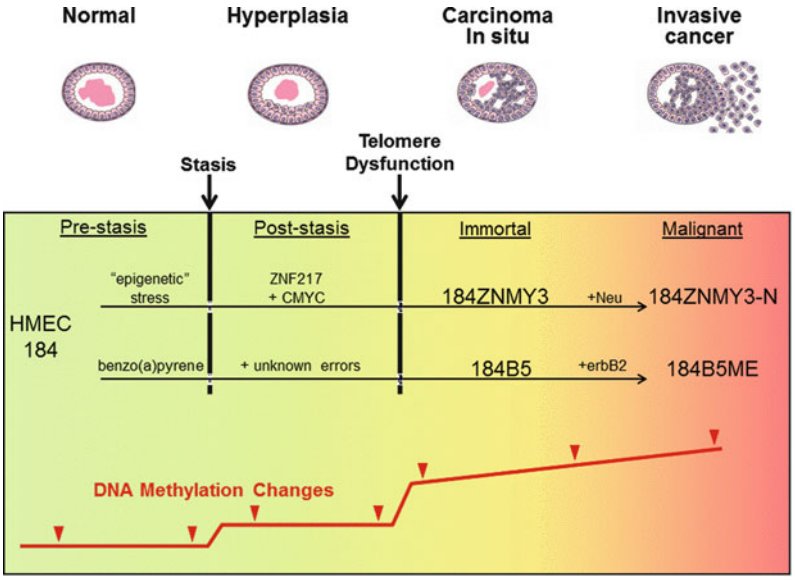

Fig. 9.2 Schematic representation of breast cancer progression and the timing of the underlying DNA methylation changes, with connections between the in vitro HMEC model system and clinical progression based on earlier work [51, 56, 65]. *Top*, the clinical correlates of the HMEC system in relation to the temporal position of the two epithelial cell proliferation barriers of stasis and telomere dysfunction that divides the timeline into pre-stasis, post-stasis, immortal, and malignant epithelial cells. *Middle*, a very simplified view and two examples of HMEC culture models, and the treatment or genetic manipulations used to generate these models. *Bottom*, the timeline of DNA methylation changes identified during the passage of finite life span HMEC through stasis, telomere dysfunction, and culminating in a malignant phenotype. *Arrows* on the DNA methylation changes *curve* show the time points analyzed for DNA methylation state

methylation events were both of the focal and long-range variety. Considering that probably 5–10% of gene promoters in malignant cancer cells show aberrant DNA methylation, a considerable number of DNA methylation changes may occur very early in multistep breast carcinogenesis, and these changes are coincident with overcoming the critical Rb/p16 cell proliferation barrier. Since a majority of the DNA methylation changes seen in the transition of HMEC from pre-stasis to post-stasis in this setting are also seen in malignant breast cancer cell lines and tumor specimens, this transition through the stasis proliferation barrier may represent a critical early event in some pathways of human breast carcinogenesis.

It is worth noting here that current commercial sources of HMEC appear to be of this post-stasis (or post-selection or variant) stage, since these HMEC are produced via the process described above—post-stasis cells that emerge from serum-free media induced stress. As such, the commercially available HMEC may have not only undergone p16 DNA methylation, but are likely to have also acquired hundreds of additional aberrant DNA methylation events [62]. As such, caution should be exercised when evaluating the epigenetic state of primary epithelial cells and considering what is epigenetically "normal."

HMEC that become post-stasis following exposure to the genotoxin and complete carcinogen benzo(a)pyrene showed more than an order of magnitude reduction in DNA differentially methylated regions when compared to the DNA methylation changes induced by stressful serum-free growth conditions. Similarly, HMEC that became post-stasis following genetic knockout of p16 using p16-targeted shRNA have very few DNA methylation changes, underscoring the functional importance of p16 in the first growth barrier. The few DNA methylation changes seen in the benzo(a)pyrene and p16 shRNA-treated cell lines suggest that different pathways through the stasis barrier will have distinct effects on the epigenetic state.

A second step of epigenetic change occurs when telomere dysfunction is overcome and cells acquire immortality. Regardless of the mechanism by which cells pass through telomere dysfunction, hundreds of DNA methylation changes occur. Similar to the DNA methylation changes acquired during the pre-stasis to post-stasis transition, changes that occur during the transition from finite life span to immortal can be focal (≤1 kb) and limited to a single gene or the changes can represent examples of long-range epigenetic silencing and cover extended regions of the genome [64].

These changes seen in the premalignant stages represented by the HMEC model show significant overlap to the DNA methylation changes seen in other human breast cancer cell lines and clinical tumor specimens. Overall, results from the studies using the HMEC model indicate that epigenetic changes occur in a stepwise fashion at critical junctions in the path to cell immortality. These results are consistent with an epigenetic progenitor model where epigenetic changes may occur early, in a stepwise fashion, can precede genetic mutation and allow for an expansion of epigenetically compromised population of cells. The large number of genes affected by epigenetic changes during the transitions through proliferation barriers can provide a foundation for the phenotypic variability and biologic heterogeneity often seen in clinical disease. The DNA methylation changes identified can potentially provide a bank of epigenetic biomarkers for assessing breast cancer risk in premalignant lesions and provide targets for therapeutic interventions.

9.6 Conclusion

In summary, complex and intertwined epigenetic changes occur during multistep carcinogenesis. These changes may be viewed as epigenetic lesions and exist in the genome in a number of forms, from focal to long range. The scope of the epigenetic lesions is likely due to multiple distinct inputs: genetic, such as mutations to chromatin modifier genes; physiologic, such as hormonal and nutritional state; and environmental, such as toxicant exposures. Experimentally tractable laboratory model systems that accurately reflect clinical cancer have been developed and allow for investigations into the causes and consequences of epigenetic change during cell transformation. Results from these systems suggest that early critical epigenetic

events occur prior to cell immortalization and coincide with the transition through well-defined barriers of cell proliferation. Following immortalization, laboratory models suggest that cells can be induced towards malignancy by a variety of stimuli, and that the epigenetic changes arise in a seemingly more progressive smoother fashion, as opposed to the stark stepwise events prior to immortalization. It is hoped that developing a clearer understanding of the identity, timing, and consequences of these epigenetic lesions will prove useful in future clinical applications that range from early disease detection to therapeutic intervention in malignant cancer.

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References

1. Clark SJ et al (1994) High sensitivity mapping of methylated cytosines. Nucleic Acids Res 22(15):2990–2997
2. Frommer M et al (1992) A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. Proc Natl Acad Sci USA 89(5):1827–1831
3. Lister R et al (2009) Human DNA methylomes at base resolution show widespread epigenomic differences. Nature 462(7271):315–322
4. Maunakea AK et al (2010) Conserved role of intragenic DNA methylation in regulating alternative promoters. Nature 466(7303):253–257
5. Drew Y et al (2011) Therapeutic potential of poly(ADP-ribose) polymerase inhibitor AG014699 in human cancers with mutated or methylated BRCA1 or BRCA2. J Natl Cancer Inst 103(4):334–346
6. Hegi ME et al (2005) MGMT gene silencing and benefit from temozolomide in glioblastoma. N Engl J Med 352(10):997–1003
7. Lorenzi PL et al (2009) DNA fingerprinting of the NCI-60 cell line panel. Mol Cancer Ther 8(4):713–724
8. Nims RW et al (2010) Short tandem repeat profiling: part of an overall strategy for reducing the frequency of cell misidentification. In Vitro Cell Dev Biol Anim 46(10):811–819
9. Hammond SL, Ham RG, Stampfer MR (1984) Serum-free growth of human mammary epithelial cells: rapid clonal growth in defined medium and extended serial passage with pituitary extract. Proc Natl Acad Sci USA 81(17):5435–5439
10. Amstad P et al (1988) Neoplastic transformation of a human bronchial epithelial cell line by a recombinant retrovirus encoding viral Harvey ras. Mol Carcinog 1(3):151–160
11. Boukamp P et al (1988) Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. J Cell Biol 106(3):761–771
12. Ke Y et al (1988) Human bronchial epithelial cells with integrated SV40 virus T antigen genes retain the ability to undergo squamous differentiation. Differentiation; research in biological diversity 38(1):60–66
13. Miller FR et al (1993) Xenograft model of progressive human proliferative breast disease. J Natl Cancer Inst 85(21):1725–1732

14. Petzoldt JL et al (1995) Immortalisation of human urothelial cells. Urol Res 23(6):377–380
15. Bello D et al (1997) Androgen responsive adult human prostatic epithelial cell lines immortalized by human papillomavirus 18. Carcinogenesis 18(6):1215–1223
16. Kiyono T et al (1998) Both Rb/p16INK4a inactivation and telomerase activity are required to immortalize human epithelial cells. Nature 396(6706):84–88
17. Dickson MA et al (2000) Human keratinocytes that express hTERT and also bypass a p16(INK4a)-enforced mechanism that limits life span become immortal yet retain normal growth and differentiation characteristics. Mol Cell Biol 20(4):1436–1447
18. Chapman E et al (2006) Expression of hTERT immortalises normal human urothelial cells without inactivation of the p16/Rb pathway. Oncogene 25(36):5037–5045
19. Chang CJ et al (2011) p53 regulates epithelial-mesenchymal transition and stem cell properties through modulating miRNAs. Nat Cell Biol 13(3):317–323
20. Junk DJ et al (2008) Different mutant/wild-type p53 combinations cause a spectrum of increased invasive potential in nonmalignant immortalized human mammary epithelial cells. Neoplasia 10(5):450–461
21. Vrba L et al (2008) p53 induces distinct epigenetic states at its direct target promoters. BMC Genomics 9:486
22. Vahidnia A, van der Voet G, de Wolff F (2007) Arsenic neurotoxicity—a review. Hum Exp Toxicol 10:823–832
23. Rohe G (1896) Arsenic. In: Foster F (ed) Reference-book of practical therapeutics. D. Appleton, New York, p 142
24. Emadi A, Gore SD (2010) Arsenic trioxide—an old drug rediscovered. Blood Rev 24(4–5):191–199
25. Wu D et al (2010) Antitumor effect and mechanisms of arsenic trioxide on subcutaneously implanted human gastric cancer in nude mice. Cancer Genet Cytogenet 2:90–96
26. Yeh K et al (2011) Tumor growth inhibition of metastatic nasopharyngeal carcinoma cell lines by low dose of arsenic trioxide via alteration of cell cycle progression and induction of apoptosis. Head Neck 5:734–742
27. Chen C et al (2007) Arsenic and diabetes and hypertension in human populations: a review. Toxicol Appl Pharmacol 3:298–304
28. Grandjean P, Murata K (2007) Developmental arsenic neurotoxicity in retrospect. Epidemiology 1:25–26
29. Smith A et al (1998) Marked increase in bladder and lung cancer mortality in a region of northern Chile due to arsenic in drinking water. Am J Epidemiol 7:660–669
30. Vahter M (2008) Health effects of early life exposure to arsenic. Basic Clin Pharmacol Toxicol 2:204–211
31. Chen C et al (1992) Cancer potential in liver, lung, bladder and kidney due to ingested inorganic arsenic in drinking water. Br J Cancer 5:888–892
32. IARC Working Group on the Evaluation of Carcinogenic Risks to Humans (2004) Some drinking-water disinfectants and contaminants, including arsenic. IARC Monogr Eval Carcinog Risks Hum 84:1–477
33. Rossman TG et al (1980) Absence of arsenite mutagenicity in *E. coli* and Chinese hamster cells. Environ Mutagen 2(3):371–379
34. Zhao C et al (1997) Association of arsenic-induced malignant transformation with DNA hypomethylation and aberrant gene expression. Proc Natl Acad Sci USA 20:10907–10912
35. Achanzar W et al (2002) Inorganic arsenite-induced malignant transformation of human prostate epithelial cells. J Natl Cancer Inst 24:1888–1891
36. Bredfeldt T et al (2006) Monomethylarsonous acid induces transformation of human bladder cells. Toxicol Appl Pharmacol 1:69–79
37. Chang Q et al (2010) Reduced reactive oxygen species-generating capacity contributes to the enhanced cell growth of arsenic-transformed epithelial cells. Cancer Res 70(12):5127–5135
38. Pi J et al (2008) Arsenic-induced malignant transformation of human keratinocytes: involvement of Nrf2. Free Radic Biol Med 45(5):651–658

39. Sens D et al (2004) Inorganic cadmium- and arsenite-induced malignant transformation of human bladder urothelial cells. Toxicol Sc 1:56–63
40. Dinney CP et al (2004) Focus on bladder cancer. Cancer Cell 6(2):111–116
41. Nishiyama H et al (2001) Negative regulation of G(1)/S transition by the candidate bladder tumour suppressor gene DBCCR1. Oncogene 23:2956–2964
42. Jensen TJ et al (2009) Arsenicals produce stable progressive changes in DNA methylation patterns that are linked to malignant transformation of immortalized urothelial cells. Toxicol Appl Pharmacol 241(2):221–229
43. Frigola J et al (2006) Epigenetic remodeling in colorectal cancer results in coordinate gene suppression across an entire chromosome band. Nat Genet 38(5):540–549
44. Novak P et al (2006) Epigenetic inactivation of the HOXA gene cluster in breast cancer. Cancer Res 66(22):10664–10670
45. Rauch T et al (2007) Homeobox gene methylation in lung cancer studied by genome-wide analysis with a microarray-based methylated CpG island recovery assay. Proc Natl Acad Sci USA 104(13):5527–5532
46. Stransky N et al (2006) Regional copy number-independent deregulation of transcription in cancer. Nat Genet 38(12):1386–1396
47. Benbrahim-Tallaa L et al (2005) Molecular events associated with arsenic-induced malignant transformation of human prostatic epithelial cells: aberrant genomic DNA methylation and K-ras oncogene activation. Toxicol Appl Pharmacol 3:288–298
48. Coppin J, Qu W, Waalkes M (2008) Interplay between cellular methyl metabolism and adaptive efflux during oncogenic transformation from chronic arsenic exposure in human cells. J Biol Chem 28:19342–19350
49. Garbe JC et al (2007) Inactivation of p53 function in cultured human mammary epithelial cells turns the telomere-length dependent senescence barrier from agonescence into crisis. Cell Cycle 6(15):1927–1936
50. Stampfer MR, Bartley JC (1985) Induction of transformation and continuous cell lines from normal human mammary epithelial cells after exposure to benzo[a]pyrene. Proc Natl Acad Sci USA 82(8):2394–2398
51. Chin K et al (2004) In situ analyses of genome instability in breast cancer. Nat Genet 36(9):984–988
52. Holst CR et al (2003) Methylation of p16(INK4a) promoters occurs in vivo in histologically normal human mammary epithelia. Cancer Res 63(7):1596–1601
53. Li Y et al (2007) Transcriptional changes associated with breast cancer occur as normal human mammary epithelial cells overcome senescence barriers and become immortalized. Mol Cancer 6:7
54. Brenner AJ, Stampfer MR, Aldaz CM (1998) Increased p16 expression with first senescence arrest in human mammary epithelial cells and extended growth capacity with p16 inactivation. Oncogene 17(2):199–205
55. Geradts J, Wilson PA (1996) High frequency of aberrant p16(INK4A) expression in human breast cancer. Am J Pathol 149(1):15–20
56. Romanov SR et al (2001) Normal human mammary epithelial cells spontaneously escape senescence and acquire genomic changes. Nature 409(6820):633–637
57. Nonet GH et al (2001) The ZNF217 gene amplified in breast cancers promotes immortalization of human mammary epithelial cells. Cancer Res 61(4):1250–1254
58. Stampfer MR et al (2001) Expression of the telomerase catalytic subunit, hTERT, induces resistance to transforming growth factor beta growth inhibition in p16INK4A(−) human mammary epithelial cells. Proc Natl Acad Sci USA 98(8):4498–4503
59. Clark R et al (1988) Transformation of human mammary epithelial cells by oncogenic retroviruses. Cancer Res 48(16):4689–4694
60. Olsen CL et al (2002) Raf-1-induced growth arrest in human mammary epithelial cells is p16-independent and is overcome in immortal cells during conversion. Oncogene 21(41): 6328–6339

61. Stampfer MR, Yaswen P (2003) Human epithelial cell immortalization as a step in carcinogenesis. Cancer Lett 194(2):199–208
62. Novak P et al (2009) Stepwise DNA methylation changes are linked to escape from defined proliferation barriers and mammary epithelial cell immortalization. Cancer Res 69(12):5251–5258
63. Ehrich M et al (2005) Quantitative high-throughput analysis of DNA methylation patterns by base-specific cleavage and mass spectrometry. Proc Natl Acad Sci USA 102(44): 15785–15790
64. Coolen MW et al (2010) Consolidation of the cancer genome into domains of repressive chromatin by long-range epigenetic silencing (LRES) reduces transcriptional plasticity. Nat Cell Biol 12(3):235–246
65. Walen KH, Stampfer MR (1989) Chromosome analyses of human mammary epithelial cells at stages of chemical-induced transformation progression to immortality. Cancer Genet Cytogenet 37(2):249–261

Chapter 10
Epigenetic Reprogramming of Mesenchymal Stem Cells

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Abstract Mesenchymal stem cells (MSCs) are multipotent stem cells of mesodermal origin that can be isolated from various sources and induced into different cell types. Although MSCs possess immune privilege and are more easily obtained than embryonic stem cells, their propensity to tumorigenesis has not been fully explored. Epigenomic changes in DNA methylation and chromatin structure have been hypothesized to be critical in the determination of lineage-specific differentiation and tumorigenesis of MSCs, but this has not been formally proven. We applied a targeted DNA methylation method to methylate a Polycomb group protein-governed gene, *Trip10*, in MSCs, which accelerated the cell fate determination of MSCs. In addition, targeted methylation of *HIC1* and *RassF1A*, both tumor suppressor genes, transformed MSCs into tumor stem cell-like cells. This new method will allow better control of the differentiation of MSCs and their use in downstream applications.

10.1 Introduction

Mesenchymal stem cells (MSCs) are somatic stem cells that can be isolated from various sources including bone marrow and fat tissue [80, 99]. Although MSCs possess more restricted pluripotency than embryonic stem (ES) cells, MSCs can still be induced to adipocytes, muscles, liver, bones, and neurons in vitro [55, 72, 73], making them a candidate for future cell therapy. From a safety consideration, there are

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debates about whether the MSCs could be transformed in vivo and whether they might be supportive or suppressive to tumoral growth [39, 88, 139]. Therefore, if the application and safety of MSCs could be monitored and well controlled, the application of MSCs will be broadened further.

Epigenetic regulation, including DNA methylation, histone modifications, and microRNAs (miRNAs), possesses the power to maintain the self-renewal or control the differentiation of stem cells [16, 32, 37, 69, 131]. Studies of ES cells have revealed the critical role of epigenetic regulation in controlling cell fate [44, 76, 107, 142]. Because there are almost no coding sequence differences between the ES cells and their derived cells, the differences between these cells are likely to come from differential gene expression [32, 47, 123]. The same rationale has prompted the use of epigenomic modifications as molecular codes to distinguish ES cells, MSCs, and their derived somatic cells. If the rationale were valid and the differences among different cell types originated from the epigenomic modifications, these distinct epigenetic states could represent the "stemness" in MSCs and ES cells, and changes of these epigenetic states might direct/interfere with the MSC differentiation.

Polycomb group proteins such as EZH2 and YY1 regulate part of the bivalent marks that represent the stemness in stem cells [119, 122]. There are loci in ES cells and MSCs associated with both active histone marks like histone 3 lysine 4 trimethylation (H3K4me3) [24, 42, 92] and repressive marks like histone 3 lysine 27 trimethylation (H3K27me3), and these are designated as bivalent loci [43, 114, 133]. These bivalent loci are often silenced [66] but are hypomethylated [134]. Among the histone marks, H3K27me3 is the substrate of Polycomb group proteins and loss of the maintenance of this histone mark is associated with the differentiation of stem cells [1, 21, 53, 70, 86]. These loci can be further activated by the association of active transcription factors and histone modifications like acetylation [61, 71, 94, 111], while their silencing could be further enhanced by DNA methylation in other lineage [5, 31, 48, 79, 87, 108, 109, 111, 118, 128, 138]. The identification of the epigenomic modifications within the bivalent loci could then reveal the ultimate fate of lineage-determining genes.

DNA methylation is one of the most dominant gene silencing mechanisms in cells and changes of methylation states correlate with the switch in cell lineages [58, 110]. It is known that changes in methylation states are inversely correlated with the expressions of corresponding genes, but the changed methylation status may not change cell fate directly. Therefore, a method that can methylate target genes and subsequently change cell fate would be an important demonstration that DNA methylation changes are sufficient to regulate cell fate decisions.

For instance, *Trip10* locus was identified as the target of Polycomb group protein and modified by DNA methylation during MSC differentiation [55]. Methylation of *Trip10* appears to be cell type specific in normal tissues as well as in cancers [55, 57]. This information suggests that *Trip10* methylation might be lineage specific and the targeted methylation of *Trip10* might then be able to direct MSC differentiation. When *Trip10* was methylated in MSCs, the MSC differentiation lineages were limited [55]. The success of the forward evaluation of the cell fate determination by

DNA methylation also opens the gateway to finetune MSC differentiation. On the other hand, the tumor suppressor genes might not have bivalent marks and methylation of these loci may lead to cell transformation. As demonstrated in a recent report, *hypermethylated in cancer* (*HIC1*) and *RassF1A* are two tumor suppressor genes that are not associated with bivalent histone marks and their methylation could transform MSCs [125].

10.2 Mesenchymal Stem Cells

MSCs can be isolated from various sources including bone marrow, adipose tissue, liver, muscle, amniotic fluid, dental pulp, placenta, and umbilical cord blood; the properties among these MSCs seem to vary accordingly [9, 49, 82, 100, 105, 113, 120, 143]. Different cell surface markers identified from different MSCs are reflective of their propensity to differentiate into different cell lineages [19, 34, 98]. Because MSCs can differentiate into different cell types in vitro, it is believed that there are common gene expression repertoires among these MSCs to maintain their stemness, but there are also different gene expression signatures that define the identities and differentiation potentials of different MSCs. Thus understanding the molecular mechanisms underlying the maintenance of cellular identities and determination of cell lineages is critical for the future clinical use of MSCs.

Unregulated differentiation is another reason to decipher the molecular codes that characterize MSCs. Different routes of transplantation make isolated clones of MSCs possess varied degrees of differentiation capacities, and dysregulation of these processes might consequently lead to disease. For example, MSCs together with or without hematopoietic stem cells (HSCs) isolated from bone marrow can be transplanted and differentiated into lung, gut, skin [68], liver and biliary epithelium [68, 97, 126, 127], skeletal myoblast [41, 46], neuroectodermal cells [18, 106], and endothelium [4, 77, 144]. These co-transplantation results imply that there are molecular machineries that might be used to define the MSCs and their derived lineages. These molecular codes also respond to neighboring cells and/or microenvironment of MSCs to maintain or differentiate cell fates. The importance of interplay with the environment is also evident by the reports that MSCs can either inhibit or support tumor growth in a cell setting-specific manner [67, 116]. The other controversy is that MSCs are proposed to both boost the immune system and suppress it [105, 129]; thus, the clinical safety of MSCs remains to be clarified.

10.3 Epigenetic Regulation and the Maintenance of MSC

Stemness needs to be maintained when the stem cells are self-renewing [50, 109, 121]. Since the coding sequences are all the same within ES cells, MSCs, and the differentiated somatic cells, there ought to be other somatic inheritable marks that

could represent the maintenance of stemness. Epigenetic marks are somatically inheritable modifications that regulate gene expression but do not change the associated gene sequence. These cellular epigenetic marks, while they can be reshaped by the environmental factors like diet and growth factors, in general are faithfully passed on to the descended lineage of cells. These properties make the epigenomic marks good candidates for the control of cellular stemness.

Bivalent loci in the stem cells are associated with both active and repressive epigenetic marks and are critical for cellular differentiation [6, 16, 32, 91]. Interactions between different epigenetic modifications can lead the governed genes to become permanently silenced or activated. The Polycomb group proteins, and associated histone modifications like H3K27me3, are one of the representative markers that are associated with stemness [20, 28, 45, 101]. Polycomb group proteins are reported to mediate the transition between the transcriptional silencing and active states of the associated gene [95] and their transitional regulatory role is evidenced by the co-existence of enhancer and suppressor genetic modifier phenotypes when the Polycomb group proteins lost their functions [85]. H3K27me3-associated loci can be further silenced by other epigenetic modifications including DNA methylation and the formation of heterochromatin [6, 62, 84, 137, 141]. On the other hand, the repressive trimethylation can be demethylated and the associated genes can then be reactivated. Loss of maintenance of these trimethylation states leads to differentiation of stem cells, which strongly suggests that maintaining these bivalent marks is critical for the maintenance of stemness [1, 21, 53, 70, 86].

Bivalent loci have been profiled in ES cells, tumors, differentiated cells, and MSCs [6]. Because the identified bivalent loci are different among these cells, the data support the hypothesis that these bivalent loci represent the unique stemness state in different cell types. From a direct comparison, there are more shared bivalent marks between ES cells and tumors than between the differentiated tissues and tumors, suggesting that tumors might be evolved from cells with more stem-like marks, and inappropriate maintenance of these marks could cause devious cell fate changes [23, 96, 132].

The bivalent loci in MSCs also mark developmentally important genes and can be further modified epigenetically [55]. The epigenetic marks on the MSC bivalent loci are distinct from those in the ES cells and differentiated cells. The bivalent loci that reside within the MSCs are often low in DNA methylation (hypomethylated) and can be further methylated or activated. The number and function of these bivalent genes might limit the lineages into which the MSCs can differentiate. It has been reported that undifferentiated MSCs contain both repressive and active chromatin marks on β-catenin-bound *c-myc* and *cyclin D* promoters [15, 35, 36]. When these MSCs became lineage committed, e.g., osteogenic, H3K4me3 was lost. This example indicates that epigenetic modifications regulate the Wnt signaling pathway in MSC, and similar epigenetic modifications are found in ES cells as well. We identified the H3K27me3-associated loci in MSCs that are differentially methylated when the MSCs are differentially induced into hepatocytes or adipocytes [55]. Loci that are not associated with DNA methylation association protein, MeCP2, were

considered hypomethylated. We found more than 383 of these bivalent loci are further associated with MeCP2 and proved to be methylated in either MSC-derived hepatocytes or adipocytes [55]. Therefore, these bivalent loci in MSCs might mark the lineages into which the MSCs are differentiated, and the later-added DNA methylation might further strengthen the cell fate evolution.

10.4 DNA Methylation and the Differentiation of MSC

DNA methylation is one of the most dominant silencing epigenetic modifications and occurs at the CpG dinucleotide in the human genome. A high frequency of CpG dinucleotides is often found at the promoter and/or first exon of genes and are named CpG islands [10, 12, 33]. Up to now, almost all the identified DNA methylation at the CpG islands silence the associated genes [11, 13, 124]. DNA methylation is a reversible event [8, 29, 60], and the removal of the silencing mark is critical for the activation of the associated genes [74, 75]. Compared with histone deacetylation inhibitors that cause less significant gene activation, demethylation induced by 5-aza-2′-deoxycytidine (5-Aza), a DNA methylation inhibitor, often causes a greater extent of restoration of gene expression [22]. Our previous results also indicated that when the estrogen receptor (ER)-targeted genes were silenced long term by DNA methylation, adding estrogen and/or overexpression of ER was insufficient to reactivate the ER target genes. Only after the DNA methylation was removed, could the expression of ER target genes be restored by the stimuli of estrogen [75]. Also, global demethylation results in global reactivation of the expression of these genes [74]. These observations all indicate that DNA methylation is a dominant silencing mark; its appearance leads to the silenced locus and the changes in methylation states reflect the changes in cellular physiology.

Altered DNA methylation status often correlates with the normal differentiation or the onset of diseases like cancer. DNA methylation is now considered a reliable biomarker and the profiling of methylation changes can be used to probe cellular or pathological events. Environmental factors relay their influence into the cells through specific signaling pathways. These influences are then recorded as epigenetic marks like DNA methylation during cell passages and are further selected in the descended population of cells. For example, when ER was knocked down by siRNA in a breast cancer cell line that once expressed ER, the downstream ER target/regulated genes were silenced gradually by various epigenetic marks, and later by DNA methylation [75]. DNA methylation also was accumulated within the ER target loci when the ER-expressing breast cancer cells were cultured long term in an estrogen-deprived environment. The recruitment and accumulation of DNA methylation within the estrogen signaling pathway left specific marks for us to track cell lineage which previously encountered the changed cellular environment. Evidence from genetic models also indicates that the environmental factors work through different signaling pathways and leave different but traceable patterns of DNA methylation. When signals like MYC or P53 were genetically manipulated, specific sets

of genes were methylated in the descended mice [93]. Therefore, the accumulated DNA methylation does not appear to occur at random.

Methylation changes caused by environmental changes like diet can be inherited and may influence cellular physiology as well as the onset of disease. The cofactor for DNA methylation reactions, *S*-adenosyl-methionine (SAM), is produced from dietary folate, and this provides the opportunity for diet to influence DNA methylation [25, 65, 104, 115, 117]. Mammals go through two genomic methylation revolutions during their development: one is during their formation of gametes, the other is directly after the fertilization is complete [64, 102, 112]. DNA methylation is erased during these two stages and re-established according to their paternal or maternal origins [136]. An elegant experiment in which pregnant mice were fed with various concentrations of food that could be converted into corresponding concentrations of SAM caused varied degrees of methylation. The newborn mice showed different degrees of fur color according to the concentration of methyl-supplemented diet consumed by the mothers, and these patterns of color lasted throughout their lives [38, 83, 89, 135]. In this example, environmental factors influenced methylation memories and changed the phenotype of the individuals in a somatically heritable way.

There is evidence indicating that changes in DNA methylation might be involved with the cell fate changes in MSCs as well. The methylation states within somatic stem/progenitor cells are different from the ones in ES cells and differentiated cells. For example, the promoter regions of *OCT4*, *NANOG,* and *SOX2* in adipose-derived MSCs display a greater extent of DNA methylation than in ES cells [6]. This methylation difference also provides an explanation for the fact that MSCs have lower differentiation capacity than the ES cells. Also, there are methylation differences within the promoters of tissue-specific genes between the bone- and adipose-derived MSCs; they correlate with their differences in lineage differentiation potential [63]. Osteoblast-specific genes such as *RUNX2* and *BGLAP* are hypermethylated in adipose-derived MSCs as compared to the bone-derived MSCs, whereas *PPARγ2*, the adipocyte-specific gene, is hypomethylated in adipose-derived MSCs [63]. Our previous data also identified a panel of genes that are differentially methylated within the differentiated hepatocytes or adipocytes when compared to the bone marrow-derived MSCs [55]. Taken together, DNA methylation status could represent the cellular identities and differentiation potentials of MSCs. It has been reported that global DNA methylation was changed in long-term cultured MSCs that might correlate with their altered differentiation capacity [17]. Changes in global methylation caused by demethylation agents have been documented to accelerate the osteogenic [3] or neuronal cell-like [2] differentiation of MSCs. However, it is unclear whether DNA methylation changes are sufficient to set the stage for MSC cell fate changes. It has been reported that predeposited DNA methylation within different isolated MSCs defined the oncogenic SYT-SSX1 fusion protein expression and limited its function in MSCs [30]. On the other hand, methylation profiling of adipogenic promoters from freshly cultured adipose stem cells to the senescence state did not correlate with their reduced differentiation potential [90, 91]. The absence of a targeted methylation method has hindered our understanding of how DNA methylation

determines the cell fate of MSCs. A solution is to find a way to methylate a bivalent gene in MSC and observe if the cell fate changed after targeting.

10.5 TRIP10 as a Model

Trip10 (also known as *CIP4*) encodes Cdc42-interacting protein 4, which was identified to be associated with Cdc42 and to regulate the cytoskeleton and membrane trafficking. Trip10 interacts with the Rho family GTPase TC-10 in adipocytes to regulate the translocation of insulin-stimulated glucose transporter 4 (Glu4) to the plasma membrane and finally to increase the uptake of glucose [26, 81]. In the brain of human Huntington's disease (HD) [52], Trip10 is reported to be a modulator of cell survival in the adjustment of DNA damage [140]. To guard against DNA damage, *Trip10* expression is decreased in hepatocyte growth factor/scatter factor (HGF/SF)-mediated cell protection, but *Trip10* level is significantly increased during hyperbaric oxygen-induced neuroprotection [51]. Overexpression of Trip10 was also observed in human HD brain striatum and the neuronal Trip10 immunoreactivity increased with neuropathological severity in the neostriatum of HD patients [52]. In addition, increased cell death was found in rat striatal neurons transfected with Trip10 [52], suggesting that Trip10 is toxic to striatal neurons. These data suggest that the effect of Trip10 in cell survival and growth is tissue specific. These diverse and sometimes contrary roles of Trip10 could be attributed in part to its splicing variants; equally important is the fact that they are the outcomes between Trip10 interaction with distinct signaling components in different cell settings.

In human bone marrow-derived MSCs, *Trip10* is hypomethylated in the undifferentiated stage and becomes hypermethylated during MSC-to-liver differentiation, but remains hypomethylated during MSC-to-adipocyte differentiation. Therefore, the methylation state of *Trip10* varies in different tissues and becomes a candidate biomarker to track MSC differentiation [55, 57]. We reasoned that the stemness state of Trip10 is maintained by the Polycomb group protein in the MSCs and that changes of chromatin structure, especially by DNA methylation, could restrict the cell lineages of MSCs. The differentiation or death of MSCs was thus predicted to be affected by *Trip10* methylation, and this model could be tested using targeted *Trip10* methylation.

10.6 Targeted DNA Methylation and MSC Differentiation

It has been hypothesized that DNA methylation within certain loci is sufficient to transform or differentiate cells, but this hypothesis had not been proved since there was no method to directly methylate specific loci [54]. Normal or abnormal methylation changes have been identified during cellular differentiation or transformation, but it remains to be elucidated whether all or any of the detected methylation changes

can affect cell fate. Moreover, if we can determine whether DNA methylation within certain loci is sufficient to determine the cell fate, this will provide additional information to evaluate the target genes that control cellular differentiation and transformation.

DNA methylation is initiated and maintained by DNA methyltransferase (DNMT) in mammalian cells [27, 40, 130]. As illustrated in Fig. 10.1a, during the cellular replication, DNMTs are recruited by the semimethylated old template and methylate the newly synthesized strand of DNAs [103]. The newly synthesized strand will then possess the same DNA methylation as the old strand. We reasoned that, by providing a methylated strand of DNA that is complementary with target loci, we might be able to recruit DNMT to the target loci and initiate targeted DNA methylation in the cell (Fig. 10.1b, [55, 56, 78]). A stretch of cloned *Trip10* promoter was in vitro methylated using commercial bacterial methylase, *Sss*I. These methylated inserts were then purified, denatured, and used to transfect MSCs. Unmethylated inserts served as the negative control; they did not induce any methylation at the *Trip10* promoter. Liposome-based transfection agents that were conjugated with florescent compounds were used for transfection in order to calculate the transfection efficiency. Also, the methylated/unmethylated inserts were labeled with Cy-dyes to track if the inserts entered the cell nuclei, because the denatured inserts need to be present and docked in the nuclei for the recruitment of DNMTs. Repeated transfection was needed to ensure the targeted DNA methylation. The promoter insert from another gene like *Casp8AP2* was used as a specificity control, as the methylated *Casp8AP2* inserts did not induce methylation at the *Trip10* promoter [55].

Targeted *Trip10* methylation was detected by semiquantitative methylation-specific PCR and bisulfite sequencing and the reduced *Trip10* expression was determined by RT-PCR and visualized by immunostaining. A two-component reporter gene system was established to validate the methylation-induced silencing at the transcription level and visualize the onset of DNA methylation in live cells. The two-component reporter system [55, 56] consists of two parts: (1) a cloned *Trip10* promoter that is linked with and regulates the expression of the *Tet* repressor (*TetR*) gene; and (2) a CMV promoter that is linked with, and regulates the expression of, the reporter gene enhanced green florescent protein (*EGFP*), with an intervening TetR binding site, $TetO_2$. Both constructs were transfected into a cell line simultaneously and colonies of cells that possess both inserted constructs were selected. Colonies of selected cells were then transfected with in vitro methylated or unmethylated *Trip10* inserts. The unmethylated *Trip10* promoter within the first construct will continue to express *TetR* that in turn represses the expression of *EGFP*. In contrast, targeted DNA methylation at the exogenous *Trip10* promoter silences the *TetR* expression which leads to the expression of the EGFP reporter. This induced EGFP expression could be reversed by adding of 5-Aza, suggesting that the original expression was caused by DNA methylation. With this reporter system, the targeted DNA methylation can be visualized in live cells.

During neuronal induction of MSCs, *Trip10* expression was greatly reduced and its distribution was confined to the peri-nuclei region in these induced cells [57]. Similar to the neuronal induction, targeted *Trip10* DNA methylation caused reduced *Trip10* expression and re-distribution and prompted the MSC-to-neuron

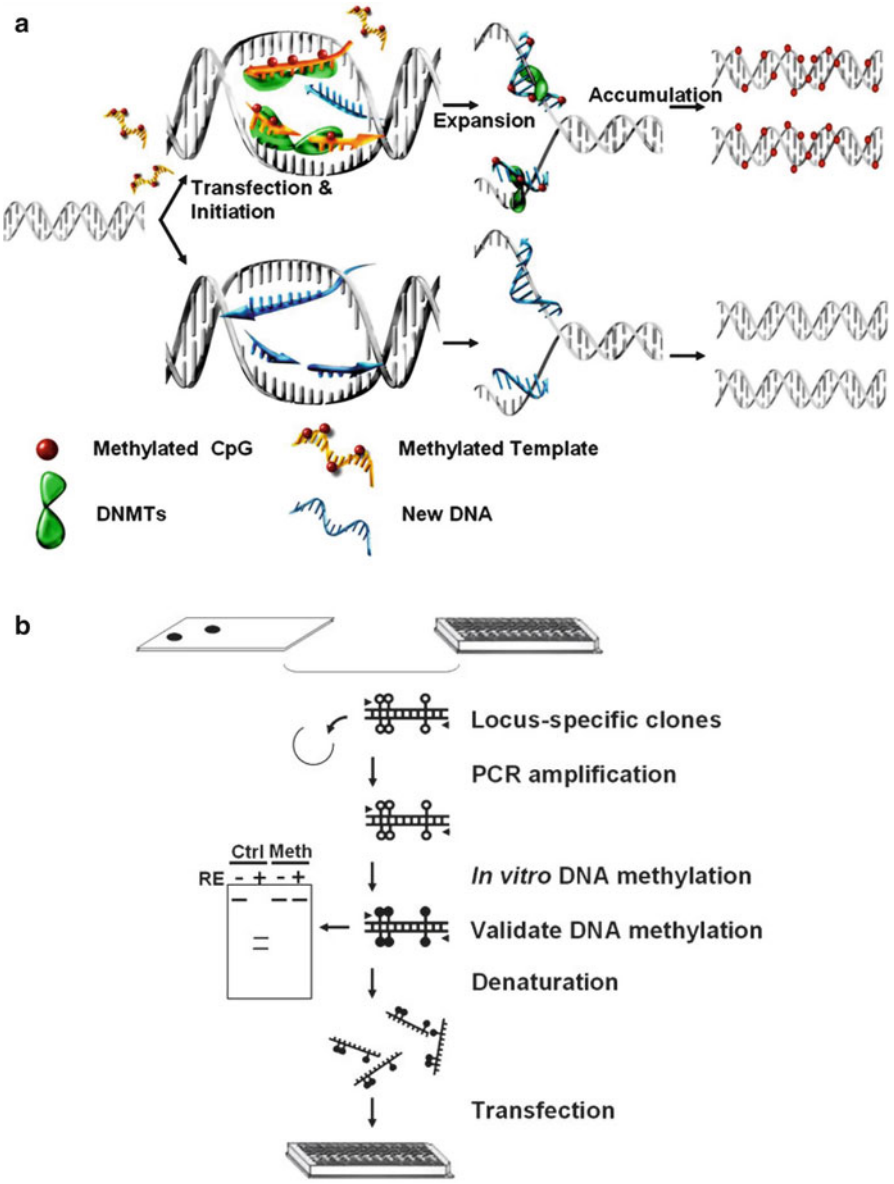

Fig. 10.1 Targeted DNA methylation. (**a**) Illustration of targeted DNA methylation. DNA methylation is maintained by DNMTs during cellular replication. *Upper*, the original unmethylated locus like *Trip10* will not recruit DNMTs to the newly synthesized strands of DNA; therefore, they remain hypomethylated. If the original strand was methylated, then the old template of DNA will recruit DNMTs to the newly synthesizing DNAs and add the methyl group to the new strand of DNAs. Targeted DNA methylation method transfects the cells with a denatured, in vitro methylated DNA with its sequence complemented to the target loci (*upper*). The provided methylated DNAs will pair with the old templates and recruit DNMTs to the newly synthesizing sites and methylate the new strands of DNAs. The seeded DNA methylation then will be spread and maintained during the following replications. (**b**) Flow of targeted DNA methylation (details in text). Templates of targeted DNA methylation could be from the CpG island library or cloned from the cultured cells. RE: methylation sensitive restriction enzymes like *Hpa*II and *BstU*I, etc

differentiation. This preferential cellular differentiation is specific since the same *Trip10* targeted DNA methylation prevented the MSC-to-adipocyte induction (Fig. 10.2a) [55]. These data indicate that DNA methylation within one of the bivalent loci is sufficient to control cellular differentiation.

10.7 DNA Methylation and Tumorigenesis of MSC

It is generally accepted that abnormal hypermethylation of tumor suppressor genes can transform normal cells [7, 12]. To support this theory, *HIC1* and *RassF1A*, two tumor suppressor genes that are methylated in several cancers but are not associated with Polycomb group protein in MSC, were in vitro methylated and then transfected into MSCs. Targeted methylation of *HIC1* or *RassF1A* alone is insufficient to transform the MSCs but concurrent *HIC1* and *RassF1A* methylation transforms the MSCs [125]. However, methylation of nine genes within the Salvador–Warts–Hippo pathway (including *RassF1A*) is insufficient to transform the MSCs [125], indicating that the *HIC1* and *RassF1A* methylation-caused transformation is not random. The transformed MSCs (named *me-H&R* MSCs) can still be differentiated into different cells including osteocytes, neurons, and adipocytes. Immunodeficient mice inoculated with a low number of *me-H&R* MSCs rapidly developed tumors. The developed tumors consisted of several clones of cells that express different cell surface markers, including mesenchymal and epithelial ones. 5-Aza treatment reversed the transformation and the tumoral properties of *me-H&R* MSCs, demonstrating that the transformation was caused by DNA methylation. Taken together, these findings suggest that the *me-H&R* MSCs become cancer stem cell (CSC)-like since they possess both tumoral and stem cell characters [125]. These results also imply that mal-maintained DNA methylation directly contributes to tumorigenesis.

10.8 Application of the Targeted DNA Methylation Technique

Epigenomic profiling in diverse cells including MSCs has revealed many cellular physiologies that are versatile and even personal [14, 17, 55, 59, 116]. Targeted DNA methylation is a direct validation of the profiling results and proves that epigenetic changes like DNA methylation are sufficient to direct MSC differentiation and tumorigenesis. As illustrated in Fig. 10.2a, MSCs could be differentiated into osteocyte, adipocyte, neuron, etc. Targeted *Trip10* methylation limits the differentiation potency of MSCs and accelerates their neural and osteogenic differentiation. On the other hand, targeted *HIC1* and *RassF1A* methylation transforms MSCs into CSC-like cells; targeted DNA methylation within nine loci in the Salvador–Warts–Hippo pathway cannot transform the MSCs but can keep the MSCs proliferating. These results indicate that CSC-like cells might arise from somatic stem cells-like MSCs (Fig. 10.2b), and the tumorigenesis and the immortalization could be dissected by the epigenetic modifications. In summary, using targeted DNA methylation, the differentiation

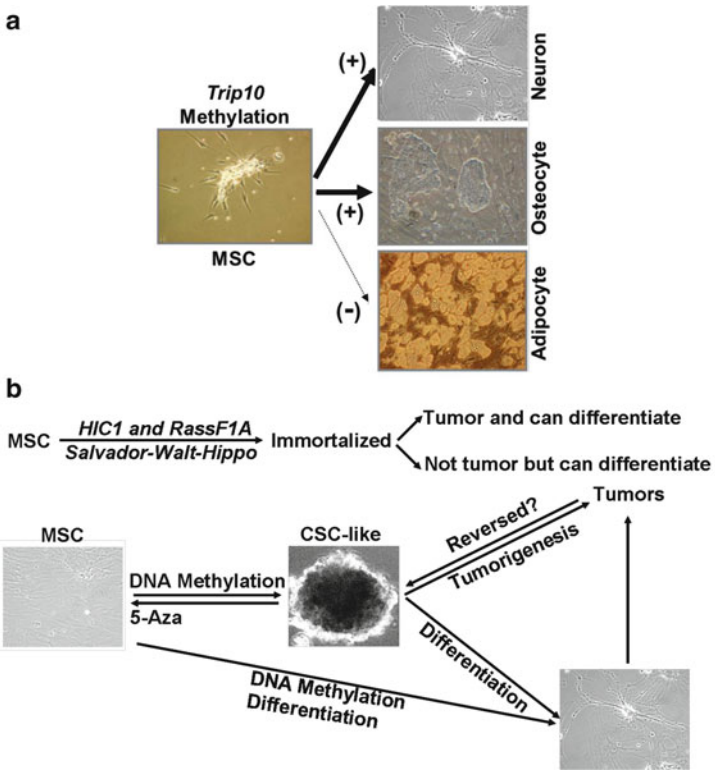

Fig. 10.2 Reprogramming of MSC. (**a**) *Trip10* methylation accelerates MSCs differentiation. Targeted DNA methylation within the *Trip10* promoter accelerates the MSCs to neuron or osteocyte differentiation but blocks their differentiation into adipocytes. (**b**) Summary of MSCs reprogramming. After methylation within the *HIC1* and *RassF1A*, the MSCs became tumors and still can differentiate. After the targeted methylation of nine genes in the Salvador–Warts–Hippo signaling pathway, the MSCs can be stably passaged and differentiated but they are not tumorigenic (*top*). Targeted methylation of *HIC1* and *RassF1A* caused the MSCs to become CSC-like (*bottom*) and this process can be reversed by 5-Aza. The CSC-like MSCs can be further developed into tumor in immunodeficient mice or differentiated into different cells like neurons. Thus, the differentiation, proliferation, and tumorigenic state of MSCs can be controlled by DNA methylation

(*Trip10*), proliferation (Salvador–Warts–Hippo), and tumorigenic (*HIC1* and *RassF1A*) characteristics of MSCs could be revealed.

References

1. Agger K, Cloos PA et al (2007) UTX and JMJD3 are histone H3K27 demethylases involved in HOX gene regulation and development. Nature 449:731–734
2. Alexanian AR (2007) Epigenetic modifiers promote efficient generation of neural-like cells from bone marrow-derived mesenchymal cells grown in neural environment. J Cell Biochem 100:362–371

3. Arnsdorf EJ, Tummala P et al (2010) The epigenetic mechanism of mechanically induced osteogenic differentiation. J Biomech 43:2881–2886
4. Asahara T, Masuda H et al (1999) Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. Circ Res 85:221–228
5. Balch C, Nephew KP et al (2007) Epigenetic "bivalently marked" process of cancer stem cell-driven tumorigenesis. Bioessays 29:842–845
6. Barrand S, Andersen IS et al (2010) Promoter-exon relationship of H3 lysine 9, 27, 36 and 79 methylation on pluripotency-associated genes. Biochem Biophys Res Commun 401:611–617
7. Baylin SB (2005) DNA methylation and gene silencing in cancer. Nat Clin Pract Oncol 2:S4–S11
8. Bender CM, Pao MM et al (1998) Inhibition of DNA methylation by 5-aza-2′-deoxycytidine suppresses the growth of human tumor cell lines. Cancer Res 58:95–101
9. Bianco P, Gehron Robey P (2000) Marrow stromal stem cells. J Clin Invest 105:1663–1668
10. Bird AP (1980) DNA methylation and the frequency of CpG in animal DNA. Nucleic Acids Res 8:1499–1504
11. Bird A (1999) DNA methylation de novo. Science 286:2287–2288
12. Bird A (2002) DNA methylation patterns and epigenetic memory. Genes Dev 16:6–21
13. Bird A, Macleod D (2004) Reading the DNA methylation signal. Cold Spring Harb Symp Quant Biol 69:113–118
14. Bloushtain-Qimron N, Yao J et al (2009) Epigenetic patterns of embryonic and adult stem cells. Cell Cycle 8:809–817
15. Boland GM, Perkins G et al (2004) Wnt 3a promotes proliferation and suppresses osteogenic differentiation of adult human mesenchymal stem cells. J Cell Biochem 93:1210–1230
16. Boquest AC, Noer A et al (2006) Epigenetic programming of mesenchymal stem cells from human adipose tissue. Stem Cell Rev 2:319–329
17. Bork S, Pfister S et al (2010) DNA methylation pattern changes upon long-term culture and aging of human mesenchymal stromal cells. Aging Cell 9:54–63
18. Brazelton TR, Rossi FM et al (2000) From marrow to brain: expression of neuronal phenotypes in adult mice. Science 290:1775–1779
19. Buhring HJ, Battula VL et al (2007) Novel markers for the prospective isolation of human MSC. Ann N Y Acad Sci 1106:262–271
20. Burdach S, Plehm S et al (2009) Epigenetic maintenance of stemness and malignancy in peripheral neuroectodermal tumors by EZH2. Cell Cycle 8(13):1991–1996
21. Burgold T, Spreafico F et al (2008) The histone H3 lysine 27-specific demethylase Jmjd3 is required for neural commitment. PLoS One 3(8):e3034
22. Cameron EE, Bachman KE et al (1999) Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. Nat Genet 21:103–107
23. Cao Q, Yu J et al (2008) Repression of E-cadherin by the polycomb group protein EZH2 in cancer. Oncogene 27:7274–7284
24. Carvin CD, Kladde MP (2004) Effectors of lysine 4 methylation of histone H3 in Saccharomyces cerevisiae are negative regulators of PHO5 and GAL1-10. J Biol Chem 279:33057–33062
25. Caudill MA, Wang JC et al (2001) Intracellular S-adenosylhomocysteine concentrations predict global DNA hypomethylation in tissues of methyl-deficient cystathionine beta-synthase heterozygous mice. J Nutr 131:2811–2818
26. Chang L, Adams RD et al (2002) The TC10-interacting protein CIP4/2 is required for insulin-stimulated Glut4 translocation in 3T3L1 adipocytes. Proc Natl Acad Sci USA 99:12835–12840
27. Cheng X, Blumenthal RM (2008) Mammalian DNA methyltransferases: a structural perspective. Structure 16:341–350
28. Christophersen NS, Helin K (2010) Epigenetic control of embryonic stem cell fate. J Exp Med 207:2287–2295

29. Chuang JC, Warner SL et al (2010) S110, a 5-Aza-2′-deoxycytidine-containing dinucleotide, is an effective DNA methylation inhibitor in vivo and can reduce tumor growth. Mol Cancer Ther 9:1443–1450
30. Cironi L, Provero P et al (2009) Epigenetic features of human mesenchymal stem cells determine their permissiveness for induction of relevant transcriptional changes by SYT-SSX1. PLoS One 4:e7904
31. Cohen NM, Dighe V et al (2009) DNA methylation programming and reprogramming in primate embryonic stem cells. Genome Res 19:2193–2201
32. Collas P (2009) Epigenetic states in stem cells. Biochim Biophys Acta 1790:900–905
33. Cross SH, Bird AP (1995) CpG islands and genes. Curr Opin Genet Dev 5:309–314
34. da Silva ML, Chagastelles PC et al (2006) Mesenchymal stem cells reside in virtually all post-natal organs and tissues. J Cell Sci 119:2204–2213
35. de Boer J, Siddappa R et al (2004) Wnt signaling inhibits osteogenic differentiation of human mesenchymal stem cells. Bone 34:818–826
36. De Boer J, Wang HJ et al (2004) Effects of Wnt signaling on proliferation and differentiation of human mesenchymal stem cells. Tissue Eng 10:393–401
37. De Miguel MP, Fuentes-Julian S et al (2010) Pluripotent stem cells: origin, maintenance and induction. Stem Cell Rev 6:633–649
38. Dolinoy DC, Weidman JR et al (2006) Maternal genistein alters coat color and protects Avy mouse offspring from obesity by modifying the fetal epigenome. Environ Health Perspect 114:567–572
39. Dwyer RM, Kerin MJ (2010) Mesenchymal stem cells and cancer: tumor-specific delivery vehicles or therapeutic targets? Hum Gene Ther 21:1506–1512
40. El-Osta A (2003) DNMT cooperativity—the developing links between methylation, chromatin structure and cancer. Bioessays 25:1071–1084
41. Ferrari G, Cusella-De Angelis G et al (1998) Muscle regeneration by bone marrow-derived myogenic progenitors. Science 279:1528–1530
42. Fingerman IM, Wu CL et al (2005) Global loss of Set1-mediated H3 Lys4 trimethylation is associated with silencing defects in Saccharomyces cerevisiae. J Biol Chem 280:28761–28765
43. Gan Q, Yoshida T et al (2007) Concise review: epigenetic mechanisms contribute to pluripotency and cell lineage determination of embryonic stem cells. Stem Cells 25:2–9
44. Gangaraju VK, Lin H (2009) MicroRNAs: key regulators of stem cells. Nat Rev Mol Cell Biol 10:116–125
45. Glinsky GV (2008) "Stemness" genomics law governs clinical behavior of human cancer: implications for decision making in disease management. J Clin Oncol 26:2846–2853
46. Gussoni E, Soneoka Y et al (1999) Dystrophin expression in the mdx mouse restored by stem cell transplantation. Nature 401:390–394
47. Hanna JH, Saha K et al (2010) Pluripotency and cellular reprogramming: facts, hypotheses, unresolved issues. Cell 143:508–525
48. Hansen KH, Bracken AP et al (2008) A model for transmission of the H3K27me3 epigenetic mark. Nat Cell Biol 10:1291–1300
49. Hematti P (2011) Human embryonic stem cell-derived mesenchymal progenitors: an overview. Methods Mol Biol 690:163–174
50. Hemberger M, Dean W et al (2009) Epigenetic dynamics of stem cells and cell lineage commitment: digging Waddington's canal. Nat Rev Mol Cell Biol 10:526–537
51. Hirata T, Cui YJ et al (2007) The temporal profile of genomic responses and protein synthesis in ischemic tolerance of the rat brain induced by repeated hyperbaric oxygen. Brain Res 1130:214–222
52. Holbert S, Dedeoglu A et al (2003) Cdc42-interacting protein 4 binds to huntingtin: neuropathologic and biological evidence for a role in Huntington's disease. Proc Natl Acad Sci USA 100:2712–2717
53. Hong S, Cho YW et al (2007) Identification of JmjC domain-containing UTX and JMJD3 as histone H3 lysine 27 demethylases. Proc Natl Acad Sci USA 104:18439–18444

54. Hsiao SH, Huang TH et al (2009) Excavating relics of DNA methylation changes during the development of neoplasia. Semin Cancer Biol 19:198–208
55. Hsiao SH, Lee KD et al (2010) DNA methylation of the Trip10 promoter accelerates mesenchymal stem cell lineage determination. Biochem Biophys Res Commun 400:305–312
56. Hsu CC, Li HP et al (2010) Targeted methylation of CMV and E1A viral promoters. Biochem Biophys Res Commun 402:228–234
57. Hsu CC, Leu YW et al (2011) Functional characterization of Trip10 in cancer cell growth and survival. J Biomed Sci 18:12
58. Ji H, Ehrlich LI et al (2010) Comprehensive methylome map of lineage commitment from haematopoietic progenitors. Nature 467:338–342
59. Jones PA, Martienssen R (2005) A blueprint for a Human Epigenome Project: the AACR Human Epigenome Workshop. Cancer Res 65:11241–11246
60. Jones PA, Taylor SM et al (1983) Inhibition of DNA methylation by 5-azacytidine. Recent Results Cancer Res 84:202–211
61. Jung JW, Lee S et al (2010) Histone deacetylase controls adult stem cell aging by balancing the expression of polycomb genes and jumonji domain containing 3. Cell Mol Life Sci 67:1165–1176
62. Kalari S, Pfeifer GP (2010) Identification of driver and passenger DNA methylation in cancer by epigenomic analysis. Adv Genet 70:277–308
63. Kang TH, Lee JH et al (2007) Epigallocatechin-3-gallate enhances CD8+ T cell-mediated antitumor immunity induced by DNA vaccination. Cancer Res 67:802–811
64. Kierszenbaum AL (2002) Genomic imprinting and epigenetic reprogramming: unearthing the garden of forking paths. Mol Reprod Dev 63:269–272
65. Kim D, Yang JY et al (2009) Overexpression of alpha-catenin increases osteoblastic differentiation in mouse mesenchymal C3H10T1/2 cells. Biochem Biophys Res Commun 382:745–750
66. Kirmizis A, Bartley SM et al (2004) Silencing of human polycomb target genes is associated with methylation of histone H3 Lys 27. Genes Dev 18:1592–1605
67. Klopp AH, Gupta A et al (2011) Concise review: dissecting a discrepancy in the literature: do mesenchymal stem cells support or suppress tumor growth? Stem Cells 29:11–19
68. Krause DS, Theise ND et al (2001) Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. Cell 105:369–377
69. Lakshmipathy U, Hart RP (2008) Concise review: microRNA expression in multipotent mesenchymal stromal cells. Stem Cells 26:356–363
70. Lan F, Bayliss PE et al (2007) A histone H3 lysine 27 demethylase regulates animal posterior development. Nature 449:689–694
71. Lau PN, Cheung P (2011) Histone code pathway involving H3 S28 phosphorylation and K27 acetylation activates transcription and antagonizes polycomb silencing. Proc Natl Acad Sci USA 108:2801–2806
72. Lee KD, Kuo TK et al (2004) In vitro hepatic differentiation of human mesenchymal stem cells. Hepatology 40:1275–1284
73. Lee OK, Kuo TK et al (2004) Isolation of multipotent mesenchymal stem cells from umbilical cord blood. Blood 103:1669–1675
74. Leu YW, Rahmatpanah F et al (2003) Double RNA interference of DNMT3b and DNMT1 enhances DNA demethylation and gene reactivation. Cancer Res 63:6110–6115
75. Leu YW, Yan PS et al (2004) Loss of estrogen receptor signaling triggers epigenetic silencing of downstream targets in breast cancer. Cancer Res 64:8184–8192
76. Li E (2002) Chromatin modification and epigenetic reprogramming in mammalian development. Nat Rev Genet 3:662–673
77. Lin Y, Weisdorf DJ et al (2000) Origins of circulating endothelial cells and endothelial outgrowth from blood. J Clin Invest 105:71–77
78. Lin YS, Shaw AY et al (2011) Identification of novel DNA methylation inhibitors via a two-component reporter gene system. J Biomed Sci 18:3

79. Liu YZ, Shao Z et al (2010) Prediction of Polycomb target genes in mouse embryonic stem cells. Genomics 96:17–26
80. Locke M, Feisst V et al (2011) Concise review: human adipose-derived stem cells (ASC): separating promise from clinical need. Stem Cells 29:404–411
81. Lodhi IJ, Chiang SH et al (2007) Gapex-5, a Rab31 guanine nucleotide exchange factor that regulates Glut4 trafficking in adipocytes. Cell Metab 5:59–72
82. Lopez MJ, Spencer ND (2011) In vitro adult rat adipose tissue-derived stromal cell isolation and differentiation. Methods Mol Biol 702:37–46
83. Martin DI, Cropley JE et al (2008) Environmental influence on epigenetic inheritance at the Avy allele. Nutr Rev 66:S12–S14
84. Mathieu O, Probst AV et al (2005) Distinct regulation of histone H3 methylation at lysines 27 and 9 by CpG methylation in Arabidopsis. EMBO J 24:2783–2791
85. Messmer S, Franke A et al (1992) Analysis of the functional role of the Polycomb chromo domain in Drosophila melanogaster. Genes Dev 6:1241–1254
86. Miller SA, Mohn SE et al (2010) Jmjd3 and UTX play a demethylase-independent role in chromatin remodeling to regulate T-box family member-dependent gene expression. Mol Cell 40:594–605
87. Mohn F, Weber M et al (2008) Lineage-specific polycomb targets and de novo DNA methylation define restriction and potential of neuronal progenitors. Mol Cell 30:755–766
88. Momin EN, Vela G et al (2010) The oncogenic potential of mesenchymal stem cells in the treatment of cancer: directions for future research. Curr Immunol Rev 6:137–148
89. Morgan HD, Sutherland HG et al (1999) Epigenetic inheritance at the agouti locus in the mouse. Nat Genet 23:314–318
90. Noer A, Boquest AC et al (2007) Dynamics of adipogenic promoter DNA methylation during clonal culture of human adipose stem cells to senescence. BMC Cell Biol 8:18
91. Noer A, Lindeman LC et al (2009) Histone H3 modifications associated with differentiation and long-term culture of mesenchymal adipose stem cells. Stem Cells Dev 18:725–736
92. Okitsu CY, Hsieh JC et al (2010) Transcriptional activity affects the H3K4me3 level and distribution in the coding region. Mol Cell Biol 30:2933–2946
93. Opavsky R, Wang SH et al (2007) CpG island methylation in a mouse model of lymphoma is driven by the genetic configuration of tumor cells. PLoS Genet 3:1757–1769
94. Pacini S, Carnicelli V et al (2010) Constitutive expression of pluripotency-associated genes in mesodermal progenitor cells (MPCs). PLoS One 5:e9861
95. Papp B, Muller J (2006) Histone trimethylation and the maintenance of transcriptional ON and OFF states by trxG and PcG proteins. Genes Dev 20:2041–2054
96. Pasini D, Malatesta M et al (2010) Characterization of an antagonistic switch between histone H3 lysine 27 methylation and acetylation in the transcriptional regulation of Polycomb group target genes. Nucleic Acids Res 38:4958–4969
97. Petersen BE, Bowen WC et al (1999) Bone marrow as a potential source of hepatic oval cells. Science 284:1168–1170
98. Pittenger MF, Mackay AM et al (1999) Multilineage potential of adult human mesenchymal stem cells. Science 284:143–147
99. Pontikoglou C, Deschaseaux F et al (2011) Bone marrow mesenchymal stem cells: biological properties and their role in hematopoiesis and hematopoietic stem cell transplantation. Stem Cell Rev 7:569–589
100. Prockop DJ (1997) Marrow stromal cells as stem cells for nonhematopoietic tissues. Science 276:71–74
101. Rajasekhar VK, Begemann M (2007) Concise review: roles of polycomb group proteins in development and disease: a stem cell perspective. Stem Cells 25:2498–2510
102. Reik W (2007) Stability and flexibility of epigenetic gene regulation in mammalian development. Nature 447:425–432
103. Robert MF, Morin S et al (2003) DNMT1 is required to maintain CpG methylation and aberrant gene silencing in human cancer cells. Nat Genet 33:61–65

104. Ross SA (2003) Diet and DNA methylation interactions in cancer prevention. Ann N Y Acad Sci 983:197–207
105. Salem HK, Thiemermann C (2010) Mesenchymal stromal cells: current understanding and clinical status. Stem Cells 28:585–596
106. Sanchez-Ramos J, Song S et al (2000) Adult bone marrow stromal cells differentiate into neural cells in vitro. Exp Neurol 164:247–256
107. Sasaki H, Matsui Y (2008) Epigenetic events in mammalian germ-cell development: reprogramming and beyond. Nat Rev Genet 9:129–140
108. Sawarkar R, Paro R (2010) Interpretation of developmental signaling at chromatin: the Polycomb perspective. Dev Cell 19:651–661
109. Schlesinger Y, Straussman R et al (2007) Polycomb-mediated methylation on Lys27 of histone H3 pre-marks genes for de novo methylation in cancer. Nat Genet 39:232–236
110. Schubeler D (2009) Epigenomics: methylation matters. Nature 462:296–297
111. Schwartz YB, Kahn TG et al (2010) Alternative epigenetic chromatin states of polycomb target genes. PLoS Genet 6:e1000805
112. Seki Y, Yamaji M et al (2007) Cellular dynamics associated with the genome-wide epigenetic reprogramming in migrating primordial germ cells in mice. Development 134:2627–2638
113. Sethe S, Scutt A et al (2006) Aging of mesenchymal stem cells. Ageing Res Rev 5:91–116
114. Shafa M, Krawetz R et al (2010) Returning to the stem state: epigenetics of recapitulating pre-differentiation chromatin structure. Bioessays 32:791–799
115. Sibani S, Melnyk S et al (2002) Studies of methionine cycle intermediates (SAM, SAH), DNA methylation and the impact of folate deficiency on tumor numbers in Min mice. Carcinogenesis 23:61–65
116. Siddiqi S, Mills J et al (2010) Epigenetic remodeling of chromatin architecture: exploring tumor differentiation therapies in mesenchymal stem cells and sarcomas. Curr Stem Cell Res Ther 5:63–73
117. Simile MM, Pascale R et al (1994) Correlation between S-adenosyl-L-methionine content and production of c-myc, c-Ha-ras, and c-Ki-ras mRNA transcripts in the early stages of rat liver carcinogenesis. Cancer Lett 79:9–16
118. Simon JA, Kingston RE (2009) Mechanisms of polycomb gene silencing: knowns and unknowns. Nat Rev Mol Cell Biol 10:697–708
119. Sparmann A, van Lohuizen M (2006) Polycomb silencers control cell fate, development and cancer. Nat Rev Cancer 6:846–856
120. Spencer ND, Lopez MJ (2011) In vitro adult canine adipose tissue-derived stromal cell growth characteristics. Methods Mol Biol 702:47–60
121. Spivakov M, Fisher AG (2007) Epigenetic signatures of stem-cell identity. Nat Rev Genet 8:263–271
122. Su Y, Deng B et al (2011) Polycomb group genes in stem cell self-renewal: a double-edged sword. Epigenetics 6:16–19
123. Surani MA, Hayashi K et al (2007) Genetic and epigenetic regulators of pluripotency. Cell 128:747–762
124. Tate PH, Bird AP (1993) Effects of DNA methylation on DNA-binding proteins and gene expression. Curr Opin Genet Dev 3:226–231
125. Teng IW, Hou PC et al (2011) Targeted methylation of two tumor suppressor genes is sufficient to transform mesenchymal stem cells into cancer stem/initiating cells. Cancer Res 71:4653–4663
126. Theise ND, Badve S et al (2000) Derivation of hepatocytes from bone marrow cells in mice after radiation-induced myeloablation. Hepatology 31:235–240
127. Theise ND, Nimmakayalu M et al (2000) Liver from bone marrow in humans. Hepatology 32:11–16
128. Tiwari VK, McGarvey KM et al (2008) PcG proteins, DNA methylation, and gene repression by chromatin looping. PLoS Biol 6:2911–2927
129. Trento C, Dazzi F (2010) Mesenchymal stem cells and innate tolerance: biology and clinical applications. Swiss Med Wkly 140:w13121

130. Turek-Plewa J, Jagodzinski PP (2005) The role of mammalian DNA methyltransferases in the regulation of gene expression. Cell Mol Biol Lett 10:631–647
131. Vincent A, Van Seuningen I (2009) Epigenetics, stem cells and epithelial cell fate. Differentiation 78:99–107
132. Wei Y, Xia W et al (2008) Loss of trimethylation at lysine 27 of histone H3 is a predictor of poor outcome in breast, ovarian, and pancreatic cancers. Mol Carcinog 47:701–706
133. Wei G, Wei L et al (2009) Global mapping of H3K4me3 and H3K27me3 reveals specificity and plasticity in lineage fate determination of differentiating CD4+ T cells. Immunity 30:155–167
134. Weinhofer I, Hehenberger E et al (2010) H3K27me3 profiling of the endosperm implies exclusion of polycomb group protein targeting by DNA methylation. PLoS Genet 6:e1001152
135. Wolff GL, Kodell RL et al (1998) Maternal epigenetics and methyl supplements affect agouti gene expression in Avy/a mice. FASEB J 12:949–957
136. Wu SC, Zhang Y (2010) Active DNA demethylation: many roads lead to Rome. Nat Rev Mol Cell Biol 11:607–620
137. Yamada Y, Watanabe A (2010) Epigenetic codes in stem cells and cancer stem cells. Adv Genet 70:177–199
138. Yamasaki-Ishizaki Y, Kayashima T et al (2007) Role of DNA methylation and histone H3 lysine 27 methylation in tissue-specific imprinting of mouse Grb10. Mol Cell Biol 27:732–742
139. Yang XF (2007) Immunology of stem cells and cancer stem cells. Cell Mol Immunol 4:161–171
140. Yuan R, Fan S et al (2001) Altered gene expression pattern in cultured human breast cancer cells treated with hepatocyte growth factor/scatter factor in the setting of DNA damage. Cancer Res 61:8022–8031
141. Zager RA, Johnson AC (2010) Progressive histone alterations and proinflammatory gene activation: consequences of heme protein/iron-mediated proximal tubule injury. Am J Physiol Renal Physiol 298:F827–F837
142. Zeng X (2007) Human embryonic stem cells: mechanisms to escape replicative senescence? Stem Cell Rev 3:270–279
143. Zheng C, Yang S et al (2009) Human multipotent mesenchymal stromal cells from fetal lung expressing pluripotent markers and differentiating into cell types of three germ layers. Cell Transplant 18:1093–1109
144. Jackson KA, Majka SM et al (2001) Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells. JCI 107:1395–1402

Part III
Clinical Implications and Analysis Methods

Chapter 11
Environmental Toxicants, Epigenetics, and Cancer

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Abstract Tumorigenesis, a complex and multifactorial progressive process of transformation of normal cells into malignant cells, is characterized by the accumulation of multiple cancer-specific heritable phenotypes triggered by the mutational and/or non-mutational (i.e., epigenetic) events. Accumulating evidence suggests that environmental and occupational exposures to natural substances, as well as man-made chemical and physical agents, play a causative role in human cancer. In a broad sense, carcinogenesis may be induced through either genotoxic or non-genotoxic mechanisms; however, both genotoxic and non-genotoxic carcinogens also cause prominent epigenetic changes. This review presents current evidence of the epigenetic alterations induced by various chemical carcinogens, including arsenic, 1,3-butadine, and pharmaceutical and biological agents, and highlights the potential for epigenetic changes to serve as markers for carcinogen exposure and cancer risk assessment.

11.1 Introduction

Tumorigenesis is a complex and multifactorial progressive process of transformation of normal cells into malignant ones. It is characterized by the accumulation of multiple cancer-specific heritable phenotypes, including persistent proliferative

Note: The views expressed in this chapter do not necessarily represent those of the U.S. Food and Drug Administration.

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signaling, resistance to cell death, evasion of growth suppression, replicative immortality, inflammatory response, deregulation of energy metabolism, genomic instability, induction of angiogenesis, and activation of invasion ultimately resulting in metastases [1]. The acquisition of these cancer-specific alterations may be triggered by the mutational and/or non-mutational (i.e., epigenetic) events in the genome which, in turn, affect gene expression and the downstream phenotypes listed above [1, 2]. Furthermore, it has been suggested that epigenetic alterations may play as important or even more prominent role in tumor development [3].

Epigenetic events, most prominently manifested by stable and heritable changes in gene expression that are not due to any alteration in the primary DNA sequence [4], signify the fundamental molecular principles in which genetic information is organized and read [5]. Epigenetic modifications include change in methylation patterns of cytosines in DNA [6, 7], modifications of the proteins that bind to DNA [8, 9], and the nucleosome positioning along DNA [4]. These epigenetic marks are tightly and interdependently connected and are essential for the normal development and the maintenance of cellular homeostasis and functions in adult organisms, particularly for X-chromosome inactivation in females, genomic imprinting, silencing of repetitive DNA elements, regulation of chromatin structure, and proper expression of genetic information [10]. The epigenetic status is well-balanced in normal cells, but may be altered in many ways in cancer cells. Additionally, growing evidence indicates that a number of lifestyle and environmental factors may disrupt this epigenetic balance and compromise the stability of the epigenome in normal cells leading to the development of a wide range of pathologies, including cancer.

11.2 Epigenetic Alterations in Cancer Cells

The unifying molecular feature of neoplastic cells is a profoundly reshaped genome characterized by global genomic *hypo*-methylation, gene-specific *hyper*- or *hypo*-methylation, and altered histone modification patterns [2, 11].

DNA demethylation signifies one of the two major DNA methylation states and refers to a state in which there is a decrease in the number of methylated cytosine bases from the "normal" methylation level. Demethylation of DNA can be achieved either passively or actively. Passive loss of methylated marks in the genome may be a consequence of limited availability of the universal methyl donor S-adenosyl-L-methionine (SAM), compromised integrity of DNA, and altered expression and/or activity of DNA methyltransferases [12]. Until recently, evidence for existence of an active replication-independent DNA demethylation process was controversial and inconclusive [7, 13]. However, recent studies provide compelling experimental evidence that active loss of DNA methylation is associated with the function of DNA repair machinery [14–17].

Global hypomethylation of DNA was the first epigenetic abnormality identified in cancer more than a quarter of century ago [18, 19]. It continues to be one of the

most common molecular alterations found in all human cancers [20, 21]; however, the molecular mechanisms behind cancer-linked global demethylation of the genome remain largely unknown. The loss of DNA methylation in cancer primarily affects stable, methylated areas of the genome composed predominantly of repetitive elements, genes, and intergenic regions [22].

There are several molecular consequences of global demethylation of DNA that may contribute to tumorigenesis. First, genomic hypomethylation causes significant elevation in mutation rates [23], activation of normally silenced tumor-promoting genes [24], and loss of imprinting [25]. Second, demethylation of the repetitive DNA sequences, such as long interspersed nucleotide elements (LINE)-1 and short interspersed nucleotide elements (SINE), retroviral intracisternal A particle (IAP), and Alu elements located at centromeric, pericentromeric, and subtelomeric chromosomal regions induces their activation and transposition leading to chromosomal instability [26–29]. For example, recent findings have demonstrated that DNA hypomethylation causes permissive transcriptional activity at the centromere [28]. Subsequently, the accumulation of small minor satellite transcripts that impair centromeric architecture and function is observed. Likewise, hypomethylation of the repetitive elements at the subtelomeric regions is associated with enhanced transcription of the telomeres [29].

Gene-specific loss of DNA methylation is also a finding for oncogenes and imprinted genes. In addition, many genes that are normally well-methylated, particularly cancer-germline genes, including B melanoma antigen family (*BAGE*), cancer testis antigen (*CAGE*), melanoma antigen family *A* (*MAGE-A*), X antigen family (*XAGE*), and other single-copy genes, including S100 calcium binding protein A4 (*S100A4*), flap endonuclease 1 (*FEN1*), and synuclein-gamma (*SNCG*), undergo progressive hypomethylation, which is accompanied by their increased expression, in human cancers [12, 21].

Despite the large body of evidence indicating that cancer-associated DNA demethylation is an important early event in tumor development, it is still less clear if the loss of DNA methylation is a cause, or a consequence of the malignant transformation [30]. The notion that DNA hypomethylation is playing a role in causation and/or promotion of cancer is based on the results of studies with nutritional "lipogenic methyl-deficient diet" [31–33], genetically engineered *Dnmt*- and *Lsh*-deficient mice [34, 35], and several models of chemical carcinogenesis [36]. In contrast, there is also evidence that cancer-linked DNA hypomethylation may be a passive inconsequential side effect of carcinogenesis [30, 37]. The latter is evidenced by facts that not all tumors exhibit DNA hypomethylation and not all carcinogenic processes are accompanied by the loss of DNA methylation [38]. Indeed, it is highly unlikely to expect that development and progression of diverse types of tumors are all associated with DNA hypomethylation. Furthermore, there is growing evidence that DNA hypomethylation suppresses development of certain tumor types, especially intestinal, gastric, and prostate carcinomas [39–41].

DNA hypermethylation is the state where the methylation of normally undermethylated DNA domains, those that predominantly consist of CpG islands [22], increases. CpG islands are defined as the genomic regions that contain the high

G + C content, have high frequency of CpG dinucleotides, are at least 400–500 bp long, and can be located either at intragenic and intergenic, or at the 5′ ends of genes [42–44]. However, only CpG islands that span 5′ promoters are mainly unmethylated. For instance, less than 3% of CpG islands in gene promoters are methylated [44].

It is well-established that hypermethylation of promoter-located CpG islands causes permanent and stable transcriptional silencing of a range of protein-coding genes [45], which, along with DNA hypomethylation, plays a critical role in cancer development [2, 11]. One of the most compelling examples of the link between DNA hypermethylation and carcinogenesis is epigenetic silencing of critical tumor-suppressor genes, including cyclin-dependent kinase inhibitor 2A (*CDKN2A*; $p16^{INK4A}$), secreted frizzled-related protein (*SFRPs*) genes, adenomatous polyposis coli (*APC*), and GATA binding protein 4 (*GATA4*). The aberrant silencing of these genes allows for survival and clonal expansion of the initiated cells. Additionally, hypermethylation of several DNA repair genes, including O^6-methylguanine-DNA methyltransferase (*MGMT*), xeroderma pigmentosum group C (*XPC*), MutL homolog 1 (*MLH1*), and breast cancer 1 and 2 (*BRCA1* and *BRCA2*) genes results in insufficient DNA repair leading to reduction in genomic stability and various genetic aberrations, particularly, the elevation of mutation rates in critical cancer-related genes [46, 47]. For example, the epigenetic silencing of *MGMT* leads to a greater mutation rate in *K-RAS* and *p53* genes during human colorectal carcinogenesis [48, 49]. Likewise, transcriptional inactivation of the *BRCA1* and *MLH1* genes caused by promoter hypermethylation results in elevated *p53* gene mutation frequency in human sporadic breast cancer [50] and microsatellite instability in sporadic colorectal cancer [51], respectively.

In addition to the vital role that DNA methylation state may play in the etiology and pathogenesis of cancer, it has been shown that disruption of normal patterns of covalent histone modifications is an epigenetic change frequently found in tumor cells. Histones are evolutionary conserved proteins that have globular carboxy-terminal domains critical to nucleosome formation, and flexible amino-terminal tails that protrude from the nucleosome core and contact adjacent nucleosomes to form higher order chromatin structures. At least eight different classes of post-translational modifications, including methylation, acetylation, phosphorylation, ubiquitination, sumoylation, biotinylation, and ADP-ribosylation have been identified on the core histones H2A, H2B, H3, H4, and the H1 family of linker histones [8, 9]. These histone marks are essential for organizing chromatin, maintaining genome stability, silencing repetitive DNA elements, regulating cell cycle progression, recognizing DNA damage sites and repair, and maintenance of proper expression of genetic information.

Accumulating evidence clearly indicates that cancer cells are characterized by a profoundly disturbed pattern of global and/or gene-specific histone modifications accompanied by alterations in the functioning of enzymes that are associated with those marks. There are various combinations of cancer-linked histone modifications that differ according to tumor type; however, one of the most characteristic examples of global changes in histone modifications is loss of histone H4 lysine 20

trimethylation and H4 lysine 16 acetylation, which is a common hallmark of human cancers [52].

Additionally, extensive studies in the past decade have indicated the existence and importance of another epigenetic mechanism of regulation of gene function by means of small non-coding microRNAs (miRNAs). Currently, miRNAs are recognized as one of the major regulatory gatekeepers of protein-coding genes in human genome [53, 54]. MiRNAs are small 16–29 nucleotide-long non-coding RNAs that primarily function as negative gene regulators at the post-transcriptional level [55]. MiRNAs are generated by RNA polymerase II or RNA polymerase III as long primary transcripts, primary miRNAs. Following transcription, primary miRNAs form a stem-loop structure, which is recognized and processed by the RNase III-type enzyme Drosha creating precursor miRNAs. These precursor miRNAs are transported from the nucleus to the cytoplasm by Exportin-5. In the cytoplasm, the pre-miRNAs are further processed by Dicer, an RNase III enzyme, generating miRNA:miRNA hybrids. After unwinding, one strand of the duplex is degraded, and another strand becomes a mature miRNA. MiRNAs can induce mRNA cleavage if complementary to 3′-untranslated region of targets is perfect or translational repression if complementarity is imperfect [53].

Currently there are more than 700 mammalian miRNAs that can potentially target up to one-third of protein-coding genes involved in the development, cell differentiation, metabolic regulation, signal-transduction, cell proliferation, and apoptosis. As the deregulation of these very same biological processes is a hallmark of cancer [1], it has been suggested that changes in miRNA expression might have significance in cancer [56–58]. In tumors, aberrant expression of miRNAs inhibits tumor suppressor genes or inappropriately activates oncogenes have been experimentally associated with most aspects of tumor biology, including tumor progression, invasiveness, metastasis, and acquisition of resistance of malignant cells to various chemotherapeutic agents [58]. This leads to the suggestion that altered expression of miRNAs is an important mechanism of carcinogenesis [57, 59].

11.3 Role of Epigenetic Alterations in Chemical Carcinogenesis

Many environmental and occupational exposures to natural substances, man-made chemical and physical agents are considered to be causative of human cancer [60–62]. In a broad sense, carcinogenesis may be induced through either genotoxic or non-genotoxic mechanisms. Genotoxic carcinogens are agents that interact directly or after metabolic activation with DNA, causing mutations and leading to tumor formation. Non-genotoxic carcinogens are a diverse group of chemical compounds that are known to cause tumors by mechanisms other than direct damage to DNA. The emphasis in carcinogenesis research, until recently, has focused mainly on the investigation of various molecular signaling events, DNA damage, DNA adduct repair, and genetic aberrations, despite the fact that the importance of epigenetic mechanisms in carcinogenic process was first suggested by Miller in 1970 [63].

Accumulating evidence suggests that regardless of the mechanism of action, both genotoxic and non-genotoxic carcinogens may also lead to prominent epigenetic abnormalities in tissues that are susceptible to carcinogenesis as a result of exposure [64–68]. The following sections present an overview of the epigenetic alterations induced by several carcinogens.

11.3.1 Arsenic

Arsenic is a naturally occurring element and a ubiquitous environmental contaminant which is a public health issue world-wide [69]. The major source of human exposure to arsenic is contaminated food and drinking water. Inorganic arsenic was one of the earliest identified human carcinogens [69, 70]. It is widely accepted that exposure to arsenic is associated with skin, lung, and bladder cancers [71]. Additionally, accumulating evidence indicates that long-term exposure to arsenic causes development of liver tumors [72].

Arsenic was classified as a known human carcinogen by the International Agency for Research on Cancer (IARC) in 2004, when sufficient evidence for human carcinogenicity became available [71]; even though limited evidence for animal carcinogenicity of arsenic existed. This may be explained mainly by the absence of adequate relevant animal models to study arsenic carcinogenesis. However, the experiments in transgenic mice, e.g., v-Ha-ras (Tg.AC), keratin VI/ornithine decarboxylase (K6/ODC), and $p53^{+/-}$, or inbred mouse strains that are prone to spontaneous cancer development provided evidence for the carcinogenicity of arsenic in animal studies. For instance, administration of arsenic to A/J mice, a strain that exhibits a susceptibility to different pulmonary pathological states including lung cancer, enhances lung tumor multiplicity and size [70, 73]. Similarly, in utero arsenic exposure of C3H/HeJ mice, which are prone to hepatocarcinogenesis, resulted in increased incidence and multiplicity of hepatocellular carcinomas in adults [74]. The most convincing evidence for the carcinogenicity of arsenic in animals has been presented in a recent report by Tokar et al. [75] that demonstrated that “whole-life” exposure of CD1 mice to arsenic causes induction of various tumors, including lung and liver.

The molecular mechanisms behind the cancer-inducing property of arsenic are not fully elucidated and remain a subject of debate. Several potential mechanisms have been proposed to explain arsenic-induced carcinogenesis, including induction of oxidative stress, DNA–protein crosslinking, chromosomal aberrations [70], disruption of signaling pathways, and epigenetic dysregulation, particularly DNA demethylation [76]. The first evidence demonstrating an association between arsenic tumorigenicity and global DNA hypomethylation was reported by Zhao et al. [77] who showed that exposure of rat liver epithelial TRL-1215 cells to arsenic in vitro led to their malignant transformation and was paralleled by global DNA demethylation. Importantly, the extent of DNA hypomethylation in the transformed cells was positively correlated with the tumorigenicity of the cells upon inoculation

into nude mice, suggesting that loss of DNA methylation may be a causative factor in arsenic-induced carcinogenesis [77]. Since then, a large amount of data has documented a substantial target organ-specific loss of global DNA methylation and repetitive element and gene-specific methylation in various in vitro and in vivo models of arsenic-induced tumorigenesis [78–80].

Several possible explanations exist for the mechanism of DNA demethylation after exposure to arsenic. First, arsenic-induced DNA hypomethylation can be explained by the absolute requirement of SAM for the biomethylation of inorganic arsenic and DNA methylation reactions [76, 81]. Therefore, the biomethylation of inorganic arsenic reduces availability of SAM for DNA and histone methylation. Second, arsenic exposure increases generation of reactive oxygen species that may cause direct damage to DNA [82, 83]. The presence of oxidative lesions in DNA (e.g., 8-oxodeoxyguanosine and 5-hydroxymethylcytosine) severely compromises the ability of DNA methyltransferases to methylate the target cytosine and leads to passive demethylation of DNA [84]. In addition, activation of DNA repair pathway promotes active demethylation of DNA [14–17]. Third, arsenic-induced oxidative stress causes depletion of the level of intracellular reduced glutathione. This consequently leads to the enhanced glutathione biosynthesis in a transsulfuration pathway, which impairs SAM biosynthesis and perturbs DNA and histone methylation reactions [85].

In addition to global and gene-specific DNA hypomethylation, arsenic exposure causes concurrent methylation-induced transcriptional silencing of a number of tumor suppressor genes, including *p53*, *CDKN2A* (*$p16^{INK4A}$*), Ras association domain family member 1 (*RASSF1A*), and death-associated protein kinase (*DAPK*) [73, 86, 87], various histone modification changes [88], and alterations in miRNA expression [89].

It is of note that growing evidence suggests that carcinogenesis induced by an environmental chronic exposure to other metals, such as nickel, chromium, cadmium, and mercury, may also involve molecular epigenetic alterations caused by the ability of these metals to induce damage to DNA and strongly influence intracellular molecular and metabolic alterations [90, 91].

11.3.2 1,3-Butadiene

The gaseous olefin 1,3-butadiene is a major industrial chemical monomer widely used in production of synthetic rubber, resins, and plastic. Additionally, this highly volatile agent is present in industrial and automobile exhaust, cigarette smoke, and ambient air in urban locations and industrial complexes [92]. Based on the results of numerous comprehensive epidemiological studies, the IARC has classified 1,3-butadiene as a known human carcinogen [92–94]. In rodents, it causes tumor formation at several target sites, including the hematopoietic system, lungs, heart, and liver [93]. Importantly, the hematopoietic system, lungs and liver are the most common sites of 1,3-butadiene-induced tumor formation in both humans and mice [93].

It is well-established that the mechanism of tumor induction caused by 1,3-butadiene-exposure is due to genotoxic reactivity of its metabolic epoxides: 1,2-epoxy-3-butene, 1,2:3,4-diepoxybutane, and 3,4-epoxy-1,2-butanediol that interact directly with DNA to form mutagenic DNA adducts [94]. However, recent evidence demonstrates that short-term inhalational exposure of C57BL/6J mice to 1,3-butadiene, in addition to DNA adduct formation, also causes extensive concurrent epigenetic changes. These include a marked reduction of global DNA and repetitive element methylation and a profound loss of histone H3K9, H3K27, and H4K20 trimethylation in the livers of C57BL/6J mice [95].

It is well-established that methylation of lysine residues 9 and 27 at histone H3 and lysine 20 at histone H4 plays a fundamental role in the formation of a condensed heterochromatin structure and transcriptional repression [96–98]. Hence, loss of H3K9 and H4K20 trimethylation induced by 1,3-butadiene-exposure may compromise genomic stability via chromatin relaxation and activation of mobile repetitive elements. Indeed, a recent report showing decondensation of chromatin and activation of main repetitive elements in the livers of 1,3-butadiene-exposed C57BL/6J mice support this suggestion [99]. Additionally, an open chromatin structure may increase further vulnerability of DNA to the genotoxicity of reactive 1,3-butadiene metabolites.

The elucidation of the mechanisms of carcinogenicity is usually carried out in genetically homogeneous in vivo models in order to fix as many variables as possible. This provides information in a single strain, yet the extrapolation of such data to the population effects is constrained by the inference from a single genome to model complex human phenotypes. To overcome this important limitation, panels of genetically defined animals may be used to determine genetic causes of inter-individual variability in cancer susceptibility [100]. In a recent study, Koturbash et al. [99] have demonstrated substantial differences in hepatic genetic and epigenetic response among mouse strains to short-term inhalational exposure to 1,3-butadiene. More importantly, the strain differences were associated with alterations in chromatin structure, mainly in the variability in histone H3K9, H3K27, and H4K20 methylation.

11.3.3 Pharmaceuticals

Diethylstilbestrol is a synthetic non-steroidal estrogen that was widely used to prevent potential miscarriages and as emergency contraceptive (morning-after pill) [101]. Currently, diethylstilbestrol is classified by the IARC as a known human carcinogen [101]. Breast is the main target organ for diethylstilbestrol-induced carcinogenesis in women who were exposed during pregnancy. Additionally, diethylstilbestrol also causes development of adenocarcinoma in the uterus and cervix of women who were exposed in utero.

In addition to the established mechanistic genotoxic and estrogen receptor-mediated carcinogenic events, epigenetic programming also plays a substantial role.

Perinatal exposure to diethylstilbestrol causes persistent demethylation and transcriptional activation of several critical cancer-related genes in the mouse uterus, including lactoferrin (*Lf*), nucleosomal binding protein 1 (*Nsbp1*), and c-*fos* [102–104]. The mechanism of these demethylation events is associated with the ability of diethylstilbestrol to inhibit expression of the maintenance (*Dnmt1*) and de novo (*Dnmt3a* and *Dnmt3b*) DNA methyltransferases in the mouse uterus [105]. Additionally, recent evidence indicates that diethylstilbestrol exposure causes epigenetically induced down-regulation of miRNA-9 in human breast epithelial cells [106], one of the frequently down-regulated miRNAs in human breast cancer [107].

Tamoxifen, a selective non-steroidal anti-estrogen, is a widely used drug for chemotherapy and for chemoprevention of breast cancer worldwide [108]. However, recently the IARC classified tamoxifen as a known human carcinogen based on evidence for endometrial cancer [101]. One of the possible mechanisms of carcinogenic effects of tamoxifen in the uterus is tamoxifen-induced gene expression changes [109], particularly, hypomethylation-linked activation of paired box 2 (*PAX2*) gene [110].

Additionally, a number of studies have demonstrated that tamoxifen is a potent hepatocarcinogen in rats with both tumor initiating and promoting properties [111]. The mechanism of tamoxifen-induced hepatocarcinogenesis is associated with its genotoxic [112, 113] and epigenetic effects [114]. These non-genotoxic epigenetic alterations include demethylation of the entire genome and the repetitive elements, loss of global histone H4 lysine 20 trimethylation [114, 115], and altered expression of miRNAs [116]. The results of these studies further emphasize the importance of non-genotoxic mechanisms in chemical carcinogenesis induced by genotoxic carcinogens.

Phenobarbital, the most widely used anticonvulsant worldwide, is a well-established mitogenic non-genotoxic rodent liver carcinogen. It is known to increase cell proliferation, alter cell cycle checkpoint control, including delaying and attenuating the G1 checkpoint, inhibit the induction of p53, thereby resulting in accumulation of DNA damage, and induce extensive epigenetic abnormalities. Treatment with phenobarbital leads to rapid and progressive accumulation of altered DNA methylation regions in the livers of C57BL/6 and B6C3F1 mice [117]. These changes were more pronounced in livers of tumor-prone B6C3F1 and CAR (constitutive androstane receptor) wild-type mice [118]. Interestingly, the number of hypermethylated regions was noticeably smaller than hypomethylated regions, among which cytochrome P450, family 2, subfamily b, polypeptide 10 (*Cyp2b10)* gene is concomitantly hypomethylated and transcriptionally activated early after phenobarbital treatment [119].

Oxazepam is widely used as a sedative-hypnotic and antianxiety drug. Chronic exposure of B6C3F1 mice to oxazepam induces development of hepatoblastoma and hepatocellular carcinoma in mice [120]. Interestingly, oxazepam, similar to phenobarbital, causes induction of *Cyp2b10* gene in the livers of B6C3F1 mice [121, 122]. Also, oxazepam-induced tumors display a decreased expression of *Apc* and phosphatase and tensin (*Pten*) homolog tumor suppressor genes and genes involved in regulation of DNA methylation and histone modification [122].

11.3.4 Biological Agents

Mycotoxins are a structurally diverse class of molecules of fungal origin that are common contaminants of the human and animal food products [123]. Three of the most ubiquitous mycotoxins, aflatoxin B_1, fumonisin B1, and ochratoxin, are classified by the IARC as known and possible human carcinogens [124, 125]. It is well-established that aflatoxin B_1, fumonisin B1, and ochratoxin A are genotoxic carcinogens [123, 126, 127]; however, accumulating evidence indicates that their carcinogenicity involves also a complex network of epigenetic alterations [128–134].

Aflatoxin B_1 induces several epigenetic abnormalities that may induce and promote tumor development. Specifically, exposure to aflatoxin B_1 causes methylation-induced transcriptional silencing of *MGMT, p16^{INK4A}*, and *RASSF1A* genes, a fundamental epigenetic event in liver carcinogenesis [128–130]. Conversely, aflatoxin B_1 is a strong inducer of epigenetically regulated *SNCG* gene [131]. Additionally, a study conducted by Hu et al. [134] has demonstrated that cytosine methylation at the CpG site at codon 14 of the *K-ras* gene is the major reason for preferential aflatoxin B_1-induced DNA-adduct formation at this codon in normal human bronchial epithelial cells.

Fumonisin B_1, in addition to various genotoxic and non-genotoxic alterations, increases the level of 5-methylcytosine in genomic DNA from 5 to 9% in human intestinal Caco-2 cells [132].

Helicobacter pylori infection is associated with development of gastric cancer, one of the most prevalent human cancers worldwide [135]. The results of several comprehensive studies indicate that *H. pylori* infection causes marked DNA methylation changes in infected normal or preneoplastic gastric mucosa. *H. pylori* infection causes significant aberrant DNA methylation in a number of the promoter CpG island-containing genes, including *p16^{INK4A}*, lipoxygenase (*LOX*), heart and neural crest derivatives expressed 1 (*HAND1*), thrombomodulin (*THBD*), and actin related protein 2/3 complex, subunit p41 (*p41ARC*) gastric cancer-associated genes in gastric mucosa [136–139]. Importantly, hypermethylation of some genes, e.g., *THBD* persisted in gastric mucosa after *H. pylori* eradication [140].

11.4 Epigenetic Alterations and the Evaluation of Cancer Risk

Recognition of the fundamental role of epigenetic alterations in cancer has resulted in the identification of numerous epigenetic abnormalities that may be used as potential biomarkers for the molecular diagnosis of cancer and prognosis of survival or treatment outcomes. Despite a lack of conclusive information to clarify whether or not epigenetic changes are involved directly in neoplastic cell transformation, evidence highlighted above suggests that epigenetic alterations may be used as early indicators of carcinogenesis for both genotoxic and non-genotoxic carcinogens. Importantly, several research groups have argued that epigenetic alterations may be

used as biomarkers in the evaluation of the carcinogenic potential of the environmental factors [5, 67, 68, 141].

Incorporation of the epigenetic biomarkers into the studies on cancer risk of exposures holds a number of advantages over traditionally used methods, such as evaluation of the carcinogen-induced DNA damage, DNA adduct formation, or bacterial mutagenicity. Specifically, we reason that the following features are in favor of greater integration of epigenetic biomarkers in studies of the carcinogenic potential of the environmental exposures: (1) early appearance; (2) stability; (3) target tissue-specificity; (4) relatively low cost of the assays needed to detect these changes; (5) applicability to both genotoxic and non-genotoxic agents; and, more importantly, (6) a greater number of detectable epigenetic changes as compared to the genetic alterations after exposure.

Also, the incorporation of epigenetic technologies into the studies of cancer risk promises to enhance substantially the efficiency of carcinogenicity testing. More importantly, the reversibility of epigenetic alterations opens novel mechanism-based approaches not only to cancer treatment but also to the timely prevention of cancer [142]. However, despite a very promising outlook on the benefits of epigenetic biomarkers, additional studies are still needed to better define the nature and mechanisms of epigenetic abnormalities with respect to carcinogenic processes [60, 143, 144]. Although extensive studies have identified a number of cancer-related epigenetic abnormalities that are associated with carcinogen exposure, there is no consensus on the role of changes in tumorigenesis.

Additionally, it is possible that not all these aberrant epigenetic events are equally important for the tumorigenic process. It is highly unlikely that all of these epigenetic changes play a causative role in tumorigenesis. For example, some epigenetic changes may drive other epigenetic events that contribute to the formation of a transformed phenotype, while others may be passenger epigenetic events that accompany the transformation process [145]. In this respect, the identification of those epigenetic events that drive cell transformation is crucially important for understanding mechanisms of tumorigenesis and for cancer prevention.

References

1. Hanahan D, Weinberg RA (2011) Hallmarks of cancer. Cell 144:646–674
2. Jones PA, Baylin SB (2007) The epigenomics of cancer. Cell 128:683–692
3. Ushijima T, Asada K (2010) Aberrant DNA methylation in contrast with mutations. Cancer Sci 101:300–305
4. Sharma S, Kelly TK, Jones PA (2010) Epigenetics in cancer. Carcinogenesis 31:27–36
5. Marlowe J, Teo SS, Chibout SD, Pognan F, Moggs JJ (2009) Mapping the epigenome—impact for toxicology. EXS 99:259–288
6. Kim JK, Samaranayake M, Pradhan S (2009) Epigenetic mechanisms in mammals. Cell Mol Life Sci 66:596–612
7. Ooi SK, O'Donnell AH, Bestor TH (2009) Mammalian cytosine methylation at a glance. J Cell Sci 122:2787–2791

8. Chen ZX, Riggs AD (2011) DNA methylation and demethylation in mammals. J Biol Chem 286:18347–18353
9. Jenuwein T, Allis CD (2001) Translating the histone code. Science 293:1074–1080
10. Kouzarides T (2007) Chromatin modifications and their function. Cell 128:693–705
11. Esteller M (2008) Epigenetics in cancer. N Engl J Med 358:1148–1159
12. Pogribny IP, Beland FA (2009) DNA hypomethylation in the origin and pathogenesis of human diseases. Cell Mol Life Sci 66:2249–2261
13. Ooi SK, Bestor TH (2008) The colorful history of active DNA demethylation. Cell 133:1145–1148
14. Ma DK, Guo JU, Ming GL, Song H (2009) DNA excision repair proteins and Gadd45 as molecular players for active DNA demethylation. Cell Cycle 8:1526–1531
15. He YF, Li BZ, Li Z, Wang Y, Tang Q, Ding J, Jiz Y, Chen Z, Li L, Sun Y, Li X, Dai Q, Song CX, Zhang K, He C, Xu GL (2011) Tet-mediated formation of 5-hydroxylcytosine and its excision by TDG in mammalian DNA. Science 333:1303–1307
16. Ito S, Shen L, Dai Q, Wu SC, Collins LB, Swenberg JA, He C, Zhang Y (2011) Tet proteins can convert 5-methylcytosine to 5-formylcytosine and 5-carboxylcytosine. Science 333: 1300–1333
17. Cortellino S, Xu J, Sannai M, Moore R, Caretti E, Cigliano A, Le Coz M, Devarajan K, Wessels A, Soprano D, Abramowitz LK, Bartolomei MS, Rambow F, Bassi MR, Bruno T, Fanciulli M, Renner C, Klein-Szanto AJ, Matsumoto Y, Kobi D, Davidson I, Alberti C, Larue L, Bellacosa A (2011) Thymine DNA glycosylase is essential for active DNA demethylation by linked deamination-base excision repair. Cell 146:67–79
18. Feinberg AP, Tycko B (2004) The history of cancer epigenetics. Nat Rev Cancer 4:143–153
19. Ehrlich M (2009) DNA hypomethylation in cancer cells. Epigenomics 1:239–259
20. Wilson AS, Power BE, Molloy PL (2007) DNA hypomethylation and human diseases. Biochim Biophys Acta 1775:138–162
21. De Smet C, Loriot A (2010) DNA hypomethylation in cancer: Epigenetic scars of a neoplastic journey. Epigenetics 5:206–213
22. Rollins RA, Haghighi F, Edwards JR, Das R, Zhang MQ, Ju J, Bestor TH (2006) Large-scale structure of genomic methylation patterns. Genome Res 16:157–163
23. Chen RZ, Pettersson U, Beard C, Jackson-Grusby L, Jaenisch R (1998) DNA hypomethylation leads to elevated mutation rates. Nature 395:89–93
24. Ehrlich M (2002) DNA methylation in cancer: too much, but also too little. Oncogene 21:5400–5413
25. Feinberg AP, Cui H, Ohlsson R (2002) DNA methylation and genomic imprinting: insights from cancer into epigenetic mechanisms. Semin Cancer Biol 12:389–398
26. Howard G, Eiges R, Gaudet F, Jaenisch R, Eden A (2008) Activation and transposition of endogenous retroviral elements in hypomethylation induced tumors in mice. Oncogene 27:404–408
27. Eden A, Gaudet F, Waghmare A, Jaenisch R (2003) Chromosomal instability and tumors promoted by DNA hypomethylation. Science 300:455
28. Wong NC, Wong LH, Quach JM, Canham P, Craig JM, Song IZ, Clark SJ, Choo KH (2006) Permissive transcriptional activity at the centromere through pockets of DNA hypomethylation. PLoS Genet 2:e17
29. Vera E, Canela A, Fraga MF, Esteller M, Blasco MA (2008) Epigenetic regulation of telomeres in human cancer. Oncogene 27:6817–6833
30. Wild L, Flanagan JM (2010) Genome-wide hypomethylation in cancer may be a passive consequence of transformation. Biochim Biophys Acta 1806:50–57
31. Wainfan E, Poirier LA (1992) Methyl groups in carcinogenesis: effects on DNA methylation and gene expression. Cancer Res 52:2071s–2077s
32. Christman JK (1995) Dietary effects on DNA methylation: do they account for hepatocarcinogenic properties of lipotrope diets? Adv Exp Med Biol 369:141–154
33. Pogribny IP, James SJ, Jernigan S, Pogribna M (2004) Genomic hypomethylation is specific for preneoplastic liver in folate/methyl deficient rats and does not occur in non-target tissues. Mutat Res 548:53–59

34. Gaudet F, Hodgson JG, Eden A, Jackson-Grusby L, Dausman J, Gray JW, Leonhardt H, Jaenisch R (2003) Induction of tumors in mice by genomic hypomethylation. Science 300:489–492
35. Fan T, Schmidtmann A, Xi S, Briones V, Zhu H, Suh HC, Gooya J, Keller JR, Xu H, Roayaei J, Anver M, Ruscetti S, Muegge K (2008) DNA hypomethylation caused by Lsh deletion promotes erythroleukemia development. Epigenetics 3:134–142
36. Counts JL, Goodman JI (1994) Hypomethylation of DNA: an epigenetic mechanism involved in tumor promotion. Mol Carcinog 11:185–188
37. Wild L, Funes JM, Boshoff C, Flanagan JM (2010) In vitro transformation of mesenchymal stem cells induces gradual genomic hypomethylation. Carcinogenesis 31:1854–1862
38. Bagnyukova TV, Tryndyak VP, Montgomery B, Churchwell MI, Karpf AR, James SR, Muskhelishvili L, Beland FA (2008) Genetic and epigenetic changes in rat preneoplastic liver tissue induced by 2-acetylaminofluorene. Carcinogenesis 29:638–646
39. Yamada Y, Jackson-Grusby L, Linhart H, Meissner A, Eden A, Lin H, Jaenish R (2005) Opposing effects of DNA hypomethylation on intestinal and liver carcinogenesis. Proc Natl Acad Sci USA 102:13580–13585
40. Tomita H, Hirata A, Yamada Y, Hata K, Oyama T, Mori H, Yamashita S, Ushijima T, Hara A (2010) Suppressive effect of global DNA hypomethylation on gastric carcinogenesis. Carcinogenesis 31:1627–1633
41. Kinney SR, Moser MT, Pascual M, Greally JM, Foster BA, Karpf AR (2010) Opposing roles of Dnmt1 in early- and late-stage murine prostate cancer. Mol Cell Biol 30:4159–4174
42. Maunakea AK, Nagarajan RP, Bilenky M, Ballinger TJ, D'Souza C, Fouse SD, Johnson BE, Hong C, Nielsen C, Zhao Y, Turecki G, Delaney A, Varhol R, Thiessen N, Shchors K, Heine VM, Rowitch DH, Xing X, Fiore C, Schillebeeckx M, Jones SJM, Haussler D, Marra MA, Hirst M, Wang T, Costello JF (2010) Conserved role of intragenic DNA methylation in regulating alternative promoters. Nature 466:253–257
43. Takai D, Jones PA (2002) Comprehensive analysis of CpG islands in human chromosomes 21 and 22. Proc Natl Acad Sci USA 99:3740–3745
44. Illingworth RS, Bird AP (2009) CpG islands—"a rough guide". FEBS Lett 583:1713–1720
45. Deaton AM, Bird A (2011) CpG islands and the regulation of transcription. Genes Dev 25:1010–1022
46. Jacinto FV, Esteller M (2007) Mutator pathways unleashed by epigenetic silencing in human cancer. Mutagenesis 22:247–253
47. Lahtz C, Pfeifer GP (2011) Epigenetic changes of DNA repair genes in cancer. J Mol Cell Biol 3:51–58
48. Esteller M, Toyota M, Sanchez-Cespedes M, Capella G, Peinado MA, Watkins DN, Issa JP, Sidransky D, Baylin SB, Herman JG (2000) Inactivation of the DNA repair gene O6-methylguanine-DNA methyltransferase by promoter hypermethylation is associated with G to A mutations in K-ras in colorectal tumorigenesis. Cancer Res 60:2368–2371
49. Esteller M, Riques RA, Toyota M, Capella G, Moreno V, Peinado MA, Baylin SB, Herman JG (2001) Promoter hypermethylation of the DNA repair gene O6-methylguanine-DNA methyltransferase is associated with the presence G:C to A:T transition mutations in p53 in human colorectal tumorigenesis. Cancer Res 61:4689–4692
50. Birgisdottir V, Stefansson OA, Bodvarsdottir SK, Hilmarsdottir H, Jonasson JG, Eyfjorf JE (2006) Epigenetic silencing and deletion of the BRCA1 gene in sporadic breast cancer. Breast Cancer Res 8:R38
51. Herman JG, Umar A, Polyak K, Graff JR, Ahuja N, Issa JP, Markowitz S, Willson JK, Hamilton SR, Kinzler KW, Kane MF, Kolodner RD, Vogelstein B, Kunkel TA, Baylin SB (1998) Incidence and functional consequences of hMLH1 promoter hypermethylation in colorectal carcinoma. Proc Natl Acad Sci USA 95:6870–6875
52. Fraga MF, Ballestar E, Villar-Garea A, Boix-Chornet M, Espada J, Schotta G, Bonaldi T, Haydon C, Ropero S, Petrie K, Lyer NG, Pérez-Rosado A, Calvo E, Lopez JA, Cano A, Calasanz MJ, Colomer D, Piris MA, Ahn N, Imhof A, Caldas C, Jenuwein T, Esteller M (2005) Loss of acetylation at Lys16 and trimethylation at Lys20 of histone H4 is a common hallmark of human cancer. Nat Genet 37:391–400

53. Bartel DP (2009) MicroRNAs: target recognition and regulatory functions. Cell 136:215–233
54. Carthew RW, Sontheimer EJ (2009) Origins and mechanisms of miRNAs and siRNAs. Cell 136:642–655
55. Guo H, Ingolia NT, Weissman JS, Bartel DP (2010) Mammalian microRNAs predominantly act to decrease target mRNA levels. Nature 466:835–840
56. Garzon R, Calin GA, Croce CM (2009) MicroRNAs in cancer. Annu Rev Med 60:167–179
57. Ventura A, Jacks ST (2009) MicroRNAs and cancer: short RNAs go a long way. Cell 136:586–591
58. Di Leva G, Croce CM (2010) Roles of small RNAs in tumor formation. Trends Mol Med 16:257–267
59. Pogribny IP (2009) MicroRNA dysregulation during chemical carcinogenesis. Epigenomics 1:281–290
60. Loeb LA, Harris CC (2008) Advances in chemical carcinogenesis: a historical review and prospective. Cancer Res 68:6863–6872
61. Wild CP (2009) Environmental exposure measurement in cancer epidemiology. Mutagenesis 24:117–125
62. Clapp RW, Jacobs MM, Loechler EL (2008) Environmental and occupational causes of cancer: new evidence 2005–2007. Rev Environ Health 23:1–37
63. Miller JA (1970) Carcinogenesis by chemicals: and overview—G.H.A. Clowes memorial lecture. Cancer Res 30:559–576
64. Herceg Z (2007) Epigenetics and cancer: towards an evaluation of the impact of environmental and dietary factors. Mutagenesis 22:91–103
65. Pogribny IP, Rusyn I, Beland FA (2008) Epigenetics aspects of genotoxic and non-genotoxic hepatocarcinogenesis: studies in rodents. Environ Mol Mutagen 49:9–15
66. Bollati V, Baccareli A (2010) Environmental epigenetics. Heredity 105:105–112
67. Nakajima T, Enomoto S, Ushijima T (2008) DNA methylation: a marker for carcinogen exposure and cancer risk. Environ Health Prev Med 13:8–15
68. Ziech D, Franco R, Pappa A, Malamou-Mitsi V, Georgakila S, Georgakitas AG, Panayiotidis MI (2010) The role of epigenetics in environmental and occupational carcinogenesis. Chem Biol Interact 188:340–349
69. Hughes MF, Beck BD, Chen Y, Lewis AS, Thomas DJ (2011) Arsenic exposure and toxicology: a historical perspective. Toxicol Sci 123:305–332
70. Rossman TG (2003) Mechanism of arsenic carcinogenesis: an integrated approach. Mutat Res 533:37–65
71. IARC Monographs on the Evaluations of Carcinogenic Risks to Humans (2004) Some drinking-water disinfectants and contaminants, including arsenic, vol 84. IARC, Lyon
72. Liu J, Waalkes MP (2008) Liver is a target of arsenic carcinogenesis. Toxicol Sci 105:24–32
73. Cui X, Wakai T, Shirai Y, Hatakeyama K, Hirano S (2006) Chronic oral exposure to inorganic arsenate interferes with methylation status of p16INK4A and RASSF1A and induces lung cancer in A/J mice. Toxicol Sci 91:372–381
74. Waalkes MP, Ward JM, Liu J, Diwan BA (2003) Transplacental carcinogenicity of inorganic arsenic in the drinking water: induction of hepatic, ovarian, pulmonary, and adrenal tumors in mice. Toxicol Appl Pharmacol 186:7–17
75. Tokar EJ, Diwan BA, Ward JM, Delker DA, Waalkes MP (2011) Carcinogenic effects of "whole-life" exposure to inorganic arsenic in CD1 mice. Toxicol Sci 119:73–83
76. Reichard JF, Puga A (2010) Effects of arsenic exposure on DNA methylation and epigenetic gene regulation. Epigenomics 2:87–104
77. Zhao CQ, Young MR, Diwan BA, Coogan TP, Waalkes MP (1997) Association of arsenic-induced malignant transformation with DNA hypomethylation and aberrant gene expression. Proc Natl Acad Sci USA 94:10907–10912
78. Chen H, Li S, Liu J, Diwan BA, Barrett JC, Waalkes MP (2004) Chronic inorganic arsenic exposure induces hepatic global and individual gene hypomethylation: implications for arsenic hepatocarcinogenesis. Carcinogenesis 25(9):1779–1786

79. Jensen TJ, Novak P, Elbin KE, Gandolfi AJ, Futscher BW (2008) Epigenetic remodeling during-arsenical-induced malignant transformation. Carcinogenesis 29:1500–1508
80. Ren X, McHale CM, Skibola CF, Smith AH, Smith MT, Zhang L (2011) An emerging role for epigenetic dysregulation in arsenic toxicity and carcinogenesis. Environ Health Perspect 119:11–19
81. Coppin JF, Qu W, Waalkes MP (2008) Interplay between cellular methyl metabolism and adaptive efflux during oncogenic transformation from chronic arsenic exposure in human cells. J Biol Chem 283:19342–19350
82. Kitchin KT, Ahmad S (2003) Oxidative stress as a possible mode of action for arsenic carcinogenesis. Toxicol Lett 137:3–13
83. Kojima C, Ramirez DC, Tokar EJ, Himeno S, Drobná Z, Stýblo M, Mason RP, Waalkes MP (2009) Requirement of arsenic biomethylation for oxidative DNA damage. J Natl Cancer Inst 101:1670–1681
84. Valinluck V, Sowers LC (2007) Endogenous cytosine damage products alter the site selectivity of human DNA maintenance methyltransferase DNMT1. Cancer Res 67:946–950
85. Lee DH, Jacobs DR Jr, Porta M (2009) Hypothesis: a unifying mechanism for nutrition and chemicals as lifelong modulators of DNA hypomethylation. Environ Health Perspect 117:1799–1802
86. Mass MJ, Wang L (1997) Arsenic alters cytosine methylation patterns of the promoter of the tumor suppressor gene p53 in human lung cells: a model for a mechanism of carcinogenesis. Mutat Res 386:263–277
87. Huang YC, Huang WC, Chen WT, Yu HS, Chai CY (2009) Sodium arsenite-induced DAPK hypermethylation and autophagy via ERK1/2 phosphorylation in human uroepithelial cells. Chem Biol Interact 181:254–262
88. Zhou X, Sun H, Ellen TP, Chen H, Costa M (2008) Arsenite alters global histone H3 methylation. Carcinogenesis 29:1831–1836
89. Beezhold K, Liu J, Kan H, Meighan T, Castranova V, Shi X, Chen F (2011) miR-190-mediated downregulation of PHLP contributes to arsenic-induced Akt activation and carcinogenesis. Toxicol Sci 123(2):411–420
90. Salnikow K, Zhitkovich A (2008) Genetic and epigenetic mechanisms in metals carcinogenesis and cocarcinogenesis: nickel, arsenic, and chromium. Chem Res Toxicol 21:28–44
91. Martinez-Zamudio R, Ha HC (2011) Environmental epigenetics in metal exposure. Epigenetics 6(7):820–827
92. IARC Monographs on the Evaluations of Carcinogenic Risks to Humans (2008) 1,3-Butadiene, ethylene oxide and vinyl halides (vinyl fluoride, vinyl chloride, and vinyl bromide), vol 97. IARC, Lyon
93. Melnick RL, Sills RC (2001) Comparative carcinogenicity of 1,3-butadiene, isoprene and chloroprene in rats and mice. Chem Biol Interact 135–136:27–42
94. Walker VE, Walker DM, Meng Q, McDonald JD, Scott BR, Selikop SK, Claffey DJ, Upton PB, Powley MW, Swenberg JA, Henderson RF, Committee HR (2009) Genotoxicity of 1,3-butadiene and its epoxy intermediate. Res Rep Health Eff Inst 144:3–79
95. Koturbash I, Scherhag A, Sorrentino J, Sexton K, Bodnar W, Tryndyak V, Latendresse JR, Swenberg JA, Beland FA, Pogribny IP, Rusyn I (2011) Epigenetic alterations in liver of C57BL/6J mice after short-term inhalational exposure to 1,3-butadiene. Environ Health Perspect 119:635–640
96. Schotta G, Lachner M, Sarma K, Ebert A, Sengupta R, Reuter G, Reinberg D, Jenuwein T (2004) A silencing pathway to induce H3-K9 and H4-K20 trimethylation at constitutive heterochromatin. Genes Dev 18:1251–1262
97. Martens JH, O'Sullivan RJ, Braunschweig U, Opravil S, Radolf M, Steinlein P, Jenuwein T (2005) The profile of repeat-associated histone lysine methylation states in the mouse epigenome. EMBO J 24:800–812
98. Jenuwein Y (2006) The epigenetic magic of histone lysine methylation. FEBS J 273:3121–3135
99. Koturbash I, Scherhag A, Sorrentino J, Sexton K, Bodnar W, Swenberg JA, Beland FA, Pardo-Manuel Devillena F, Rusyn I, Pogribny IP (2011) Epigenetic mechanisms of mouse

interstrain variability in genotoxicity of the environmental toxicant 1,3-butadiene. Toxicol Sci 122:448–456
100. Rusyn I, Gatti DM, Wilshire T, Kleeberger SR, Threadgill DW (2010) Toxicogenomics: population-based testing of drug and chemical safety in mouse models. Pharmacogenomics 11:1127–1136
101. IARC Monographs on the Evaluations of Carcinogenic Risks to Humans (2011) A review of human carcinogens, vol 100, Part A: Pharmaceuticals. IARC, Lyon
102. Li S, Washburn KA, Moore R, Uno T, Teng C, Newbold RR, McLachlan JA, Negishi M (1997) Developmental exposure to diethylstilbestrol elicits demethylation of estrogen-responsive lactoferrin gene in mouse uterus. Cancer Res 57:4356–4359
103. Li S, Hansman R, Newbold R, Davis B, McLachlan JA, Barrett JC (2003) Neonatal diethylstilbestrol exposure induces persistent elevation of c-fos expression and hypomethylation in its exon-4 in mouse uterus. Mol Carcinog 38:78–84
104. Tang WY, Newbold R, Mardilovich K, Jefferson W, Cheng RY, Medvedovic M, Ho SM (2008) Persistent hypomethylation in the promoter of nucleosomal binding protein 1 (Nsbp1) with overexpression of Nsbp1 in mouse uteri neonatally exposed to diethylstilbestrol or genistein. Endocrinology 149:5922–5931
105. Sato K, Fukata H, Kogo Y, Ohgane J, Shiota K, Mori C (2009) Neonatal exposure to diethylstilbestrol alters expression of DNA methyltransferases and methylation of genomic DNA in the mouse uterus. Endocr J 56:131–139
106. Hsu PY, Detherage DE, Rodriguez BA, Liyanarachchi S, Weng YI, Zuo T, Liu J, Cheng AS, Huang TH (2009) Xenoestrogen-induced epigenetic repression of microRNA-9-3 in breast epithelial cells. Cancer Res 69:5936–5945
107. Lehmann U, Hasemeier B, Christgen M, Müller M, Römermann D, Länger F, Kreipe H (2008) Epigenetic inactivation of microRNA gene has-mir-9-1 in human breast cancer. J Pathol 214:17–24
108. Jordan VC (2006) Tamoxifen (ICI146,474) as a target therapy to treat and prevent breast cancer. Br J Pharmacol 147:S269–S276
109. Shang Y (2006) Molecular mechanisms of oestrogen and SERMs in endometrial carcinogenesis. Nat Rev Cancer 6:360–368
110. Wu H, Chen Y, Liang J, Shi B, Wu G, Zhang Y, Wang D, Li R, Yi X, Zhang H, Sun L, Shang Y (2005) Hypomethylation-linked activation of PAX2 mediates tamoxifen-stimulated endometrial carcinogenesis. Nature 438:981–987
111. Wogan GN (1997) Review of the toxicology of tamoxifen. Semin Oncol 24:S87–S97
112. Phillips DH (2001) Understanding the genotoxicity of tamoxifen? Carcinogenesis 22:839–849
113. Gamboa da Costa G, McDaniel-Hamilton LP, Heflich RH, Margues MM, Beland FA (2001) DNA adduct formation and mutant induction in Sprague–Dawley rats treated with tamoxifen and its derivatives. Carcinogenesis 22:130701315
114. Tryndyak VP, Muskhelishvili L, Kovalchuk O, Rodriguez-Juarez R, Churchwell MI, Ross SA, Beland FA, Pogribny IP (2006) Effect of long-term tamoxifen exposure on genotoxic and epigenetic changes in rat liver: implications for tamoxifen-induced hepatocarcinogenesis. Carcinogenesis 27:1713–1720
115. Tryndyak VP, Kovalchuk O, Muskhelishvili L, Montgomery B, Rodriguez-Juarez R, Melnyk S, Ross SA, Beland FA, Pogribny IP (2007) Epigenetic reprogramming of liver cells in tamoxifen-induced rat hepatocarcinogenesis. Mol Carcinog 46:187–197
116. Pogribny IP, Tryndyak VP, Boyko A, Rodriguez-Juarez R, Beland FA, Kovalchuk O (2007) Induction of microRNAome deregulation in rat liver by long-term tamoxifen exposure. Mutat Res 619:30–37
117. Bachman AN, Phillips JM, Goodman JI (2006) Phenobarbital induces progressive patterns of GC-rich and gene-specific altered DNA methylation in the liver of tumor-prone B6C3F1 mice. Toxicol Sci 91:393–405
118. Phillips JM, Goodman JI (2009) Multiple genes exhibit Phenobarbital-induced constitutive active/androstane receptor-mediated DNA methylation changes during liver tumorigenesis and in liver tumors. Toxicol Sci 108:273–289

119. Lempiäinen H, Müller A, Brasa S, Teo SS, Roloff TC, Morawiec L, Zamurovic N, Vicart A, Funhoff E, Couttet P, Schübeler D, Grenet O, Marlowe J, Moggs J, Terranova R (2011) Phenobarbital mediates an epigenetic switch at the constitutive androstane receptor (CAR) target gene Cyp2b10 in the liver of B6C3F1 mice. PLoS One 6:e18216
120. Bucher JR, Shackelford CC, Haseman JK, Johnson JD, Kurtz PJ, Persing RL (1994) Carcinogenicity studies of oxazepam in mice. Fundam Appl Toxicol 23:280–297
121. Iida M, Anna CH, Hartis J, Bruno M, Wetmore B, Dubin JR, Sieber S, Bennett L, Cunningham ML, Paules RS, Tomer KB, Houle CD, Merrick AB, Sills RC, Devereux TR (2003) Changes in global gene and protein expression during early mouse liver carcinogenesis induced by non-genotoxic model carcinogens oxazepam and Wyeth-14,643. Carcinogenesis 24:757–770
122. Lahousse SA, Hoenerhoff M, Collins J, Ton TV, Massinde T, Olson D, Rebolosso Y, Koujitani T, Tomer KB, Hong HH, Bucher J, Sills RC (2011) Gene expression and mutation assessment provide clues of genetic and epigenetic mechanisms in liver tumors of oxazepam-exposed mice. Vet Pathol 48:678–699
123. Ferguson LR, Philpott M (2008) Nutrition and mutagenesis. Annu Rev Nutr 28:313–329
124. IARC Monographs on the Evaluations of Carcinogenic Risks to Humans (2002) Some traditional herbal medicines, some mycotoxins, naphthalene and styrene, vol 82. IARC, Lyon
125. Wild CP, Gong YY (2010) Mycotoxins and human disease: a largely ignored global health issue. Carcinogenesis 31:71–82
126. Knasmüller S, Bresgen N, Kassie F, Mersch-Sundermann V, Gelderblom W, Zöhrer E, Eckl PM (1997) Genotoxic effects of three Fusarium mycotoxins, fumonisin B1, moniliformin and vomitoxin in bacteria and primary cultures of rat hepatocytes. Mutat Res 391:39–48
127. Wang JS, Groopman JD (1999) DNA damage by mycotoxins. Mutat Res 424:167–181
128. Zhang YJ, Ahsan H, Chen Y, Lunn RM, Wang LY, Chen SY, Lee PH, Chen CJ, Santella RM (2002) High frequency of promoter hypermethylation of RASSF1A and p16 and its relationship to aflatoxin B1-DNA adduct levels in human hepatocellular carcinoma. Mol Carcinog 35:85–92
129. Zhang YJ, Chen Y, Ahsan H, Lunn RM, Chen SY, Santella RM (2003) Inactivation of the DNA repair gene O6-methylguanine-DNA methyltransferase by promoter hypermethylation and its relationship to aflatoxin B1-DNA adducts and the p53 mutations in hepatocellular carcinoma. Int J Cancer 103:440–444
130. Su H, Zhao J, Xiong Y, Xu T, Zhou F, Yuan Y, Zhang Y, Zhuang SM (2008) Large-scale analysis of the genetic and epigenetic alterations in hepatocellular carcinoma from Southeast China. Mutat Res 641:27–35
131. Zhao W, Liu H, Liu W, Wu Y, Chen W, Jiang B, Zhou Y, Xue R, Luo C, Wang L, Jiang JD, Liu J (2006) Abnormal activation of the synuclein-gamma gene in hepatocellular carcinomas by epigenetic alteration. Int J Oncol 28:1081–1088
132. Kouadio JH, Dano SD, Moukha S, Mobio TA, Creppy EE (2007) Effects of combinations of Fusarium mycotoxins on the inhibition of macromolecular synthesis, malondialdehyde levels, DNA methylation and fragmentation, and viability in Caco-2 cells. Toxicon 49:306–317
133. Marin-Kuan M, Cavin C, Delatour T, Schilter B (2008) Ochratoxin A carcinogenicity involves a complex network of epigenetic mechanisms. Toxicon 52:195–202
134. Hu W, Feng Z, Tang M (2003) Preferential carcinogen-DNA adduct formation at codons 12 and 14 in the human K-ras gene and their possible mechanisms. Biochemistry 42: 10012–10023
135. IARC Monographs on the Evaluations of Carcinogenic Risks to Humans (2011) A review of human carcinogens, vol 100, Part B: Biological agents. IARC, Lyon
136. Maekita T, Nakazawa K, Mihara M, Nakajima T, Yanaoka K, Iguchi M, Arii K, Kaneda A, Tsukamoto T, Tatematsu M, Tamura G, Saito D, Sugimura T, Ichinose M, Ushijima T (2006) High levels of aberrant DNA methylation in Helicobacter pylori-infected gastric mucosae and its possible association with gastric cancer risk. Clin Cancer Res 12:989–995
137. Nakajima T, Yamashita S, Maekita T, Niwa T, Nakazawa K, Ushijima T (2009) The presence of methylation fingerprint of Helicobacter pylori infection in human gastric mucosae. Int J Cancer 124:905–910

138. Niwa T, Tsukamoto T, Toyoda T, Mori A, Tanaka H, Maekita T, Ichinose M, Tatematsu M, Ushijima T (2010) Inflammatory processes triggered by Helicobacter pylori infection cause aberrant DNA methylation in gastric epithelial cells. Cancer Res 70:1430–1440
139. Shin CM, Kim N, Jung Y, Park JH, Kang GH, Kim JS, Jung HC, Song IS (2010) Role of Helicobacter pylori infection in aberrant DNA methylation along multistep gastric carcinogenesis. Cancer Sci 101:1337–1346
140. Nakajima T, Enomoto S, Yamashita S, Ando T, Nakanishi Y, Nakazawa K, Oda I, Gotoba T, Ushijima T (2010) Persistence of a component of DNA methylation in gastric mucosa after Helicobacter pylori eradication. J Gastroenterol 45:37–44
141. LeBaron MJ, Rasoulpour RJ, Klapacz J, Ellis-Hutchings RG, Hollnagel HM, Gollapudi BB (2010) Epigenetics and chemical safety assessment. Mutat Res 705:83–95
142. Huang YW, Kuo CT, Stoner K, Huang TH, Wang LS (2011) An overview of epigenetics and chemoprevention. FEBS Lett 585:2129–2136
143. Goodman JI, Augustine KA, Cunningham ML, Dixon D, Dragan YP, Falls JG, Rasoulpour RJ, Sills RC, Storer RD, Wolf DC, Pettit SD (2010) What do we need to know prior to thinking about incorporating an epigenetic evaluation into safety assessments? Toxicol Sci 116:375–381
144. Rasoulpour RJ, LeBaron MJ, Ellis-Hutchings RG, Klapacz J, Gpllapudi BB (2011) Epigenetic screening in product safety assessment: are we there yet? Toxicol Mech Methods 21:298–311
145. Kalari S, Pfeifer GP (2010) Identification of driver and passenger DNA methylation in cancer by epigenomic analysis. Adv Genet 70:277–308

Chapter 12
Blood-Derived DNA Methylation Markers of Cancer Risk

Carmen Marsit and Brock Christensen

Abstract The importance of somatic epigenetic alterations in tissues targeted for carcinogenesis is now well recognized and considered a key molecular step in the development of a tumor. Particularly, alteration of gene-specific and genomic DNA methylation has been extensively characterized in tumors, and has become an attractive biomarker of risk due to its specificity and stability in human samples. It also is clear that tumors do not develop as isolated phenomenon in their target tissue, but instead result from altered processes affecting not only the surrounding cells and tissues, but other organ systems, including the immune system. Thus, alterations to DNA methylation profiles detectable in peripheral blood may be useful not only in understanding the carcinogenic process and response to environmental insults, but can also provide critical insights in a systems biological view of tumorigenesis. Research to date has generally focused on how environmental exposures alter genomic DNA methylation content in peripheral blood. More recent work has begun to translate these findings to clinically useful endpoints, by defining the relationship between DNA methylation alterations and cancer risk. This chapter highlights the existing research linking the environment, blood-derived DNA methylation alterations, and cancer risk, and points out how these epigenetic alterations may be contributing fundamentally to carcinogenesis.

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12.1 Introduction

Epigenetic alterations within cells that give rise to tumors are believed to be causal contributors to the development of malignancy [27, 38]. The most widely studied epigenetic mechanism in cancer is DNA methylation and it is well recognized that cancer cells concomitantly exhibit both gene-specific increases in DNA methylation and genome-wide hypomethylation compared to their normal tissue counterparts. Because DNA methylation is tissue-specific, perhaps it is no surprise that a multitude of studies seeking to detect tumor-specific DNA methylation for early detection/diagnosis have used cell-free fractions (serum, plasma) of peripheral blood. Studies measuring DNA methylation in serum and plasma aim to reduce the potential noise contributed by leukocyte methylation patterns in whole blood and to specifically detect tumor-derived DNA methylation. However, interindividual variability in leukocyte methylation patterns may be—akin to genetic variation—related to an individual's cancer risk while acquired alterations to leukocyte methylation may represent both a cause and consequence of carcinogenesis in solid tissues. As new measurement technologies and analytic strategies are being developed, and there is an improved understanding of the contribution of the immune system to solid tumor development, there may be great utility in peripheral blood methylation analysis for predicting cancer risk.

This chapter will cover evidence from human studies that peripheral blood DNA methylation states can inform cancer risk. First, investigations of repetitive element and global DNA methylation will be presented. Then, epimutation and gene-specific methylation markers of cancer risk will be discussed, followed by more recent and larger-scale investigations of blood methylation and cancer risk. Notably, as mentioned above, cancer epigenetics includes a large body of research on cell-free (plasma, serum) DNA methylation for diagnostic and prognostic purposes that is not within this chapter's scope. To end, potential mechanisms underlying the basis for blood-based methylation markers of cancer risk, and future directions for this avenue of research will be covered.

12.2 Repetitive Elements, Global Methylation, and Cancer Risk

12.2.1 Introduction to Global Methylation and Repetitive Elements

While the classic example of altered DNA methylation in cancer would likely describe promoter hypermethylation-induced gene silencing of a tumor suppressor gene, before this phenomenon was understood it was recognized that tumors are heavily hypomethylated relative to their normal tissue counterparts. In contrast to tumor suppressor gene promoters, moderately to highly repetitive,

non-coding sequences of the genome are normally methylated [26, 63]. Indeed, generally non-specific methylation of repeat and non-coding elements is considered an important part of normal development, cellular differentiation, and X-chromosome inactivation. Hence, changes in this methylation can lead to specific human disease states including cancer. In fact, genomic or global hypomethylation is now thought to occur early in tumorigenesis, including in pre-cancerous lesions [60, 66, 67], and may promote cancer development by contributing to genomic instability.

A few studies have directly assessed the relationship between total genomic methyl-cytosine in blood and cancer risk. Pufulete and colleagues measured genome-wide reduction in 5-methylcytosine content with a (relatively insensitive) ^{3}H-thymidine incorporation assay in peripheral blood lymphocytes and found that hypomethylation significantly increased risk for colon adenoma and indicated a trend in risk of colon cancer [60]. In an investigation of colorectal adenoma among women, Lim et al. measured total genomic leukocyte methylation utilizing DNA digestion followed by liquid chromatography and mass spectrometry for quantitation in 230 cases and controls. When setting the referent group as the women in the *lowest tertile of methylation*, women in the second tertile had a reduced risk of colorectal adenoma (odds ratio (OR): 0.72, 95% CI: 0.34–1.52), and women in highest methylation tertile had a significantly decreased risk of colorectal adenoma (OR: 0.17, 95% CI: 0.06–0.49) [47]. Around the same time, a hospital-based case–control study in Spain found that reduced total percent methyl-cytosine content (using high-performance capillary electrophoresis, *HpaII* digestion, and densitometry) was significantly associated with bladder cancer risk [54]: compared to the quartile of subjects with the highest percent 5-methyl-cytosine, the adjusted OR for subjects in the lowest quartile of methylation was 2.67 (95% CI: 1.8–4.0). Further, when stratifying by smoking status, global hypomethylation was a strong risk factor for bladder cancer in never smokers (OR: 6.4, 95% CI: 2.4–17.2).

Early links between genomic hypomethylation and pathogenesis generated great interest in developing additional methods to determine global DNA methylation. Total genomic methyl-cytosine content can be directly measured, though large amounts of substrate and highly specialized equipment are required. In the mid 1990s, founded on the basis of chemical modification of DNA with sodium bisulfite, a PCR-based method for measuring DNA methylation was developed: methylation-specific PCR (MSP) [30]. Later, a quantitative version of MSP known as MethyLight was developed in Peter Laird's lab [21]. Using MethyLight to measure *LINE-1*, *Alu*, and satellite element repeats, Weisenberger et al. showed that methylation of repetitive elements were reasonably well correlated with total methyl-cytosine content [74]. Around the same time, the first report of bisulfite sequencing for *LINE-1* and *Alu* elements was published and was claimed as a simple method to estimate global DNA methylation [80]. Alone, *LINE-1* and *Alu* elements comprise about 30% of the human genome, making them an attractive target for a surrogate measure of global methylation [80]. With these more accessible methods to measure "global methylation", many groups began evaluating global methylation. As a result, the term "global methylation" lost its specific meaning

and started being used to describe any of these assays even though their measures are potentially non-comparable.

Because repetitive elements such as *LINE-1* and *Alu* are used to signify global methyl-cytosine content, it is important to clarify what these elements are and to point out potential drawbacks of using these as surrogate measures of global methylation. Long interspersed nuclear elements (*LINE-1*) and short interspersed nuclear elements (SINEs, which include *Alu* elements and mammalian interspersed repeats (MIR)), and long terminal repeats (LTRs) are retrotransposons. Collectively, with tandem repeats such as satellite elements (SAT), LINE, SINE, and LTR retrotransposons comprise approximately half of the human genome. The majority of these elements are evolutionary remnants that are truncated or mutated and even if transcribed would have no phenotype. For instance, there are approximately 500,000 *LINE-1* elements in the genome; very few of these are full-length (6 kb) complete with an internal RNA polymerase II promoter in the 5′ UTR, two open reading frames that encode an RNA-binding protein and elements for retrotransposon activity, and a 3′ UTR with a polyadenylation signal [17]. Unlike *LINE-1*, *Alu* elements use an internal RNA polymerase III promoter and lack any coding sequence. For retrotransposition, *Alu* elements require the retrotransposon machinery encoded by *LINE-1* elements [19]. LTRs are considered endogenous retroviruses, and with over 400,000 copies, these repeat elements account for 8% of the human genome [43]. Lastly, satellite repeated sequences (SAT) are small DNA transposons that are the oldest type of transposable element, having arisen as a result of simple repeat amplification [39, 43].

Because repeat elements can have transposition activity, largely outnumber coding genes and make up a large fraction of the genome, it is critical that they are appropriately regulated. Hence, in normal cells repeat elements are maintained as silenced with relatively high levels of DNA methylation in their promoter regions. However, if methylation is lost at repeat elements they may be re-expressed and insert into various regions of the genome, possibly leading to the inactivation of tumor suppressor genes, or activation of oncogenes, thereby contributing to cancer as well as other human diseases [18, 41].

12.2.2 Satellite Elements and Long Terminal Repeats

Although satellite elements and long terminal repeats are numerous and make up a considerable portion of the human genome, their potential role in carcinogenesis remains understudied. Nonetheless, initial investigations into LTR repeats in tumors have indicated that inappropriate activation of LTR repeats is linked to cancers. The methylation status of one type of LTR, the endogenous retrovirus type K (HERV-K) was hypomethylated in bladder tumor tissue compared to normal bladder [23]. Similarly, in a small number of ovarian tumors, HERV-W was hypomethylated compared to non-tumor tissue [53]. More recently, an examination of satellite repeat expression in pancreatic ductal adenocarcinomas revealed that HSATII

transcripts were highly cancer-specific, alpha satellite transcripts were abundantly expressed, and that increased satellite expression in these cancers was likely due to loss of methylation [71]. Unfortunately, to our knowledge there have not yet been any studies examining methylation of satellite or LTRs in blood to test for association with risk of cancer. However, as large-scale sequencing efforts continue, non-coding elements are becoming better annotated and may allow for better-informed approaches to investigate the potential role of satellite and LTR repeat methylation in blood as it relates to cancer risk [1].

12.2.3 Long Interspersed Nuclear Elements and Alu elements

Using bisulfite pyrosequencing assays, a number of studies on *LINE-1* methylation in human peripheral blood have now been conducted. First, it is interesting to note that there are several studies investigating the association of *LINE-1* methylation in blood DNA with exposures that are etiologically relevant to human cancers. Examples of exposures that are associated with *LINE-1* hypomethylation include benzene [10], particulate matter including traffic particles [4, 68], polycyclic aromatic hydrocarbons [58], and persistent organic pollutants [62].

One of the first case–control studies of cancer to measure *LINE-1* methylation in blood was conducted in head and neck squamous cell carcinoma (HNSCC) [36]. Hsiung et al. measured *LINE-1* methylation with a modified version of combined bisulfite restriction analysis in over 800 HNSCC cases and controls. The between-subject variability in *LINE-1* methylation ranged from 54 to 87%, with a significant ($P<0.002$) increase in the *LINE-1* methylation in males compared to females, and significant increases in *LINE-1* methylation associated with positive HPV16 antibody serology and for subjects of non-Caucasian race compared to Caucasians ($P<0.02$ and $P<0.03$, respectively). In cases, controlling for age, gender, race, lifetime average drinks per week, and HPV16 serology, dietary folate in the lowest tertile, compared to the upper two tertiles, had a borderline significant reduction in *LINE-1* methylation. Similarly, subjects with the *MTHFR* 677 variant had a significant ($P<0.04$) reduction in *LINE-1* methylation; whereas, among cases, smoking was significantly associated ($P<0.04$) with increased *LINE-1* methylation. With respect to risk of HNSCC, patients in the lowest tertile of *LINE-1* methylation had a significant relative risk of HNSCC (OR: 1.6, 95% CI: 1.1–2.4), while those in the mid tertile showed an elevated OR of 1.3 (95% CI: 0.9–2.0) when controlling for age, gender, race, smoking, drinking, and HPV16 serology. Across tertiles there was a significant trend ($P<0.03$) for increased HNSCC risk with lower *LINE-1* methylation, and suggested that epigenetic variation, in this case extent of repetitive region methylation, is associated with disease risk [36].

In a study of breast cancer risk, Choi et al. measured *both* total methyl-cytosine content and *LINE-1* methylation in blood DNA from cases and controls [15]. With 176 cases and 173 controls, the authors first measured methyl-cytosine content and *LINE-1* methylation in a pilot subset of 19 cases and 18 controls, and found that

cases had significantly reduced methyl-cytosine content ($P=0.001$) compared to controls, whereas *LINE-1* methylation was not associated with case status or correlated ($r=-0.2$, $P=0.23$). Based on the results from the pilot cases and controls, the remaining cases and controls were evaluated for total methyl-cytosine only. Among several demographic factors examined (including age, race, BMI, smoking, parity, and menopausal status), high alcohol intake (>median) was the only factor significantly associated with reduced methyl-cytosine, and this was true in each of the case ($P<0.04$) and control groups ($P<0.04$). Further, among all cases and controls total methyl-cytosine content in blood DNA was significantly lower in cases than controls: when compared to women in the highest tertile of methylation, women in the lowest tertile of methylation had a significantly increased risk of breast cancer (OR: 2.9, 95% CI: 1.7–4.9). Despite the association between methyl-cytosine levels and alcohol intake, alcohol consumption did not affect the association between methyl-cytosine content and breast cancer risk. However, when stratifying on demographic and lifestyle factors, the authors found that risk was further increased by lower methyl-cytosine content in women with a family history of disease, as well as among women who were never smokers.

Studying the risk of gastric cancer in relation to repeat element methylation, Hou et al. used pyrosequencing and measured both *LINE-1* and *Alu* methylation in blood DNA from 302 gastric cancer cases and 421 age- and gender-matched controls [35]. This population-based case–control study enrolled participants from Warsaw, Poland. Methylation data were stratified into tertiles and in an analysis adjusted for age, sex, education level, smoking, and alcohol there were borderline significant associations between reduced methylation and gastric cancer risk for *LINE-1* (OR: 1.4, 95% CI: 0.9–2.0) and *Alu* (OR: 1.3, 95% CI: 0.9–1.9). Yet, in stratified analyses the association between *LINE-1* hypomethylation and gastric cancer risk was stronger for individuals with a family history of disease (OR: 3.1, 95% CI: 1.4–7.0), current drinkers of alcohol (OR: 1.9, 95% CI: 1.0–3.6), current smokers (OR: 2.3, 95% CI: 1.1–4.6), subjects who rarely or never consumed fruit, as well as carriers of either of two polymorphisms in 5-methyltetrahydrofolate-homocysteine methyltransferase reductase (*MTRR*). However, associations between *LINE-1* methylation and cancer risk were not modified by sex, infection with *Helicobacter pylori*, or intake of protein, vitamin B6, or folate.

An investigation of *LINE-1* blood DNA methylation and bladder cancer risk in a population-based case–control study in New Hampshire also indicated that reduced *LINE-1* methylation is associated with cancer risk [76]. Among 285 cases and 465 controls, *LINE-1* methylation values from bisulfite pyrosequencing ranged from 58 to 92%. Comparing subjects in the lowest methylation decile to all other subjects, controlling for age, gender, and smoking status indicated a significantly increased risk of bladder cancer for the lowest decile subjects (OR: 1.8, 95% CI: 1.1–2.9). In addition, these authors stratified their analysis by invasive and non-invasive disease and found that the lowest decile of *LINE-1* methlyation was associated with a significantly increased risk of non-invasive disease, but not invasive disease. Similar to the results from Hsiung et al., which showed that males had significantly higher *LINE-1* methylation levels, Wilhelm et al. stratified their analysis by gender and

found the association between *LINE-1* hypomethylation and bladder cancer to be stronger in women than in men (OR_{women}: 2.5, 95% CI: 1.2–5.2; OR_{men}: 1.5, 95% CI: 0.8–2.7). Finally, recalling the studies of *LINE-1* methylation and environmental exposures with etiologic relevance to cancer, these authors showed a significant association between high exposure to arsenic and reduced *LINE-1* methylation in control subjects.

Along with the studies of global and repeat element hypomethylation and bladder cancer risk in Europeans from Moore et al., and in American Caucasians from Wilhelm et al., a third study in Chinese subjects has also been published [13]. Among 510 cases and 528 controls from a case–control study based in Shanghai China, *LINE-1* methylation values from bisulfite pyrosequencing ranged from 73 to 93%. Notably, the low-end of *LINE-1* methylation in these subjects was higher than studies of Caucasians, 73% compared to 58% from [36], and 53% from [76]. Nonetheless, similar to previous research, men had significantly higher *LINE-1* methylation than women ($P = 0.004$), and perhaps some of the disparity in low-range methylation among studies may be attributable to a higher prevalence of men in this study (77%) compared to the studies from Hsiung et al. (69%) and Wilhelm et al. (69%). Among all cases and controls in adjusted models comparing tertiles of *LINE-1* methylation, the lowest methylation tertile compared to the highest revealed an elevated risk of bladder cancer that did not reach statistical significance (OR: 1.3, 95% CI: 0.95–1.7). However, when stratifying by smoking status, never smokers in the lowest tertile of *LINE-1* methylation had a significantly increased risk of bladder cancer (OR: 1.9, 95% CI: 1.2–3.1). Further, lifelong non-smokers with *GSTM1* and/or *GSTT1* null genotypes had an even higher risk of bladder cancer (OR: 2.4, 95% CI: 1.3–4.1).

12.2.4 Challenges and Caveats

Despite recent advances in measuring repetitive element methylation with bisulfite pyrosequencing, a full understanding of the biology of these elements is still lacking, and there are technical limitations that should be carefully considered. Although reported in numerous studies, the relatively greater extent of methylation of *LINE-1* elements in men compared to women is not understood, and may represent fundamental differences that need to be further explored. Though the CpGs targeted for methylation measurement in pyrosequencing assays are generally 3–6 CpG sites in the 5′ UTR, because it is unclear how many of the 500,000 *LINE-1* elements are full length (6 kb) it is not known *how many* copies of *LINE-1* elements are actually being measured in any given individual. From an evolutionary standpoint the newer *LINE-1* sequences are more likely to be fully intact, though the 5′ end of the repeat can be deleted and it is not yet known what the prevalence of these deletions are. In addition, the total number of newer *LINE-1* sequence elements is polymorphic in the population. Together, these issues make it difficult if not impossible to know how many *LINE* elements are being measured and whether the number is similar across samples

or individuals. So, although a number of studies are now identifying and confirming associations between epigenetic alterations to these elements and cancer, the biological mechanism towards carcinogenesis that these observations represent is not understood. A more in-depth treatment of the challenges associated with repeat element and global methylation measures is available in Nelson et al. [56].

12.3 Gene-Specific Methylation and Epimutation

12.3.1 Epimutation

There is growing recognition that gene-specific soma-wide and/or germline DNA methylation, often called epimutation, can predispose individuals to cancer [20, 31]. Initial work in the area of epimutation identified changes to gene imprinting status that was phenotypically equivalent to disease attributable to genetic alterations. For example, Wilm's tumor can derive from inherited mutation in the *IGF2* gene leading to a change in the imprinting status and therefore the biallelic expression of this gene. A change in the DNA methylation status of the maternally imprinted allele without change to the underlying sequence can lead to loss of imprinting (LOI) at the *H19/IGF2* locus, which similarly results in biallelic expression and risk for Wilm's tumor [52, 65]. A number of other imprinting disorders have been identified and have been linked not only with genetic etiology but also epimutations, including Beckwith–Wiedeman, Silver–Russel, Prader–Willi, and Angelman syndromes [28, 57]. Epimutations resulting in LOI are relatively rare due to the scarcity of imprinted genes in the genome.

Epimutations also have been shown to occur in the context of biallelic expression and such epimutations have been linked to cancer. For example, 37% of individuals presenting with Cowden's syndrome or with Cowden-like features, but without genetic alteration to the *PTEN* gene, harbor germline allele-specific DNA methylation upstream of *PTEN*. This leads to reduced expression of the *KILLIN* gene and a greatly increased risk of breast and renal cancer [7]. Similarly, a subset of familial breast and ovarian cancer without *BRCA1* or *BRCA2* mutation is linked to mosaic epimutation of *BRCA1* [78], and hereditary non-polyposis colorectal cancer (HNPCC) without germline mutation is observed with allele-specific mosaic methylation of *MLH1* or *MSH2* [14, 32–34]. In some cases, multiple generations of individuals within these HNPCC families could be identified [33, 55]. In other cases, the aberrant methylation present in the affected individuals germline (i.e., spermatozoa), could not be identified in family members, suggesting a potential de novo germline or early embryonic event [32, 66, 67]. This lack of a consistent direct inheritance of the epimutation itself, but the potential for familial transmission suggests that it may, in fact, be a predisposition to epimutation in general that is truly being inherited.

These are examples of highly penetrant but rare epimutation in genes known to contribute to specific disease. Such findings are analogous to decades of work in

genetic susceptibility to cancer which originated with studies of highly penetrant, rare mutations leading to rare genetic disease and provided the profound understanding of the key genes involved in tumorigenesis. As genetic susceptibility studies later evolved into the investigation of more common, polymorphic variation associated with sporadic cancer, so has the study of epimutation begun to move beyond these rare variants to studies of common epigenetic variability association with common disease.

12.3.2 Gene-specific methylation

A number of investigations of peripheral blood DNA methylation have focused on the examination of candidate tumor suppressor gene methylation, taking cues from the alterations detected in targeted solid tissues to define candidates. Often these studies are based upon the assumption that the alterations driving carcinogenesis in a target tissue will be identified in blood and potentially other non-target tissues, although the somatic nature of methylation would argue against such assumption. Nevertheless, there is a large and growing literature utilizing candidate approaches to examine in populations single and multi-gene panels of candidate tumor suppressor genes in peripheral blood as markers of solid tumor risk.

Wong and colleagues [77] measured *CDKN2B* methylation in buffy coat DNA from 15 hepatocellular carcinoma (HCC) patients, 15 patients without cancer but with chronic hepatitis/cirrhosis, and 20 healthy controls with MSP. Among the 15 HCC patients, eight had *CDKN2B* methylation buffy coat DNA, whereas none of the healthy controls or individuals with hepatitis/cirrhosis had methylated *CDKN2B*. Further, the eight HCC patients with *CDKN2B* methylation in blood DNA also had *CDKN2B* methylation in their tumor tissue.

In colon cancer, Ally and colleagues measured methylation in blood DNA from 27 cases, 30 individuals with adenoma, 16 with hyperplastic polyps, and 57 disease-free controls [2]. Using bisulfite pyrosequencing the authors examined seven CpG sites in the promoter region of the estrogen receptor alpha gene (*ESR1*) and across all subjects the methylation of *ESR1* ranged from 0 to 13% (median, 4.3%). Across disease groups there was not a difference in *ESR1* methylation ($P > 0.05$). However, *ESR1* methylation was 60% lower in peripheral blood samples than in normal colonic tissues. Further, the authors observed a correlation between colonic tissue methylation and blood methylation of *ESR1* that was independent of age, gender, disease status, and body mass index (BMI).

Another interesting example comes from studies of *BRCA1* methylation in peripheral blood DNA of cases with breast cancer. Germline *BRCA1* mutations are related to the development of hereditary breast cancers, which account for ~5–10% of cases, and generally present at a younger age and with a more aggressive phenotype. Although mRNA levels of *BRCA1* have been shown to be reduced in a subset of sporadic breast cancer cases [70], mutations of *BRCA1* in sporadic breast cancer are rarely (if ever) present [25, 42]. As *BRCA1* is known to contain a CpG island in

its promoter region, it was hypothesized that DNA methylation-induced silencing may be present in a subset of sporadic breast tumors. It has been shown that up to 44% of sporadic breast tumors are methylated at *BRCA1*, and tumors with methylated *BRCA1* share pathologic features of tumors with mutated *BRCA1* [9, 12]. In 2008, Snell and colleagues measured *BRCA1* methylation in blood DNA from seven familial breast cancer cases that did not have detectable *BRCA1* or *BRCA2* mutations and seven age-matched controls. These authors used several techniques to measure *BRCA* methylation including MethyLight, methylation-sensitive high-resolution melting, and a digital version of the latter. Three of the seven patients studied had >0% methylation of *BRCA1* in peripheral blood DNA and the corresponding tumors were found to be heavily methylated. Among the control subjects, six of seven had no detectable *BRCA1* methylation, only one subject had low-level *BRCA1* (0.1%) methylation [64].

Al-Moghrabi et al. measured *BRCA1* methylation with MSP in 47 breast tumor tissues, and in peripheral blood from seven breast cancer cases and 73 disease-free controls [3]. Among tumor tissues, 13 (27%) had *BRCA1* methylation. Similarly, two (29%) of the seven blood samples from breast cancer cases were methylated at *BRCA1*. Further, there was a significant association between a younger age at diagnosis (≤40 years) and *BRCA1* methylation ($P < 0.004$). However, 8 of the 73 (11%) disease-free controls also had *BRCA1* methylation in blood, which was not a significantly lower prevalence of *BRCA1* methylation than in cases. Nonetheless, with only seven breast cancer cases providing blood DNA, this study may have been underpowered to detect a significant association between *BRCA1* methylation in blood and risk of breast cancer. In addition, it is possible that the disease-free women with *BRCA1* methylation in blood are still at an increased risk of developing breast cancer.

Iwamoto and colleagues presented a similar study of *BRCA1* methylation in peripheral blood DNA from 200 cases and 200 controls [37]. In peripheral blood samples from cases and controls, *BRCA1* methylation was measured with quantitative MSP and found to be associated with a significantly increased risk of breast cancer (OR: 1.7, 95% CI: 1.01–2.96), controlling for age, family history, age at menarche, parity, menopausal status, and BMI. In addition, these authors also measured *BRCA1* methylation in 162 breast tumors where 31 (19%) were *BRCA1* methylation-positive and these tumors were more likely to be estrogen receptor and progesterone receptor-negative. When stratifying by presence of *BRCA1* methylation in tumors (and controlling for covariates above), peripheral blood methylation of *BRCA1* was highly associated with risk of developing *BRCA1* methylation-positive breast cancer (OR: 17.8, 95% CI: 6.7–47.1).

Blood DNA methylation of *BRCA1* in relation to the risk of ovarian cancer has also been reported. Bosivel and colleagues [11] measured blood DNA methylation of both *BRCA1* and *BRCA2* promoter regions in 51 ovarian cancer cases (without *BRCA* mutation) and 349 controls using quantitative analysis of methylated alleles. Although they did not observe an association between *BRCA2* methylation level and case status, these authors reported significantly *reduced BRCA1* methylation in ovarian cancer cases compared to controls. However, the implications of a significantly

hypomethylated *BRCA1* promoter region in association with ovarian cancer are somewhat counterintuitive and warrant further investigation.

In a case–control study of lung cancer, Li et al. measured methylation of the putative tumor suppressor gene *FHIT* in peripheral blood DNA samples from Han Chinese subjects with MSP [46]. Among 123 lung cancer cases, 42 (34%) had *FHIT* promoter methylation, whereas none of the 105 control subjects' blood DNAs were methylated, indicating a significantly increased risk of lung cancer associated with peripheral blood methylation of *FHIT* (OR: 2.3, 95% CI: 2.0–2.7). Additionally, these authors reported that blood methylation of *FHIT* was significantly associated with cases who had early stage (I) disease ($P < 0.05$), and not cases with high-stage (IV) disease.

12.3.3 Panels of candidate genes

Some groups have reported blood methylation data for panels of candidate genes. The heterogeneity in molecular alterations of specific tumor types could be motivation for studies that examine multiple gene-loci, and the results from Iwamoto et al. are apropos: peripheral blood methylation of *BRCA1* was highly associated with risk of developing *BRCA1* methylation-positive breast cancer [37]. Of course, within a particular tumor type, not every tumor will have the same repertoire of molecular alterations. Hence, a more comprehensive approach to study blood-based methylation markers of cancer risk would measure methylation of several genes known (or suspected) to be methylated in a moderate to high proportion of tumors.

One such study from Liu et al. used an approach directed at six genes on chromosome 3p because a previous report from these authors had demonstrated a CpG island methylator phenotype (CIMP) associated with genes on 3p in lung tumors [48, 49]. Here, the authors used peripheral blood DNA from 80 cases of non-small cell lung cancer (NSCLC) and 80 matched controls and measured methylation of six genes (*OGG1*, *RARB*, *SEMA3B*, *RASSF1A*, *BLU*, *FHIT*) on chromosome 3p with MSP. If at least three of these genes were methylated the sample was considered 3pCIMP+. The prevalence of methylation in blood DNA from cases was higher than controls for all genes except *FHIT* where the same number of cases and controls were methylated. Further, almost all case blood samples (78/80, 98%) had at least one methylated gene, whereas 78% (62/80) of control blood samples had at least one methylated gene. When comparing 3pCIMP status in cases and controls, 44% of NSCLC cases were 3pCIMP + and only 6% of control blood DNA samples were 3pCIMP + ($P < 0.001$). In a model adjusting for age, sex, and smoking status, subjects with 3pCIMP + blood DNA were at a significantly increased risk of NSCLC (OR: 12.8, 95% CU: 4.4–37.4) [49].

Another gene-panel approach to investigate the role of blood-based DNA methylation markers of lung cancer risk was recently published by Vineis and colleagues using nested cases and controls from the European Prospective Investigation into Cancer and Nutrition (EPIC) [72]. This group measured methylation of multiple

CpGs in five genes: *CDKN2A*, *RASSF1A*, *GSTP1*, *MTHFR*, and *MGMT* with a bisulfite pyrosequencing approach in 93 lung cancer cases and 99 controls. Stratifying pyrosequencing methylation data for each gene on the median, adjusted models revealed that increased *RASSF1A* methylation was associated with a significantly increased risk of lung cancer (OR: 1.9, 95% CI: 1.0–3.5), though none of the other genes, or combination thereof were associated with disease. The authors also reported that serum levels of B vitamins and one-carbon metabolites were associated with methylation; increased folate was associated with increased *RASSF1A* and *MTHFR* methylation, whereas increased methionine was associated with decreased *RASSF1A* methylation [72].

Prior to these works, a group in France published a comparison of blood DNA methylation of ten genes in a study of prostate cancer [61]. Using prostate cancer cases with disease relapse ($n=20$), patients without relapse ($n=22$), as well as control subjects ($n=22$), the authors measured methylation of ten genes; *RASSF1A*, *CDH1*, *APC*, *DAPK*, *MGMT*, *CDKN2A* (*p16* and *p14*), *GSTP1*, *RARB*, and *TIMP3* using quantitative MSP. Compared to all cases, methylation levels of all ten genes were lower in control subject blood DNA, and five were significantly lower; *DAPK* ($P=0.04$) *RASSF1A*, *GSTP1*, *APC*, and *RARB* (all $P<0.0001$).

An interesting final example of small gene-panel studies comes from Flanagan and colleagues who developed a tiling microarray with a methylation-sensitive enzyme-based approach to study 17 breast cancer susceptibility genes [22]. With the tiling array the authors took an unbiased approach to examining the promoter and gene-coding regions for the 17 candidate genes. In the pilot phase, 14 cases with bilateral breast cancer and 14 control subjects had their blood DNA methylation measured. Notably, the authors described 181 regions in the 17 genes analyzed that had significantly variable methylation ($P<0.001$) across all 28 individuals, and the majority of these regions were significantly closer (within 200 bp) to repetitive elements than would be expected ($P=7.4e-07$). As a follow up, the authors validate two regions of variable methylation 4 kb downstream of the *ATM* gene in 190 cases and 190 controls and observed significantly increased methylation of *ATM* variable region 2 in cases compared to controls ($P=0.002$). In an inter-quartile analysis of the methylation data from this same region, subjects in the highest quartile of methylation were at a significantly increased risk of breast cancer (OR: 3.2, 95% CI: 1.8–5.9) compared to subjects in the lowest quartile [22]. One of the key facets of this particular study is that unlike most other investigations, these authors did not restrict their methylation measurements to promoter regions and argues that future studies should consider the distribution of regions measured for methylation.

12.4 Larger Gene-Panels and Commercial Methylation Arrays

A separate class of studies that has undertaken larger-scale approaches (25 genes to genome-wide) to investigate blood-based markers of DNA methylation and cancer risk will be covered here. One such study from Widschwendter et al. used a

three-step approach to investigate blood DNA methylation and the risk of breast cancer [75]. First, these authors chose 49 estrogen receptor target (ERT) and polycomb group target (PCGT) genes and second, used MethyLight to measure methylation in 83 healthy post-menopausal women. Thirdly, based on the distribution of methylation in these individuals 25 of the 49 genes were selected for measurement in 353 cases and 730 controls. After controlling for age and family history of breast cancer, methylation of 5 of the 25 genes (*ZNF217*, *NEUROD1*, *SFRP1*, *TITF1* (officially *NKX2-1* as of 8/14/11), *NUP155*) was associated with a significantly increased risk of breast cancer (ORs range: 1.40–1.49, median OR: 1.48) [75]. This study provides further proof of principle for the utility of blood-based methylation markers of cancer risk. However, because methylation of five separate genes were independently associated with breast cancer, it would have been interesting to know whether an analytic approach that combined the methylation markers would have increased the effect estimate.

A similar study of small cell lung cancer (SCLC) risk from Wang et al. also used a multi-step approach to curate a group of genes measured for methylation in a small pilot group of cases and controls before expanding into additional cases and controls [73]. This study took advantage of recent technologic advances that allow for the simultaneous resolution of hundreds to hundreds of thousands of methylation events, providing an epigenotyping platform for rapid epigenetic profiling [8]. First, bisulfite-modified blood DNA from 44 cases and 44 controls was applied to the Illumina GoldenGate methylation array which measures 1,505 CpG sites associated with >800 cancer-related genes. Testing 1,332 CpGs (those with methylation states not associated with cancer treatment) the authors observed 62 CpG sites associated with 52 genes to be significantly associated with cases status (FDR $P<0.05$). To follow up, the authors chose nine of these 62 CpGs for validation by bisulfite pyrosequencing in 138 cases and 138 controls. Controlling for age, sex, and smoking history, the methylation status of the nine CpG sites collectively were able to correctly classify 86% of cases as being at a higher risk of SCLC. Further, when considering specific CpGs, for the risk of SCLC increased ~4-fold for each 5% decrease in *ERCC1* methylation (OR: 3.9, 95% CI: 2.0–6.1) and ~1.5-fold for each 5% decrease in *CSF3R* methylation (OR: 1.5, 95% CI: 1.1–2.0) [73].

A group from the Mayo Clinic in Minnesota also used a two-phase study and the GoldenGate array to study blood methylation and risk of cancer, though they focused on pancreatic cancer [59]. First, these authors measured blood DNA methylation with the array in 132 cases and 60 controls and reported 110 CpGs with significantly differential methylation between cases and controls (FDR $P<0.05$). Then, using analogous technology in a custom platform from Illumina (VeraCode), the top 96 CpGs associated with case control status were subjected to validation in a further 240 cases and 240 matched controls. Leveraging the potential of combining methylation measures a prediction model was built and included five CpG sites associated with five genes: *IL10*, *LCN2*, *ZAP70*, *AIM2*, and *TAL1*. Collectively, these five CpGs demonstrated good discrimination between pancreatic cancer cases and controls (c-statistic phase I = 0.85, phase II = 0.72) [59].

Teschendorff et al. published an investigation of blood methylation profiles to predict ovarian cancer using a more comprehensive array platform, the Illumina Infinium 27K array [69]. Following exclusions for batch effects and quality control, methylation array data from 148 controls, 113 pre-treatment, and 122 post-treatment cases from the UK Ovarian Cancer Population Study were included in the analysis. Comparing methylation among controls to pre-treatment cases, the authors identified 2,714 CpG sites that were significantly (FDR $P<0.05$) associated with ovarian cancer. Notably, among the top 50 CpGs, 87% were hypomethylated in cases compared to controls ($P=9e-09$). To construct a DNA methylation signature associated with ovarian cancer, these authors used a supervised approach to the data with 100 iterations of training and testing sets (each with 90 controls and 70 pre-*t* cases) and multivariate logistic regression. With these iterations and a cross-validation step, the top 100 CpG sites were determined to be an optimal number of CpG sites for their classifier. The performance of these 100 CpGs as a classifier for ovarian caner in a blinded test set was very good (AUC: 0.8, 95% CI: 0.74–0.87), and was validated in the post-treatment cases (AUC: 0.76, 95% CI: 0.72–0.81) [69].

In a New Hampshire population-based bladder cancer case–control study, Marsit et al. examined peripheral blood DNA methylation profiles using the Infinium 27K array. Using a novel, semi-supervised recursively partitioned mixture modeling (SS-RPMM) strategy [40] involving classifier training in a series of subjects consisting of 118 controls and 112 cases, and validation in an independent series of 119 controls and 111 cases, Marsit et al. identified a panel of 9 CpG loci whose profile of DNA methylation was significantly associated ($P<0.0001$) with bladder cancer [50]. Membership in any of the three classes of DNA methylation associated with risk demonstrated a 5.2-fold increased risk of bladder cancer (95% CI 2.8, 9.7), when controlled for subject age, gender, smoking status, and family history of bladder cancer. Notably, the methylation classes whose membership was predominantly bladder cancer cases had higher levels of mean methylation across the 9 CpG loci. Gene-set enrichment analysis of the loci most associated with bladder cancer demonstrated that transcription-factor binding sites related to immune modulation and forkhead family transcription were over-represented among regions whose methylation differed in bladder cases compared to controls. The key role of immune modulation in both aging and carcinogenesis, and particularly bladder carcinogenesis, lends mechanistic significance to these findings.

Using the same array platform and SS-RPMM analytical approach, the association between peripheral blood methylation profiles and HNSCC was assessed by Langevin et al. [44] in 96 HNSCC cases and 96 cancer-free control subjects. In this study, cases and controls were best differentiated by a methylation profile of six CpG loci (associated with *FGD4*, *SERPINF1*, *WDR39*, *IL27*, *HYAL2,* and *PLEKHA6*), and after adjustment for subject age, gender, smoking, alcohol consumption, and HPV16 serostatus, the AUC was 0.85 (95% CI: 0.76–0.92). Notably, the methylation classes whose membership was predominantly head and neck cancer cases had lower mean methylation across the 6 CpG loci. Although this is not yet adequate for use in clinical settings, these results further demonstrate the poten-

tial of DNA methylation measured in blood for development of non-invasive applications for detection of head and neck cancer and the utility of the proposed methods for the analysis of the array-based methylation data.

12.5 Mechanisms

Just as normal genetic variation is now understood to be associated with a predisposition to a vast array of human diseases [51], it is important to consider interindividual variation in tissue-specific DNA methylation to better understand the ability of this variation to inform disease risk. Epigenetic variation has been hypothesized to cause underlying differences in disease susceptibility among monozygotic twins, and young twin-pairs have been shown to be more epigenetically similar than older monozygotic twins [24]. The aging process and differences in environment have been hypothesized to influence clinically significant changes in methylation profiles as individuals accumulate varying exposures with age.

Marks of DNA methylation are entirely reprogrammed during in-utero development. This reprogramming, during the pre-implantation period, necessitates a rapid de-methylation of the genome, thought to be accomplished through an active process [29, 45], followed by appropriate, cell and tissue-specific methylation of the genome. The mechanisms through which these processes of de-methylation and reprogramming of the DNA methylation marks and particularly, the appropriate targeting of enzymes responsible for establishing those marks remains unclear. Importantly, epigenetic reprogramming during in-utero development constitutes a critical period during which environmental stimuli and insults can alter the establishment of cell-type-specific DNA methylation profiles and may constitute one point at which variation in methylation profiles is established. Therefore, alteration to epigenetic profiles has been posited as the molecular basis of the developmental origins of health and disease phenomenon, which links the environment (taken broadly) in-utero, with outcomes throughout the life course of the individual [5, 6].

Beyond the variation in DNA methylation profile which is established in-utero, additional variation may arise resulting from exposures and the environment encountered throughout life, or from the process of aging itself. Work from Christensen et al. [16] demonstrated that features of the patterns of age-associated methylation were conserved irrespective of tissue-type, suggesting a common mechanism or dysregulation to explain these alterations. Potential mechanisms include reduced fidelity of maintenance methyltransferases with aging leading to hypomethyation events. Although age-related methylation alterations may not functionally result in a pathologic process, drifts of normal epigenomes may nonetheless confer significantly increased risk of conversion to a pathologic phenotype by enhancing both the likelihood and frequency of subsequent methylation events that ultimately result in aberrant expression or altered genomic stability.

Particularly when considering profiles of methylation in a heterogeneous tissue sample such as blood, it should be recognized that the quantitative measure of

methylation truly represents the fraction of cells within the sampled population exhibiting a methyl-group at any CpG site. Therefore, differences in DNA methylation profiles could and likely do indicate aging or exposure-related changes to the underlying populations of cells comprising that mixture. In the case of blood these shifts may indicate changes to the profile of immune cells and thus alterations to the immune system permissive to or resulting from carcinogenesis. In fact, comparing *LINE1*, Sat2, and Alu methylation levels in whole blood, granulocytes, mononuclear cells, and lymphoblastoid lines with multiple methylation assays (MethyLight, luminometric methylation assay, and a methyl acceptance assay) Wu et al. have demonstrated differences in methylation dependent upon substrate and assay used [79]. As additional studies are conducted to identify differentially methylated regions among various leukocyte subtypes, it may soon be possible to identify proportional shifts in specific leukocyte subtypes that may contribute to cancer, or indicate immune response to an existing tumor.

12.6 Conclusions

The extent of variability of the cellular epigenome in non-pathologic tissues, particularly at gene promoter regions, remains a critical question; the amount of variation in genomic methylation across the population is not currently known. It is clear that epigenetic variability detectable in human blood is influenced, in part, by aging and exposures, and in turn, specific profiles of methylation in blood are associated with cancer risk (Fig. 12.1). The ease of collection of blood samples and the rapidly advancing technologies to assess DNA methylation in genomic DNA from this tissue make this an ideal focus of study for novel biomarkers of disease risk and of disease prognosis. Additionally, as we better understand functional consequences of altered methylation profiles, there will be an improved understanding at the systems level of the contribution of non-target tissues and systems on carcinogenesis, likely yielding novel approaches not only of diagnosis but treatment as well.

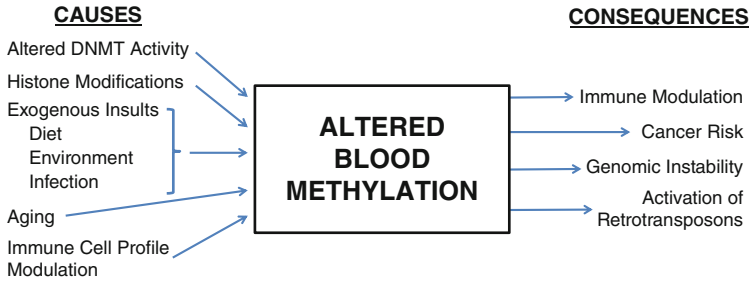

Fig. 12.1 Causes and consequences of altered blood DNA methylation

References

1. Alexander RP, Fang G et al (2010) Annotating non-coding regions of the genome. Nat Rev Genet 11(8):559–571
2. Ally MS, Al-Ghnaniem R et al (2009) The relationship between gene-specific DNA methylation in leukocytes and normal colorectal mucosa in subjects with and without colorectal tumors. Cancer Epidemiol Biomarkers Prev 18(3):922–928
3. Al-Moghrabi N, Al-Qasem AJ et al (2011) Methylation-related mutations in the BRCA1 promoter in peripheral blood cells from cancer-free women. Int J Oncol 39(1):129–135
4. Baccarelli A, Wright RO et al (2009) Rapid DNA methylation changes after exposure to traffic particles. Am J Respir Crit Care Med 179(7):572–578
5. Barker DJ (2004) Developmental origins of adult health and disease. J Epidemiol Community Health 58(2):114–115
6. Barker DJ (2004) The developmental origins of well-being. Philos Trans R Soc Lond B Biol Sci 359(1449):1359–1366
7. Bennett KL, Mester J et al (2010) Germline epigenetic regulation of KILLIN in Cowden and Cowden-like syndrome. JAMA 304(24):2724–2731
8. Bibikova M, Lin Z et al (2006) High-throughput DNA methylation profiling using universal bead arrays. Genome Res 16(3):383–393
9. Birgisdottir V, Stefansson OA et al (2006) Epigenetic silencing and deletion of the BRCA1 gene in sporadic breast cancer. Breast cancer research: BCR 8(4):R38
10. Bollati V, Baccarelli A et al (2007) Changes in DNA methylation patterns in subjects exposed to low-dose benzene. Cancer Res 67(3):876–880
11. Bosviel R, Michard E et al (2011) Peripheral blood DNA methylation detected in the BRCA1 or BRCA2 promoter for sporadic ovarian cancer patients and controls. Clin Chim Acta 412(15–16):1472–1475
12. Butcher DT, Rodenhiser DI (2007) Epigenetic inactivation of BRCA1 is associated with aberrant expression of CTCF and DNA methyltransferase (DNMT3B) in some sporadic breast tumours. Eur J Cancer 43(1):210–219
13. Cash HL, Tao L et al (2011) LINE-1 hypomethylation is associated with bladder cancer risk among nonsmoking Chinese. Int J Cancer 130(5):1151–1159
14. Chan TL, Yuen ST et al (2006) Heritable germline epimutation of MSH2 in a family with hereditary nonpolyposis colorectal cancer. Nat Genet 38(10):1178–1183
15. Choi JY, James SR et al (2009) Association between global DNA hypomethylation in leukocytes and risk of breast cancer. Carcinogenesis 30(11):1889–1897
16. Christensen BC, Houseman EA et al (2009) Aging and environmental exposures alter tissue-specific DNA methylation dependent upon CpG island context. PLoS Genet 5(8):e1000602
17. Cordaux R, Batzer MA (2009) The impact of retrotransposons on human genome evolution. Nat Rev Genet 10(10):691–703
18. Deininger PL, Batzer MA (1999) Alu repeats and human disease. Mol Genet Metab 67(3): 183–193
19. Dewannieux M, Esnault C et al (2003) LINE-mediated retrotransposition of marked Alu sequences. Nat Genet 35(1):41–48
20. Dobrovic A, Kristensen LS (2009) DNA methylation, epimutations and cancer predisposition. Int J Biochem Cell Biol 41(1):34–39
21. Eads CA, Danenberg KD et al (2000) MethyLight: a high-throughput assay to measure DNA methylation. Nucleic Acids Res 28(8):E32
22. Flanagan JM, Munoz-Alegre M et al (2009) Gene-body hypermethylation of ATM in peripheral blood DNA of bilateral breast cancer patients. Hum Mol Genet 18(7):1332–1342
23. Florl AR, Lower R et al (1999) DNA methylation and expression of LINE-1 and HERV-K provirus sequences in urothelial and renal cell carcinomas. Br J Cancer 80(9):1312–1321
24. Fraga MF, Ballestar E et al (2005) Epigenetic differences arise during the lifetime of monozygotic twins. Proc Natl Acad Sci USA 102(30):10604–10609

25. Futreal PA, Liu Q et al (1994) BRCA1 mutations in primary breast and ovarian carcinomas. Science 266(5182):120–122
26. Gama-Sosa MA, Wang RY et al (1983) The 5-methylcytosine content of highly repeated sequences in human DNA. Nucleic Acids Res 11(10):3087–3095
27. Gaudet F, Hodgson JG et al (2003) Induction of tumors in mice by genomic hypomethylation. Science 300(5618):489–492
28. Gicquel C, Rossignol S et al (2005) Epimutation of the telomeric imprinting center region on chromosome 11p15 in Silver-Russell syndrome. Nat Genet 37(9):1003–1007
29. Hajkova P, Erhardt S et al (2002) Epigenetic reprogramming in mouse primordial germ cells. Mech Dev 117(1–2):15–23
30. Herman JG, Graff JR et al (1996) Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci USA 93(18):9821–9826
31. Hitchins MP (2010) Inheritance of epigenetic aberrations (constitutional epimutations) in cancer susceptibility. Adv Genet 70:201–243
32. Hitchins M, Williams R et al (2005) MLH1 germline epimutations as a factor in hereditary nonpolyposis colorectal cancer. Gastroenterology 129(5):1392–1399
33. Hitchins MP, Wong JJ et al (2007) Inheritance of a cancer-associated MLH1 germ-line epimutation. N Engl J Med 356(7):697–705
34. Hitchins M, Owens S et al (2011) Identification of new cases of early-onset colorectal cancer with an MLH1 epimutation in an ethnically diverse South African cohort(dagger). Clin Genet 80(5):428–434
35. Hou L, Wang H et al (2010) Blood leukocyte DNA hypomethylation and gastric cancer risk in a high-risk Polish population. Int J Cancer 127(8):1866–1874
36. Hsiung DT, Marsit CJ et al (2007) Global DNA methylation level in whole blood as a biomarker in head and neck squamous cell carcinoma. Cancer Epidemiol Biomarkers Prev 16(1):108–114
37. Iwamoto T, Yamamoto N et al (2011) BRCA1 promoter methylation in peripheral blood cells is associated with increased risk of breast cancer with BRCA1 promoter methylation. Breast Cancer Res Treat 129(1):69–77
38. Jones PA, Laird PW (1999) Cancer epigenetics comes of age. Nat Genet 21(2):163–167
39. Jordan IK, Rogozin IB et al (2003) Origin of a substantial fraction of human regulatory sequences from transposable elements. Trends Genet 19(2):68–72
40. Koestler DC, Marsit CJ et al (2010) Semi-supervised recursively partitioned mixture models for identifying cancer subtypes. Bioinformatics 26(20):2578–2585
41. Kolomietz E, Meyn MS et al (2002) The role of Alu repeat clusters as mediators of recurrent chromosomal aberrations in tumors. Genes Chromosomes Cancer 35(2):97–112
42. Lancaster JM, Wooster R et al (1996) BRCA2 mutations in primary breast and ovarian cancers. Nat Genet 13(2):238–240
43. Lander ES, Linton LM et al (2001) Initial sequencing and analysis of the human genome. Nature 409(6822):860–921
44. Langevin SM, Koestler DC et al (2012) Peripheral blood DNA methylation profiles are predictive of head and neck squamous cell carcinoma: an epigenome-wide association study. Epigenetics 7(3):291–299
45. Lee J, Inoue K et al (2002) Erasing genomic imprinting memory in mouse clone embryos produced from day 11.5 primordial germ cells. Development 129(8):1807–1817
46. Li W, Deng J et al (2010) Association of 5′-CpG island hypermethylation of the FHIT gene with lung cancer in southern-central Chinese population. Cancer Biol Ther 10(10):997–1000
47. Lim U, Flood A et al (2008) Genomic methylation of leukocyte DNA in relation to colorectal adenoma among asymptomatic women. Gastroenterology 134(1):47–55
48. Liu Z, Zhao J et al (2008) CpG island methylator phenotype involving tumor suppressor genes located on chromosome 3p in non-small cell lung cancer. Lung Cancer 62(1):15–22
49. Liu Z, Li W et al (2010) CpG island methylator phenotype involving chromosome 3p confers an increased risk of non-small cell lung cancer. J Thorac Oncol 5(6):790–797

50. Marsit CJ, Koestler DC et al (2011) DNA methylation array analysis identifies profiles of blood-derived DNA methylation associated with bladder cancer. J Clin Oncol 29(9): 1133–1139
51. McCarthy MI, Hirschhorn JN (2008) Genome-wide association studies: potential next steps on a genetic journey. Hum Mol Genet 17(R2):R156–R165
52. McKay JD, Truong T et al (2011) A genome-wide association study of upper aerodigestive tract cancers conducted within the INHANCE consortium. PLoS Genet 7(3):e1001333
53. Menendez L, Benigno BB et al (2004) L1 and HERV-W retrotransposons are hypomethylated in human ovarian carcinomas. Mol Cancer 3:12
54. Moore LE, Pfeiffer RM et al (2008) Genomic DNA hypomethylation as a biomarker for bladder cancer susceptibility in the Spanish Bladder Cancer Study: a case–control study. Lancet Oncol 9(4):359–366
55. Morak M, Schackert HK et al (2008) Further evidence for heritability of an epimutation in one of 12 cases with MLH1 promoter methylation in blood cells clinically displaying HNPCC. Eur J Hum Genet 16(7):804–811
56. Nelson HH, Marsit CJ et al (2011) "Global methylation" in exposure biology and translational medical science. Environ Health Perspect 119(11):1528–1533
57. Netchine I, Rossignol S et al (2007) 11p15 imprinting center region 1 loss of methylation is a common and specific cause of typical Russell-Silver syndrome: clinical scoring system and epigenetic-phenotypic correlations. J Clin Endocrinol Metab 92(8):3148–3154
58. Pavanello S, Bollati V et al (2009) Global and gene-specific promoter methylation changes are related to anti-B[a]PDE-DNA adduct levels and influence micronuclei levels in polycyclic aromatic hydrocarbon-exposed individuals. Int J Cancer 125(7):1692–1697
59. Pedersen KS, Bamlet WR et al (2011) Leukocyte DNA methylation signature differentiates pancreatic cancer patients from healthy controls. PLoS One 6(3):e18223
60. Pufulete M, Al-Ghnaniem R et al (2003) Folate status, genomic DNA hypomethylation, and risk of colorectal adenoma and cancer: a case control study. Gastroenterology 124(5): 1240–1248
61. Roupret M, Hupertan V et al (2008) Promoter hypermethylation in circulating blood cells identifies prostate cancer progression. Int J Cancer 122(4):952–956
62. Rusiecki JA, Baccarelli A et al (2008) Global DNA hypomethylation is associated with high serum-persistent organic pollutants in Greenlandic Inuit. Environ Health Perspect 116(11): 1547–1552
63. Sano H, Imokawa M et al (1988) Detection of heavy methylation in human repetitive DNA subsets by a monoclonal antibody against 5-methylcytosine. Biochim Biophys Acta 951(1): 157–165
64. Snell C, Krypuy M et al (2008) BRCA1 promoter methylation in peripheral blood DNA of mutation negative familial breast cancer patients with a BRCA1 tumour phenotype. Breast Cancer Res 10(1):R12
65. Steenman MJ, Rainier S et al (1994) Loss of imprinting of IGF2 is linked to reduced expression and abnormal methylation of H19 in Wilms' tumour. Nat Genet 7(3):433–439
66. Suter CM, Martin DI et al (2004) Germline epimutation of MLH1 in individuals with multiple cancers. Nat Genet 36(5):497–501
67. Suter CM, Martin DI et al (2004) Hypomethylation of L1 retrotransposons in colorectal cancer and adjacent normal tissue. Int J Colorectal Dis 19(2):95–101
68. Tarantini L, Bonzini M et al (2009) Effects of particulate matter on genomic DNA methylation content and iNOS promoter methylation. Environ Health Perspect 117(2):217–222
69. Teschendorff AE, Menon U et al (2009) An epigenetic signature in peripheral blood predicts active ovarian cancer. PLoS One 4(12):e8274
70. Thompson ME, Jensen RA et al (1995) Decreased expression of BRCA1 accelerates growth and is often present during sporadic breast cancer progression. Nat Genet 9(4):444–450
71. Ting DT, Lipson D et al (2011) Aberrant overexpression of satellite repeats in pancreatic and other epithelial cancers. Science 331(6017):593–596

72. Vineis P, Chuang SC et al (2011) DNA methylation changes associated with cancer risk factors and blood levels of vitamin metabolites in a prospective study. Epigenetics 6(2):195–201
73. Wang L, Aakre JA et al (2010) Methylation markers for small cell lung cancer in peripheral blood leukocyte DNA. J Thorac Oncol 5(6):778–785
74. Weisenberger DJ, Campan M et al (2005) Analysis of repetitive element DNA methylation by MethyLight. Nucleic Acids Res 33(21):6823–6836
75. Widschwendter M, Apostolidou S et al (2008) Epigenotyping in peripheral blood cell DNA and breast cancer risk: a proof of principle study. PLoS One 3(7):e2656
76. Wilhelm CS, Kelsey KT et al (2010) Implications of LINE1 methylation for bladder cancer risk in women. Clin Cancer Res 16(5):1682–1689
77. Wong IH, Lo YM et al (2000) Frequent p15 promoter methylation in tumor and peripheral blood from hepatocellular carcinoma patients. Clin Cancer Res 6(9):3516–3521
78. Wong EM, Southey MC et al (2011) Constitutional methylation of the BRCA1 promoter is specifically associated with BRCA1 mutation-associated pathology in early-onset breast cancer. Cancer Prev Res (Phila) 4(1):23–33
79. Wu HC, Delgado-Cruzata L et al (2011) Global methylation profiles in DNA from different blood cell types. Epigenetics 6(1):76–85
80. Yang AS, Estecio MR et al (2004) A simple method for estimating global DNA methylation using bisulfite PCR of repetitive DNA elements. Nucleic Acids Res 32(3):e38

Chapter 13
Epigenetic Therapies in MDS and AML

Elizabeth A. Griffiths and Steven D. Gore

Abstract The use of low dose hypomethylating agents for patients with myelodysplastic syndrome (MDS) and secondary acute myeloid leukemia (AML) has had made a significant impact. In the past, therapies for these diseases were limited and patients who elected to receive treatment were subject to highly toxic, inpatient chemotherapeutics, which were often ineffective. In the era of hypomethylating agents (azacitidine and decitabine), a patient with high grade MDS or AML with multilineage dysplasia can be offered the alternative of outpatient, relatively low-toxicity therapy. Despite the fact that CR (CR) rates to such agents remain relatively low at 15–20%, a much larger percentage of patients will have clinically significant improvements in hemoglobin, platelet, and neutrophil counts while maintaining good outpatient quality of life. As our clinical experience with azanucleotides expands, questions regarding patient selection, optimal dosing strategy, latency to best response and optimal duration of therapy following disease progression remain, but there is no question that for some patients these agents offer, for a time, an almost miraculous clinical benefit. Ongoing clinical trials in combination and in sequence with conventional therapeutics, with other epigenetically active agents, or in conjunction with bone marrow transplantation continue to provide promise for optimization of these agents for patients with myeloid disease. Although the mechanism(s) responsible for the proven efficacy of these agents remain a matter of some controversy, activity is thought to stem from induction of DNA hypomethylation, direct DNA damage, or possibly even immune modulation; there is no question that they have become a permanent part of the armamentarium against myeloid neoplasms.

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13.1 Introduction

Myelodysplastic syndromes (MDS) are a heterogenous group of malignant myeloid disorders characterized by peripheral blood cytopenias in association with bone marrow hypercellularity and dysplasia [1]. Patients with high grade MDS (int-2 or high by IPSS criteria, Fig. 13.1) have a high rate of transformation to acute myeloid leukemia (AML) and poor long-term survival with a life expectancy in the absence of treatment between 0.4 and 1.8 years [2]. The International Prognostic Scoring System (IPSS) was developed as a tool for stratifying patient outcomes based upon readily available clinical characteristics. Figure 13.1 details the components necessary for the generation of an IPSS score and the expected survival for each designation [2]. "Secondary" AMLs such as those arising in patients with an antecedent MDS diagnosis are generally resistant to traditional chemotherapeutics and the overall survival (OS) in this group of patients is universally poor [3–5]. Both MDS and AML are diseases of the elderly with a majority of patients diagnosed when they are older than 60 years [5]. Although a small minority of patients with MDS will present with mild cytopenias and low grade disease, a majority do not [2]. Patients with MDS associated with multilineage cytopenias (anemia, thrombocytopenia, and neutropenia), high bone marrow blast percentages, or characteristic adverse chromosomal features often progress rapidly to AML and in the absence of bone marrow transplantation, ultimately die of their disease [2].

For these patients, and for a large number of older people who present with putatively de novo myeloid leukemias, but with unrecognized low grade cytopenias and

Prognostic Variable	Score value				
	0	0.5	1.0	1.5	2.0
Bone Marrow Blast %	<5	5-10		11-20	21-30
Karyotype	Good= normal,-y, del(5q), del(20q)	Intermediate= all others	Poor= ≥3 or any 7 abnormality		
Cytopenias	0/1	2/3			

IPSS risk group	Score	Median Survival
Low	0	5.7 years
Int-1	0.5-1	3.5 years
Int-2	1.5-2.0	1.2 years;
High	≥2.5	0.4 year

Fig. 13.1 Clinical criteria for and IPSS risk group classification of patients with myelodysplasia, from ref. [2]

bone marrow dysplasia, conventional induction chemotherapeutics (IC, with daunorubicin and cytarabine) have been in large measure disappointing [6]. Furthermore many such patients are unfit for intensive treatment and are offered instead low dose cytarabine, clinical trials or supportive care [7]. In this group the OS rates at 2 and 5 years remain only 10% and 2% respectively [3, 4]. Patients who are fit to receive traditional IC require long periods of time (often 4–6 weeks) in the hospital, and this treatment offers a complete remission rate of only 20–30%, with median survivals ranging between 5 and 13 months [6, 8, 9]. In addition to induction failure and early relapse, even in those who achieve remission, prolonged hospitalization can have the side effect of physical deconditioning and the 3 or more weeks of neutropenia resulting from this treatment can result in resistant bacterial and fungal infections [6]. These burdens create patients who are unable to return to good quality of life and who become ineligible for salvage therapy or clinical trials upon relapse due to poor performance status, organ dysfunction or infection. Even in those who retain an excellent performance status following induction, primary refractory AML remains a significant quality of life problem, requiring frequent blood transfusions, extensive prophylactic antibiotic regimens, and regular hospital visits [9].

Until recently, toxic traditional IC was the only real option for fit patients with high grade MDS or AML with MDS related changes [1]. Recently however, the epigenetically active drugs azacitidine (Aza, Vidaza, Celgene, Concord OH) and decitabine (Dac, Dacogen, Esai Inc., Mars, PA) have been approved both in the United States and Europe for the treatment of MDS and low blast count (<30%) AML [7, 10]. These drugs, both of which are incorporated into DNA resulting in the depletion of the intracellular methyltransferases (DNMTs) when given at low dose, were the first epigenetically active therapy to be approved for cancer. They have resulted in a significant change in the approach to patients with MDS and required the development of the International Working Group (IWG) response criteria in MDS in order to measure meaningful improvements in cytopenias that did not fit into the traditional response assessment which designated only complete (CR) or partial (PR) responses as meaningful [11, 12]. A summary of the IWG response criteria in MDS are provided in Table 13.1. In particular, Aza has been shown to improve OS, delay the transformation to AML in high-grade MDS patients, and produce significant responses in patients with low blast count AML [7]. Although a statistically significant survival benefit has not been demonstrated following treatment with Dac, this drug has been shown to produce both CRs and hematological improvements in both MDS and AML patients who receive it [10, 13]. Taken together these drugs offer an effective alternative to induction chemotherapy and have become the standard of care for patients with MDS as well as selected patients with AML.

As with conventional chemotherapeutic strategies for these patients, responses are usually limited to a year or two, but therapy is largely outpatient, with minimal end organ toxicity and few side effects [14]. Despite notable limitations, these drugs have made a significant impact upon quality of life for a large number of patients with high grade MDS and AML. Ongoing work to understand the mechanism responsible for the efficacy of these drugs and the ultimate loss of response observed

Table 13.1 Selected clinical trials with azacitidine (aza) or decitabine (dac) in MDS

Trial	CALGB 9221	D-0007	ICD03-180	AZA-001	US Oncology	ADOPT	EORTC 06011
Author (publication year)	Silverman (2002) [27]	Kantarjian (2006) [10]	Kantarjian (2007) [55]	Fenaux (2009) [7]	Lyons (2009) [36]	Steensma (2009) [56]	Lubbert (2011) [13]
Number enrolled	191	170	95	358	151	99	233
Number treated with study drug	150 (99 upfront, 51) crossovers	89	95	179	151	99	119
Phase	III	III	II	III	II	II	III
Study regimen	Aza SQ 75 mg/m^2×7 days	Dac IV 15 mg/m^2 q8h×3 days	Dac IV 10 mg/m^2×10 days IV 20 mg/m^2×5 days SQ 20 mg/m^2×5 days	Aza SQ 75 mg/m^2×7 days	Aza SQ 75 mg/m^2 ×5days–2 days off-2days ×5 days–2days off-5 days ×5 days	Dac IV 20 mg/m^2×5 days	Dac IV 15 mg/m^2 q8h×3days
Int-2 or high IPSS (%)	46	70	66	87	Not reported	46	93
Median cycles administered	4	3	7	9	6	5	4
CR % (by IWG 2000)	9	9	37	17	Not reported	15	13
CR+PR+HI% (by IWG 2000)	48	30	73	49	48	43	34

clinically is ongoing. Furthermore, the development of novel dosing strategies, combinations, and the appropriate use of allogenic transplantation provide hope for improving response duration and possibly even providing an opportunity for long-term remission to these unfortunate patients.

13.2 Single Agent "Hypomethylating" Therapy for MDS and AML

13.2.1 Azacitidine

Aza is a nucleoside analog of cytidine in which the carbon 5 position of the pyrimidine ring has been substituted with nitrogen (Fig. 13.2a) [15]. It is imported into cells by the action of nucleotide transporters, where it is activated by uridine–cytidine kinase and incorporated into RNA (Fig. 13.3) [15]. Sixty to 80% of the Aza dose given is incorporated into RNA and this has impacts upon protein synthesis and RNA metabolism [15]. Twenty to 40% of the dose is converted into the deoxyribonucleoside Dac by the action of ribonucleotide reductase [15]. This deoxyribonucleoside base is then phosphorylated and incorporated into DNA where it acts as a suicide substrate for DNMTs and induces DNA hypomethylation during cellular replication as well as DNA damage due to adduct formation [15]. Aza was first synthesized and tested in 1960s and 1970s [16, 17]. In early clinical trials as a traditional chemotherapeutic, it was demonstrated to be effective in myeloid malignancy, however its efficacy was limited by significant gastrointestinal toxicity and prolonged cytopenias [16–18]. Cytarabine or AraC was developed at about the same time. This drug, another nucleoside analog of cytidine whose activity is thought to result in chain termination, is among the most active drugs used for myeloid malignancy. Ultimately the toxicity of 5-Aza limited its further

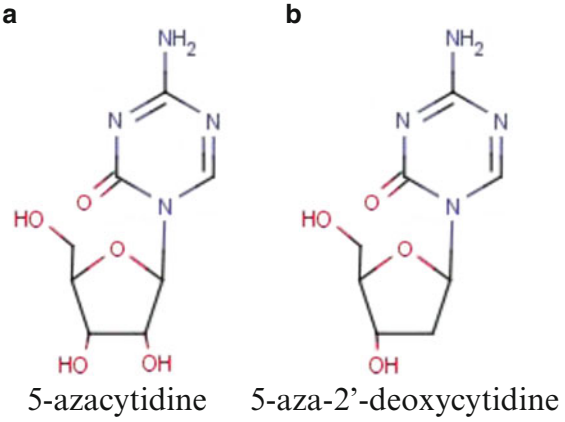

Fig. 13.2 Molecular structure of Aza (**a**) and Dac (**b**)

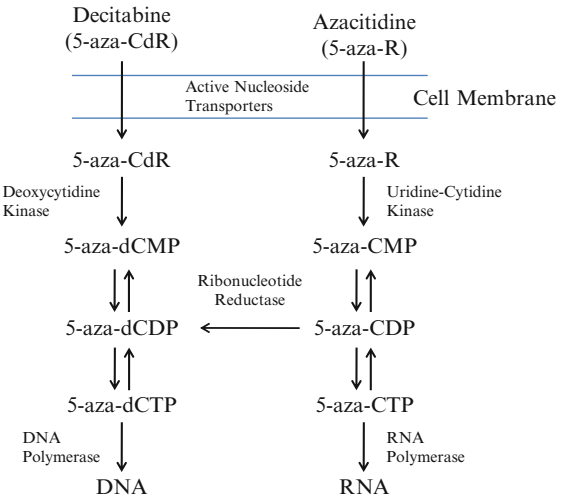

Fig. 13.3 Uptake and serial steps for the incorporation of Aza and Dac into RNA and DNA

clinical development, and cytarabine became the nucleoside analog of choice in myeloid malignancy [17, 18].

In 1978, Peter Jones and colleagues demonstrated that treatment of mouse embryo cells in vitro with Aza and its deoxy analog 5-aza-2′-deoxycytitine (Dac) could induce differentiation into functional myotubes [19]. Jones and Taylor went on to show that this differentiation resulted from changes in DNA methylation elicited by treatment with azanucleosides [20, 21]. Further work, by Dr. Jones and others, identified methylation as a common event in many malignancies, including the pre-leukemic condition known as MDS, a disease for which no treatment was available [22]. Although initially used as a laboratory tool to test gene and chromosome specific methylation changes, the identification of methylation as a potentially reversible cancer specific event spurred interest in the possibility that cancers treated with these drugs might be induced to differentiate and potentially to apoptose and die.

Ultimately in the 1990s, insights into methylation events common to MDS, specifically identification of recurrent methylation of tumor suppressor genes such as p15INK4B, resulted in the development of a number of phase I and II clinical trials of azanucleotides in this disease [23, 24]. Table 13.2 reviews the key published trials with single agent azanucleotides in MDS.

Among the first published trials with Aza for the treatment of MDS delivered the drug at 75 mg/m^2 as a continuous intravenous infusion for 7 days every 4 weeks [25]. This trial enrolled high grade MDS patients with symptomatic disease characterized by red cell and platelet transfusion dependence and poor life expectancy (refractory anemia with excess blasts (10–20%), or refractory anemia with excess blasts in transformation (20–30%). Forty three patients were evaluable and responses were seen in 21 (49%) of these patients [25]. Five patients (12%) achieved a CR, 11 (25%) achieved a partial response (PR), and 5 “improved” (a response characterized in the study as a ≥50% reduction in transfusion requirements, or improvement in platelets,

Table 13.2 Selected clinical trials with azacitidine (aza) or decitabine (dac) in MDS

Trial	CALGB 9221	D-0007	ICD03-180	AZA-001	US Oncology	ADOPT	EORTC 06011
Author (publication year)	Silverman (2002) [27]	Kantarjian (2006) [10]	Kantarjian (2007) [55]	Fenaux (2009) [7]	Lyons (2009) [36]	Steensma (2009) [56]	Lubbert (2011) [13]
Number enrolled	191	170	95	358	151	99	233
Number treated with study drug	150 (99 upfront, 51) crossovers	89	95	179	151	99	119
Phase	III	III	II	III	II	II	III
Study regimen	Aza SQ 75 mg/m^2×7 days	Dac IV 15 mg/m^2 q8h×3 days	Dac IV 10 mg/m^2×10 days IV 20 mg/m^2×5 days SQ 20 mg/m^2×5 days	Aza SQ 75 mg/m^2×7 days	Aza SQ 75 mg/m^2 ×5d–2 days off-2 days ×5 days–2 days off-5 days ×5 days	Dac IV 20 mg/m^2/days×5 days	Dac IV 15 mg/m^2 q8h×3 days
Int-2 or high IPSS (%)	46	70	66	87	Not reported	46	93
Median cycles administered	4	3	7	9	6	5	4
CR % (by IWG 2000)	9	9	37	17	Not reported	15	13
CR+PR+HI% (by IWG 2000)	48	30	73	49	48	43	34

hemoglobin or neutrophils) [25]. OS in these high risk patients was 13.3 months and for those achieving CR or PR was 14.7 months, and the chief toxicities were mild to moderate nausea [25]. A number of other clinical trials using this drug were published suggesting that Aza had significant activity in MDS and these results were sufficient to prompt two larger, randomized trials of Aza in MDS [26].

In 2004, the FDA approved Aza for the treatment of MDS based upon results from a single phase III clinical trial (described in detail below) [27]. A second trial demonstrating survival was required by European regulators, and this was published formally in 2009 [7]. These trials established Aza as the standard of care approach to patients with int-2 and high risk MDS by demonstrating a prolongation in the time to progression to AML, decreased transfusion requirements, improvements in neutropenia, and ultimately, improvements in OS.

13.2.1.1 CALGB 9221

The first phase III trial of Aza in patients with MDS was published by investigators from the CALGB [27]. CALGB 9221 enrolled 191 patients of median age 68 with French American British-defined MDS (reference for FAB classification), to receive either supportive care or Aza at a dose of 75 mg/m^2/day subcutaneously for 7 of 28 days. Patients were maintained on their randomized arm for 4 months, after which patients who were deemed to have progressed on the supportive care arm could crossover to the Aza arm. Patient characteristics were distributed evenly across both arms with 59% of the patients overall having RAEB or RAEB-T by FAB criteria (46% Int-2 or high by IPSS) [27]. Sixty five percent of the enrolled patients were red blood cell transfusion dependent (69% Aza arm, 61% supportive care arm) [27].

Responses were evaluated in both arms. Among patients randomized to receive supportive care, 5% met criteria for improvement; no patients on this arm achieved a CR or PR. Of the 99 patients randomized to receive Aza, 60% ($n=60$) achieved a response ($p<0.0001$) [28]. Responses were classified as CR in 7% ($n=7$), PR in 16% ($n=16$), and improvement in 37% ($n=37$). Of those patients demonstrating "improvement," 35% had increases in three cell lines inadequate to qualify as a PR, 30% had improvement in two cell lines, and 35% had improvement in only one cell line. Responses did not depend upon MDS sub-classification. Forty nine patients crossed over to receive Aza, of these 47% ($n=23$) responded and 10% ($n=5$) achieved a CR [27]. Patients treated with Aza had a median time to progression to AML or death of 21 months vs. 12 months in those patients treated with supportive care alone, and this was statistically significant ($p=0.007$), median OS in an intention to treat analysis was 20 months in the Aza treated patients vs. 14 months for those randomized to supportive care, although this difference was not statistically significant ($p=0.10$) [27].

Due to the design of this study, the survival analysis was confounded by the 49 patients who crossed over to receive Aza. In order to eliminate this bias, a landmark analysis at the 6 month date was performed. Three subgroups were identified, the

first included patients randomized to supportive care who did not crossover, or who crossed over after the six 6 month time point, the second were patients who were randomized to Aza, and the third were patients who crossed over after 4 months, but before 6 months [27]. This analysis excluded 36 patients who died before the landmark date. The median survival in these three groups was 11 (supportive care only), 14 (early crossover), and 18 (randomized to Aza) months respectively. A statistically significant difference in survival was observed between the Aza treated and supportive care groups ($p=0.03$), but not between supportive care and early crossovers [27].

Transfusion requirements were tracked in both groups. In the Aza treated group transfusion needs increased during the first cycle, and thereafter declined, whereas in the supportive care arm transfusion requirements remained stable or increased. Of the 99 patients initially randomized to receive Aza, 51% had an improvement in hemoglobin, 45% (29) became RBC transfusion independent, and 6 (9%) had a reduction in transfusion dependence by at least 50%. Improved platelet counts were observed in 47%, and increased white cell counts were seen in 40% of the Aza treated patients [27].

In addition to objective improvements in transfusion requirements, white cell counts, survival and prolonged time to AML transformation, patients treated with Aza on this trial experienced significant improvements in quality of life. These were reported as improvements in fatigue, physical functioning, dyspnea, psychological distress, and positive effect, all of which demonstrated statistical significance when compared to patients treated with supportive care alone with a p value ≤ 0.01 [27]. Similar results were observed in the patients who crossed over to Aza. Toxicities among the Aza treated patients were most frequently related to myelosuppression and were difficult to distinguish from the underlying disease. It was notable that treatment with Aza did not appear to increase the infection or bleeding rates above background, and furthermore only one treatment related death was reported on the study, emphasizing the safety of this therapy, even for older patients [27].

13.2.1.2 AZA-001

Although the data from CALGB 9221 was compelling, this study did not, in the final analysis, demonstrate a difference in OS between the patients randomized to receive Aza and those randomized to supportive care, likely as a result of the crossover trial design. The AZA-001 study was designed to address the question of whether Aza provided an OS benefit for high grade MDS patients [7]. This cleverly conceived, international, randomized trial definitively demonstrated that Aza 75 mg/m^2 given subcutaneously for 7 days of a 28 day schedule prolonged OS when compared with conventional care regimens (CCRs) as selected by the patients physician. The investigators aimed to provide at least six cycles of Aza to those patients randomized to the experimental arm. Conventional care was assigned by the patient's physician prior to randomization depending upon the patient's age, performance status co-morbidities and patient preference. CCR consisted of the three most

common treatments for patients with int-2 or high risk MDS: IC including cytarabine 100–200 mg/m^2/day × 7 days plus, daunorubicin 45–60 mg/m^2 × 3 days or idarubicin 9–12 mg/m^2/day × 3 days or mitoxantrone 8–12 mg/m^2/day, low dose cytarabine (LDAC) at a dose of 20 mg/m^2 for 14 days every 28 days, or best supportive care (BSC). All patients randomized received CCR as selected by their physician or Aza on trial. A total 358 patients were randomized. In this way a pre-specified subgroup analysis based upon physician assignment was possible and helped to eliminate differences in outcome based upon issues of performance status and patient fitness.

The primary OS endpoint of this study was met after a median follow-up of 21.1 months [7]. At this analysis the OS in the Aza treated patients was 24.5 months vs. 15 months for patients assigned to CCR and this result was found to be statistically significant ($p \leq 0.0001$). Two year OS also favored Aza, at 51% vs. 25% for CCRs ($p \leq 0.0001$) [7]. Predefined subgroup analysis was also done in order to compare Aza responses with each of the CCRs selected and within specific cytogenetic and IPSS risk groups. There were significant differences between Aza and BSC with an OS benefit for azacytidine treatment of 9.6 months (HR 0.58, $p = 0.0045$), as well as between Aza and LDAC with an OS benefit of 9.2 months (HR 0.36, $p = 0.0006$) [7]. No statistically significant differences in OS were seen when Aza was compared with IC; OS was prolonged by 9.4 months with a hazard ratio of 0.76, but the p value was not significant at 0.51 [7]. This apparent discrepancy was likely due to the low numbers in this subgroup ($n = 42$); 17 patients in this group were randomized to Aza and 25 to intensive chemotherapy.

No differences in response to Aza were seen across the IPSS risk groups enrolled (although most patients were int-2 or high risk $n = 313$ (87%)), nor within the cytogenetic risk groups identified by the IPSS (good, intermediate, poor). Patients with del-7 or del(7q), a group recognized to have particularly poor prognosis, had an OS of 13.1 months vs. 4.6 months in the CCR group [7, 29].

Responses on this trial were similar to those observed in CALGB 9221. Overall, 29% of those assigned to Aza achieved either CR (17%) or PR (12%) compared with 12% (8% CR, 4% PR) assigned to CCR ($p = 0.0001$) [7]. Any hematological improvement (HI) was observed in 49% of those treated with Aza vs. 29% of those treated with CCR ($p = 0.0001$) [7]. In addition, for those treated with Aza, major erythroid responses were seen in 40% of patients, major platelet responses in 33% and major neutrophil responses in 19%. By contrast, for those receiving CCR major erythroid responses were seen in 11% ($p < 0.0001$), major platelet responses were seen in 14% ($p < 0.0003$) and major neutrophil responses were seen in 18% ($p = 0.58$, not statistically significant) [7]. Patients treated with Aza experienced a statistically significant reduction in the need for intravenous antibiotics (33% relative risk reduction vs. CCR; RR 0.66 95% CI:0.49–0.87 $p = 0.0032$). Furthermore of the 111 patients with red cell transfusion dependence at the time of study enrollment, 50 (45%) became transfusion independent vs. 13 (11.4%) of the 114 patients randomized to receive CCR (p value significant at 0.0032) [7].

Secondary endpoints in this trial included time to AML transformation and hematological response according to the IWG 2000 criteria for MDS [11]. Treatment with Aza in the entire group was associated with delayed leukemic transformation;

the median time to transformation was 17.8 months in the Aza treated group vs. 11.5 months in the CCR group ($p < 0.0001$) [7].

Among the most notable findings on this trial was that achievement of CR or PR was not necessary in order to achieve an improvement in OS; any patient who achieved a hematological response showed a survival benefit.

13.2.1.3 AZA in AML

Changes in the diagnostic criteria for AML based upon the WHO guidelines published in 2008 resulted in the reclassification of patients enrolled on both the CALGB and AZA-001 from the previous FAB classification of Refractory Anemia with Excess Blasts in Transformation (RAEB-T; 20–30% bone marrow blasts) to a new diagnosis of AML [1, 30, 31]. The WHO now defines any patients with ≥20% blasts as having AML [30].

A pooled analysis of previously published CALGB studies including 9221, 8921, and 8421, in which enrolled patients treated with Aza would now be re-assigned as AML was published in 2006 [28]. This reported the response to Aza given either intravenously or subcutaneously at a dose of 75 mg/m^2/day for 7 days of a 28 day cycle in 103 patients who would now be classified as having AML, 91 of whom received Aza [28]. Of these patients 33 (36%) developed a response (8 CRs, 2 PRs, 23 HIs), with a median duration of response of 7.3 months (range 2.2–25.9 months) [28]. Formal comparison with supportive care alone across the three studies was not possible, but 27 patients enrolled in 9221 were randomized to upfront Aza and a further 13 crossed over to receive Aza before the 6 month analysis. Of these, 7% in the Aza group achieved CR or PR compared with 0% in the observation-only group [28]. Median survival time for the 27 patients assigned upfront to Aza was 19.3 months compared with 12.9 months for the 25 AML patients randomly assigned to observation. Of 13 patients with WHO AML at the time of study entry who crossed over to receive Aza, one achieved a PR, and one HI.

Of the 358 patients originally enrolled on AZA-001, a third would now be identified as having AML. A second analysis of these patients was undertaken in order to assess outcome in this group of older adults treated with either Aza or CCR [7, 32]. Of the 113 patients now designated as AML, 63 were assigned to BSC, 34 to LDAC and 16 to IC [32]. The median age in all groups was 70 years with a range of 58–80. Patients were evenly distributed with respect to age, cytogenetic risk group, and ECOG scores. Bone marrow blast percentages were similar in both groups at 23% with a range of 20–34%. In all, 55 patients were randomized to the Aza arm and 53 to CCR. After a median follow-up of 20.1 months, OS was significantly ($p = 0.005$) longer in those patients treated with Aza (24.5 months) than in those receiving CCR (16 months). The 2 year survival was also superior in the Aza group at 50% compared with16% in the CCR group ($p = 0.001$) [32]. Adverse events in this group of patients were primarily grade 3 and 4 cytopenias, which remain difficult to distinguish from the underlying disease. Four patients in the Aza group and three patients in the CCR group discontinued treatment as a result of adverse events.

Several prospective studies of Aza given on the conventional schedule of 75 mg/m^2/day for 7 days in patients identified as AML at diagnosis have been reported. One such study enrolled 82 patients with AML (27 (33%) with secondary disease) and a median age of 72 years (range, 29–87 years) [33]. Thirty-five patients (43%) received Aza as their first treatment, and 47 patients (57%) had previously received 1 or more lines of chemotherapy. The overall response rate in this group was 32% (26/82 patients) with 16 patients (20%) achieving a CR or a CR with incomplete count recovery, and 10 patients (12%) achieving a PR [33]. Untreated patients responded more often than those previously treated with 31% of untreated patients achieving either a CR or a CR with incomplete count recovery compared with only 9 (19%) such responses in the previously treated group ($p=0.006$). The response duration in untreated patients who achieved a response was 13 months with 1 and 2 year survivals of 58 and 24% respectively [33]. Another study from Germany evaluated medically unfit ($n=20$) or relapsed/refractory ($n=20$) patients with AML and a median bone marrow blasts count of 42% [34]. This study showed similar statistically significant differences in response between untreated patients, who demonstrated overall responses (CR + PR + HI) of 50%, and patients with relapsed or refractory disease, who had an overall response rate of only 10% ($p=0.008$) [34]. These response rates are striking and compare favorably with responses seen with induction therapy although additional data are necessary in order to determine whether Aza or Dac will end up the therapeutic agent of choice in this context [6, 35].

Results from the CALGB trials were sufficient in the United States and the AZA-001 trial satisfied the European regulators for the approval of Aza as standard therapy for patients with MDS and low blast count AML. In the United States, approval was granted for all IPSS defined MDS subtypes, while in Europe approval is confined to patients with Int-2 and high risk IPSS scores not eligible for bone marrow transplantation, those with CMML-2 and those with WHO defined AML with 20–30% blasts or multilineage dysplasia.

Both large phase III trials demonstrated this drugs activity in MDS and AML, and further showed that unlike previous therapies, DNMTi require prolonged exposure to elicit a clinical benefit. In the CALGB trials most responses were seen by cycle 4 (75%), with a median number of cycles to any response (CR, PR, HI) of three cycles [27]. The range for this response was 1–17 cycles, however and although 90% of patients achieved a response by cycle 6, some patients got their response as late as cycle 17 [27]. In the AZA-001 trial where the goal was to provide at least six cycles of therapy and there was no predefined stopping point, the investigators demonstrated that continuing the Aza dosing as long as possible can result in improvements in the observed responses, and these results were re-iterated by additional analysis of the studies conducted by the CALGB [28, 32]. The secondary analysis of CALGB studies demonstrated a response in 91 of 179 patients, and responders received a median of 14 cycles of therapy (range 2–30) [28]. The median time to first response in this study was slightly shorter than that seen in 9221, at 2 cycles (but with a range of 1–16) and although most responses (91%) were achieved by the sixth cycle, continuation of Aza was able to improve the quality of the first response in 48% of those treated, and this best response was seen in most patients (92%) by

the 12th cycle [28]. Overall 30 patients achieved a best response of CR 3.5 cycles beyond the first response (with a 95% CI of 3.0–6.0 cycles), and in 21 patients whose best response was PR, this was seen as a median of 3.0 cycles after the first response (95% CI was 1.0–3.0) [28].

13.2.1.4 Other Considerations of Dose and Schedule

Additional questions which remain about the use of single agent Aza therapy are related to administration schedule (to weekend or not to weekend, are 7 days enough) and optimal drug delivery (subcutaneous vs. intravenous vs. oral).

In community practice there is often difficulty in giving this drug on the FDA approved schedule due to inadequate availability of personnel to administer the drug on weekends. This practical consideration resulted in a trial of several schedules of Aza administered in a community setting during weekdays only [36]. In this trial, 151 patients, for the most part with lower risk MDS (low, int-1 in 63% of patients), were randomized to receive Aza on one of the three schedules: 75 mg/m^2 daily for 5 days, off 2 days and then on 2 days (5-2-2), 50 mg/m^2 daily for 5 days, off 2 days and then on for 5 further days (5-2-5), and lastly 75 mg/m^2 daily for 5 days alone (5-0-0) [36]. These schedules seemed to result in similar hematological improvement rates (44%, 45%, 56%, respectively), but this study was not designed to produce statistically significant results, nor have these schedules been directly compared with the approved 7 day schedule. Thus it is difficult to condone alteration of the schedule at this time, based upon the lack of survival data in these schedules and the demonstrated survival benefit with administration of these drugs on the approved schedule. One additional schedule question has been raised by the preliminary data from the Eastern Cooperative Oncology Group trial 1905, which was a randomized phase II trial comparing Aza 50 mg/m^2/day subcutaneously for 10 days to the same Aza schedule given in combination with the Histone deacetylase (HDAC) inhibitor entinostat (4 mg/m^2/day PO days 3 and day 10) [37]. This abstract reported only on patients with baseline cytogenetic abnormalities ($n = 40$ evaluable) but demonstrated complete cytogenetic responses of 13% and a partial cytogenetic responses of 23% for an overall response in this subgroup of 51% (21/40) [37]. No differences in response were seen between the two treatment groups. Notably the responses observed were significantly higher than those reported with conventional Aza dosing raising the question of whether a lower dose, longer administration schedule may be of some benefit. At present these data are insufficient to change practice, however as additional groups publish the results of ongoing clinical trials of different dosing schedules, practice changes may be in order.

With respect to optimal drug delivery there is only a single study which directly compares the pharmacokinetics of intravenous to subcutaneous dosing within individual patients. In this study the pharmacokinetic profile of intravenous administration was almost identical to that seen with subcutaneous dosing, although the peak drug concentration was higher in patients receiving intravenous drug [38]. Despite these

data, published clinical trials using 20 min IV infusion schedules are limited to two studies, one which gave Aza for 5 days and the other for 7 [39, 40]. Both of these studies demonstrated response rates which were similar to those seen with subcutaneous dosing (27% in the 5 day and 56% for the 7 day schedule), but neither of them was powered to detect a survival benefit [39, 40]. Despite the dearth of published response data, it seems reasonable to switch to intravenous administration in patients who suffer significant injection site reactions with subcutaneous dosing, and the FDA approved a New Drug Application for intravenous Aza in January 2007, supporting this practice [41].

Initial studies with oral Aza were limited by rapid catabolism of the compound in aqueous environments but the development of a film-coated formulation improved stability [42, 43]. Since that time the first phase I study of oral Aza has been published, demonstrating activity for the oral drug in patients with both MDS and CMML, with promising response rates [44]. Six of 17 (35%) previously treated patients had a response (CR + PR + HI) and 11 of 15 (73%) untreated patients responded (CR + PR + HI). This study demonstrated no overall response in the 8 patients with AML, however two patients had stable disease for 14 and 15 cycles [44]. Overall these results suggest that oral Aza may be a real possibility for the future and clinical trials of this drug are ongoing.

13.2.2 Dac

5-Aza-2′-deoxycytidine (Dac) is a deoxynucleoside analog of cytidine in which the carbon 5 position of the pyrimidine ring has been substituted with nitrogen (Fig. 13.2b) [15]. It is imported into cells by the action of nucleotide transporters, where it is activated by deoxycytidine kinase and then phosphorylated (Fig. 13.3) [15]. After its phosphorylation to the triphosphate form, 100% of the drug is incorporated into DNA, where it interrupts the action of DNA methyltransferases as described above for Aza. Similar to Aza, Dac has been demonstrated to cause both DNA hypomethylation and DNA damage, albeit at lower concentrations [45]. The identification of DNA hypomethylation as a functional consequence of exposure to both Aza and Dac, in conjunction with the recognition of DNA methylation changes as a frequent abnormality in cancer, spurred significant clinical interest in the development of these drugs for clinical use [20, 45].

Although effects upon DNA methylation were recognized and noted early in its development, initial clinical trials focused on conventional dosing strategies aimed at developing a maximum tolerated dose schedules [46–48]. These studies demonstrated considerable activity but with toxicity not significantly superior to cytarabine, with several studies performed investigating combinations with other chemotherapeutics in the salvage setting [49, 50].

Several early studies showed promising results with “low dose” Dac regimens, however these studies provided the drug at doses of 40–50 mg/m^2/day, and toxicity remained a serious problem [51–53]. The first study to investigate the “optimal”

lower dose Dac schedule for maximal demethylation was published in Blood in 2002 by Jean-Pierre Issa and colleagues [54]. This trial enrolled 48 patients at doses ranging from 5 to 20 mg/m^2/day for 10–20 days of a 6 week schedule depending upon count recovery. Most interestingly in this study, responses appeared to be superior for the lower dose schedules studied, prompting the authors to suggest further investigations of the drug be undertaken at truly lower dose schedules [54].

Based upon extensive phase I/II data at moderate to higher doses, the first large scale trial of Dac enrolled 170 patients with MDS between 2001 and 2004 and randomized them to either Dac (89 patients), given at 15 mg/m^2 iv every 8 h (45 mg/m^2/day) for 5 days, or BSC (81 patients) [10]. Patients were removed from the study for disease progression, transformation to AML, failure to achieve a PR after six cycles of therapy, or failure to achieve a CR after eight cycles of therapy. Additionally, patients who did achieve a CR were removed from therapy after two cycles of sustained CR. The groups were well matched for all important variables with a median age of 70 years (range, 30–85 years). A majority of the patients (71%) had int-2 or high risk disease by IPSS criteria. The primary study endpoints were overall response rate and time to AML transformation or death. Overall 30% ($n=27$) of patients experienced improvement on the study (CR + PR + HI) compared with 7% ($n=6$) patients randomized to BSC, and this difference was statistically significant $p=0.001$ [10]. In a retrospective central review of pathology nine patients enrolled on Dac and three patients on the supportive care arm were designated as having AML (by FAB criteria, >30% bone marrow blasts). Response rates in these nine patients were 56% (5/9), while none of the patients enrolled on the supportive care arm developed a response [10]. It is important to note that in this randomized controlled non-crossover trial there was no survival benefit for the use of Dac, although one might argue that the dose used (45 mg/m^2/day × 5 days) was not low enough to maximize hypomethylation over cytotoxicity and the median number of cycles administered was low (3).

Following the results of this trial (which were disappointing from a survival perspective, but represented the first active agent for patients with high grade myelodysplasia), in 2006 the FDA approved Dac for all MDS subtypes. Based upon the results of earlier studies suggesting that lower dose Dac dosing might be superior, two pivotal phase II studies were performed aimed at identifying the "optimal" hypomethylating dose for Dac [55, 56]. The first of these was published in 2007 and enrolled 95 patients, again with a majority (66%) of patients having int-2 or high risk disease [55]. All patients were randomized to receive one of the three different Dac schedules, 10 mg/m^2 intravenously over 1 h daily for 10 days, 20 mg/m^2 intravenously over 1 h daily for 5 days, or 20 mg/m^2 subcutaneously daily for 5 days. Patients received a median of seven cycles of treatment and the CR rate overall was significantly better than anticipated at 37%, and an overall improvement (including CR + PR + HI) was observed in a staggering 73% of patients [55]. The 5 day schedule was deemed superior with 25/64 patients on this arm achieving CR and this schedule was selected for further investigation in subsequent trials [55]. The second analogous trial published in 2009 by Steensma and colleagues enrolled 99 patients in a single arm trial of Dac 20 mg/m^2 over 1 h daily for 5 days [56]. A lower percentage of patients on this trial were high grade (46%), and the median number of administered courses were slightly

lower (5) than in the prior investigation. These authors observed a 15% CR rate and an overall response rate of 43% (CR + PR + HI) [56]. Both trials demonstrated that the lower dose schedule of Dac 20 mg/m^2/day for 5 days had at least equivalent efficacy when compared with the FDA approved schedule, and furthermore that maintaining 4 week dosing intervals and repeated cycles of therapy were important in order to maximize response.

One additional phase III study of Dac has been published [13]. It is important to note that this study did not employ the 5 day, 20 mg/m^2/day schedule described above. This trial was designed to demonstrate a survival benefit for the use of Dac in patients with MDS, comparable to that observed with Aza. Two-hundred and thirty-three patients with a median age of 70 years (range 60–90) were enrolled; 53% had poor-risk cytogenetics and 33% fulfilled WHO AML diagnostic criteria (≥20% blasts) [13]. The primary end point for this trial was OS. Patients were stratified by IPSS risk group, cytogenetics and enrollment site, and were randomly assigned to receive either Dac or BSC. This study design specifically prohibited patient crossover to the experimental arm in an effort to eliminate crossover bias. The Dac was given intravenously at a dose of 15 mg/m^2 every 8 h for 3 days. Cycles were scheduled to repeat every 6 weeks, but the interval could be extended up to 10 weeks for failure of count recovery, eight cycles of treatment were planned. In total 119 patients were randomized to receive decitabine and 114 patients were randomized to the control arm; only 21% of patients received the planned eight cycles of treatment. At the planned analysis point of 2 years, OS in the Dac treatment cohort was 10.1 months vs. 8.5 months in the supportive care arm, this difference was not statistically significant ($p = 0.38$, HR, 0.88; 95% CI, 0.66–1.17) [13]. Sixteen patients on the Dac arm (13%) achieved a CR and 25 patients (21%) improved (PR + HI), for an overall response rate of 34%. The median time to best response was 3.8 months (range, 1.4–11.8 months) for all responders, with a median of 5.8, 2.9, and 3.8 months to reach CR, PR, and HI, respectively. Two patients (2%) in the supportive care arm had a HI, there were no CRs or PRs in this group. Dac did not have a statistically significant impact upon time to AML transformation; patients on Dac transformed to AML after 8.8 months vs. 6.1 months in the supportive care arm (HR 0.85; 95% CI 0.64–1.12; $p = 0.24$) [13].

Disappointing results, in terms of survival benefit, from two large phase III trials of Dac in MDS have resulted in a significant shift in terms of practice away from Dac in this population [10, 13]. Despite these results, some clinicians continue to use Dac in the first line treatment of MDS patients, and it is certainly notable that none of the three phase III studies of Dac used the most common low dose schedule of Dac at 20 mg/m^2/day for 5 days, a dose schedule which is pharmacologically more consistent with the 75 mg/m^2 Aza dose demonstrated to prolong survival. Additionally, the European phase III trial delayed subsequent Dac cycles based upon cytopenias, a strategy which is increasingly recognized as inferior. As a result of these caveats it is likely that Dac has similar efficacy to Aza, although at present the data have not definitively demonstrated this equivalence.

13.2.2.1 DAC in AML

Despite disappointing results in patients with MDS, many clinicians favor Dac in patients presenting with AML, particularly in those with very proliferative disease, as a result of its relative cytotoxicity when compared with Aza. A dosing strategy employing 20 mg/m^2 for 10 days has been studied by investigators at the Ohio State James Cancer Center [35, 57]. This dose schedule was initially developed in a phase I trial designed to assess combination therapy with valproic acid, however a single agent response of 73% in a group of very elderly (median age 70) patients with high risk AML prompted phase II investigation (see below) [57]. The Phase II trial enrolled 53 patients of median age 74 years (range 60–85) with AML (16 complex karyotype, 19 with an antecedent hematological diagnosis) and produced a response rate of 64% (34/53) composed of 25 CRs and 9 CRs without count recovery [35]. Patients enrolled on study had a median survival of more than a year, suggesting that this strategy is similarly effective to conventional chemotherapeutics in this patient population [6, 8, 9]. These very promising results have produced an ongoing cooperative trial using this dose schedule in older patients with AML and may yet demonstrate statistically significant improvements in survival for this particular subgroup of elderly AML patients.

13.3 Azanucleotides and CMML

Dac remains the most studied drug in patients with CMML, a distinct entity within the WHO diagnostic criteria form MDS. Several studies have examined the activity of Dac both prospectively and retrospectively in this group. One recently published phase II study enrolled 39 patients of median age of 71 years with advanced CMML to receive Dac on the 20 mg/m^2/day intravenous schedule for 5 days of a 28 day cycle [58]. Enrolled patients received a median of ten cycles of drug (range, 1–24) and the overall response rate was 38%, composed of 4 (10%) CRs, 8 (21%) marrow responses, and 3(8%) His [58]. With a median on trial follow-up of 23 months the OS was 48%. Another study examined the response to Dac in 31 patients diagnosed with CMML who were treated on two phase II and one phase III clinical trials [59]. Patients included in the analysis had similar demographics and disease characteristics across the three studies. The median age was 70 and patients were predominantly male (71%). The overall response rate in this group was 36% (14% CR + 11% PR + 11% HI) [59]. Although Aza has also been shown to have activity in this disease, the number of published reports in this group are limited, and thus most experts would likely favor the use of Dac for patients with CMML outside the context of a clinical trial [60]. An ongoing clinical trial designed to prospectively enroll patients with CMML is ongoing in order to address the efficacy of Aza in this disease.

13.4 Outcomes Following Azanucleotide Failure

As we develop our experience with azanucleotides it has become clear that patients who lose their response to azanucleotides have a dismal prognosis [14]. As a result of these poor outcomes, current standard practice is to maintain patients on therapy with hypomethylating drugs on a monthly schedule indefinitely and to stop only in the context of overt progression. Unfortunately, analysis of patients enrolled on early studies of Aza who develop disease progression have now been published, showing that in patients who fail azanucleotides, survival is remarkably short with a median life expectancy of 5.6 months and a 2-year survival probability of 15% [61]. Similar results have been reported in patients who fail Dac [14, 62]. Outcomes in these reports suggest that enrollment on clinical trials and bone marrow transplantation may result in superior outcome in these patients, however in the absence of successful bone marrow transplantation the OS reported at 1 year remains a mere 28% [14, 61, 62].

13.5 Histone Deacetylase Inhibitors

Histone deacetylase inhibitors (HDACis) are a novel class of drugs whose putative mechanism of action depends upon the ability to alter gene expression. Intracellularly, DNA is stored in the form of "beads on a string" in which the DNA duplex winds around a nucleosome composed of eight histones (two each of H2A, H2B, H3 and H4) [63]. The DNA/histone unit (the nucleosome) is condensed to form higher order chromatin structures such as heterochromatin, which has densely packed nucleosomes and euchromatin, which has loosely packed nucleosomes [63]. Modifications, including ubiquitination, methylation, phosphorylation, poly(ADP) ribosylation, and acetylation, of specific amino acid residues within each histone make up the "histone code" which determines the state of the regional chromatin at specific genes and thus their transcriptional activity [63]. DNA methylation events are thought to induce changes within the local "histone code" which promote gene silencing, although whether methylation events or histone marks are primary remains a matter of some controversy. Perhaps the most studied histone modification is acetylation of lysine N-terminal tails which are common to most histones. Acetylation of lysine results in an open chromatin conformation and promotes gene transcription while deacetylation of lysine residues promotes gene silencing [63].

HDACs are enzymes that remove acetyl groups from a variety of different protein targets including histones. Increased HDAC activity has been described in cancer cells, and aberrant HDAC activity is characteristic of a number of well recognized recurrent genetic anomalies characteristic of leukemia including the core binding factor gene fusions (t(8;21)(q22;22) and inv(16)), and the sine qua non of acute promyelocytic leukemia t(15;17)(q24;21) [64–66]. The gene products of such fusions result in aberrant recruitment of HDACs to genes important for myeloid

differentiation. Recognition of HDACi as a potential novel therapy in myeloid malignancy resulted from the observation that drugs known to induce differentiation in vitro induced histone hyperacetylation, potentially leading to re-expression of epigenetically silenced genes [67]. Many different diverse chemical compounds can inhibit HDACs, including short chain fatty acids (e.g., phenylbutyrate), hydroxamic acid derivatives (e.g., vorinostat), non-hydroxamate small molecules (e.g., entinostat), and cyclic peptides (e.g., romidepsin) [68].

Most of the published clinical trials of HDACi in MDS and AML are phase I. As single agents the response rates observed have been relatively low, usually between 10 and 20% [68]. Toxicities with these agents demonstrate a common pattern and include fatigue, nausea, vomiting, and diarrhea. Although most of these studies evaluated the correlative endpoint of histone acetylation, no associations between hyperacetylation of histones and response to therapy have been demonstrated. For a more complete review of HDACi in cancer please see Chap. 3, Sect. 3.5 of this book.

13.6 Azanucleotides and HDACis

There has been significant enthusiasm for a combination strategy which includes azanucleotides in conjunction with HDACis. This stems from the observation in vitro that sequential exposure to Dac or Aza followed by HDACi result in synergistic re-expression of DNA methylation silenced genes [69]. Several studies evaluating such combinations have been published to date and the results remain mixed. Although some studies suggest a higher response rate than for single agent azanucleotides, most data are in the phase I or II setting, at a single center, and employ alternative dosing strategies for the azanucleotide making it difficult to distinguish whether these responses are truly superior. In those studies where a single agent arm was also enrolled response rates do not appear to be consistently superior [37, 57]. Although early correlative endpoints did demonstrate evidence to support a connection between reversal of methylation events and response to therapy, subsequent studies (even at the same institution by the same investigators) have failed to substantiate a correlation between gene specific reversal of methylation and response [70, 71].

The first two studies published reports on a combination of Aza at doses between 25 and 75 mg/m^2/day subcutaneously for 5–10 days [70, 72]. These studies enrolled a total of 42 patients with MDS (16) and AML (26), of median age 66. These studies reported that the combination was well tolerated and resulted in response rates of 34 (11/32, 5 CRs) and 50% (5/10, no CRs) respectively (CR + PR + stable disease) [70, 72]. The second study reported correlative epigenetic data in three responders and three non-responders, with those patients who developed a response showing robust demethylation of the tumor suppressor gene *p15^{INK4B}* while those who did not retained methylation at this locus, suggesting that changes in methylation were indeed a marker for responsiveness [70].

Two phase I/II studies have evaluated the combination of Dac with valproic acid. The first employed Dac 15 mg/m^2/day for 10 days with a dose escalation of valproic acid from 20 to 50 mg/kg/day for 10 days in patients with high grade MDS or AML [73]. Fifty four patients of median age 60 (range 5–80 years) were enrolled, 48 patients had AML and 6 had MDS, 11 patients were previously untreated. Twelve patients responded to therapy; 10 developed a CR and 2 a CR with incomplete platelet recovery. Median responses were seen after 2 months (range 29–130 days) and responders survived a median of 15.3 (range 4.6–20.2+) months vs. 4.9 (0.6–17.8+) months in non-responders [73]. Responders were more likely to have been randomized to a higher dose of valproic acid. Although changes in methylation (both gene specific events, including $p15^{INK4B}$, and genome wide methylation, by LINE-1 pyrosequencing) and gene expression were analyzed in the patients on this study no correlations with response were observed [73]. All patients experienced a decrease in genome wide methylation which correlated with Dac exposure. In a second study, this one employing Dac 20 mg/m^2/day for 10 days intravenously, responses were also encouraging with an overall response rate of 44% in 11 of 25 enrolled patients [57]. This trial enrolled 25 AML patients, in whom the median age was 70 years; 12 patients were untreated and 13 had relapsed disease. In this group of slightly older patients, encephalopathy was the principal toxicity and this was dose limiting at 20–25 mg/kg/day. In an intent-to-treat analysis, the response rate was 52% (13). CR was observed in 8 patients and PR in 4. Responses appeared similar for patients who received Dac alone and for those who received valproic acid in addition. In this study, re-expression of estrogen receptor was statistically significantly associated with clinical response ($p=0.05$), however although the investigators also demonstrated ER promoter demethylation, global DNA hypomethylation, depletion of DNA methyltransferase enzyme, and histone hyperacetylation, these markers did not correlate with response [57].

The combination of Aza with vorinostat (SAHA) has also been explored. In one phase I trial in patients with MDS and AML this combination produced an impressive overall response rate of 64%[74]. A second phase II trial of this combination in patients with MDS and AML has also been reported [75]. This trial enrolled 17 untreated patients and demonstrated an overall response rate of 41% ($n=7$) [75]. Similar outcomes (overall response of 37%) were observed in patients receiving a combination of Aza with the compound MGCD0103, an oral isotype-selective HDACi [76]. Although these responses appear to be encouraging, a majority of these combination studies have been published to date only in abstract form and larger studies are necessary in order to verify their superiority.

Data from one of the first randomized phase II studies to enroll patients either on single or double agent therapy was presented at the 2010 ASH meeting and reviewed in detail earlier in this manuscript (see Aza section under Sect. 5.2.1.4), this study, at least, suggests that combination therapy may not be superior [37]. In this trial patients with either MDS or AML with MDS related changes were randomized to receive either Aza at 50 mg/m^2 for 10 days subcutaneously alone or Aza in combination

with entinostat 4 mg/m^2 orally on days 3 and 10. Although the final results of this trial have not yet been published, it is important to note that the response rates for patients enrolled to receive Aza alone were indistinguishable from those who got the combination.

These results and others with a variety of HDACis may underestimate the value of combined therapy. It is important to note that among the many mechanisms postulated to be responsible for the efficacy of HDACis are induction of apoptosis and cell cycle arrest [77]. Since azanucleotides require DNA replication in order to produce DNA demethylation, it may be that administration of HDACi simultaneously or even in advance of the azanucleotide may result in diminished incorporation and limit responsiveness. Presently, a multi-institution phase II sequence study designed to address this question is open for enrollment [78].

13.7 Azanucleotides and Conventional Chemotherapy

One study has been published which explores the possible role of azanucleotide in "priming" leukemia cells for death [79]. This open label, phase I study was designed to address the safety and feasibility of Dac at a dose of 20 mg/m^2 either as a continuous infusion or a short infusion for 3, 5, or 7 days followed by standard dose 7 + 3 IC (cytarabine 100 mg/m^2/day continuous intravenous infusion for 7 days + daunorubicin 60 mg/m^2/day for 3 days). The study enrolled 30 patients of median age 55 (range 23–60) with newly diagnosed AML and a less than favorable karyotype (inv(16), t(8;21) and APL patients were excluded). Thirteen patients had complex, 11q23 or chromosome 7 abnormality associated leukemias and 8 had an antecedent hematological diagnosis. Toxicity was not dissimilar to that seen with 7 + 3 alone, although there appeared to be slightly more gastrointestinal toxicity in the group treated with 7 days of Dac priming, and there were no deaths. All subjects received consolidation, 20 patients went on to receive allogeneic bone marrow transplantation. Overall 27 (90%) of patients responded to one course of induction therapy, 17 patients achieved a CR and 10 a PR, patients scored as a PR all achieved hematological remission, but went on to receive a second course of induction resulting in a CR in 8/10 patients [79]. The overall CR rate following 1 or 2 cycles of induction therapy was therefore 83%. With a median follow-up of 32 months, 53% of patients (16/30) remained alive and in CR, 14 subjects died, 3 of complications related to allogeneic bone marrow transplant and the remainder died of relapsed or refractory AML [79]. The correlative DNA methylation analysis of this study revealed universal demethylation at both gene specific and genome wide loci with all schedules of Dac. The most potent hypomethylation was observed in patients treated with bolus, rather than continuous infusion schedules of Dac.

Although preliminary, this phase I trial demonstrated a remarkably good CR rate and a randomized phase II study designed to assess the two most potent demethylation schedules of Dac priming identified by this study should begin accrual in 2012.

13.8 Azanucleotides and Bone Marrow Transplantation

Allogeneic bone marrow transplant (allo-transplant) is the only curative strategy currently available for patients with MDS and high risk AML. Presently the role of hypomethylating agents both prior to and following transplant is under investigation.

Several small retrospective studies of azanucleotide induction prior to allo-transplant have been reported, two using Dac and two using Aza. The first of these reported outcomes in 17 patients with MDS of median age 55.5 (range 36–66) years undergoing allo-transplant (12 sibling donor, 5 unrelated donor) after prior therapy with Dac (various dosing regimens) [80]. These patients received predominantly reduced intensity conditioning and peripheral blood stem cells (13/17). With median follow-up of 12 (range 3–35) months, 8 patients remained in CR [80]. A second prospective study performed in Europe reported similar results in 15 patients of median age 69 (range 60–75) years with either MDS ($n=10$) or AML ($n=5$) [81]. All patients were treated with upfront Dac followed by reduced intensity allo-transplant (4 sibling donor, 11 unrelated donor). Fourteen patients achieved a CR (93%), with a median duration of 5 (range 1–51) months [81]. The relapse rate in this group was similar (4/15) to that reported retrospectively. The third study examined outcomes in 54 patients with MDS or CMML who either received (30) or did not receive (24) prior therapy with Aza [82]. Patients treated with Aza received a median of 4 (range 1–7) courses prior to transplant. The overall, relapse free and cumulative relapse 1 year following transplant were 47, 41, and 20%, for those patients treated with Aza and 60, 51, and 32% for untreated patients and these results were not statistically significantly different [82]. The final trial using Aza was a retrospective review of 68 patients undergoing allo-transplant for MDS or AML arising from MDS [83]. Thirty five patients received Aza followed by either myeloablative (40%) or reduced intensity (60%) conditioning. Thirty three patients received IC followed by allo-transplant. In these two, albeit somewhat different groups, the OS at 1 year was 57% in those treated with Aza and 36% in the IC group [83]. Overall these data suggest that Dac and Aza are a reasonable pre-transplantation strategy that does not adversely affect outcome when compared with high dose induction or supportive care. A phase II clinical trial of Dac prior to allo-transplant is ongoing in Singapore using the currently favored schedule of 20 mg/m^2/day for 5 days intravenously.

Post-transplant relapse remains a significant problem in MDS and high risk AML patients. Traditionally relapses in this population have been managed with donor lymphocyte infusions (DLI) (in those who do not demonstrate graft vs. host disease) or re-induction with traditional chemotherapeutic agents. Although limited prospective data exist on the use of azanucleotides for salvage of patients relapsing following allogeneic transplant, or as a preventive strategy following transplant, several small studies have been published, suggesting that these agents may have a significant role to play.

The first of these examined the efficacy of Aza at a flat dose of 100 mg subcutaneously days 1–3 followed by planned DLI on day 10 [84]. Cycles were repeated

every 22 days for a median of 2 (range 1–10) courses to 26 patients with relapsed AML ($n=24$) or CMML ($n=2$) following allo-transplant. Toxicity with this combination was as expected and consisted of infections and GVHD. Four patients (15%) were salvaged with a complete and lasting CR following this combination [84].

A second study, this one retrospective, described the results of salvage with Aza 100 mg/m^2 for 5 days in 22 patients of median age of 50 (range 28–69) years, with either AML (17) or MDS (5) relapsed following allo-transplant [85]. A majority (20/23) of these patients had received a myeloablative conditioning regimen and half (10/23) had a sibling donor. On average two cycles of Aza were administered (range 1–8). Most patients also received DLI (18/23). In this group, 5 patients (23%) achieved a CR lasting a median of 433 days (range 114–769) with a 2-year survival rate of 23%[85].

A third single institution study, retrospectively reviewed Aza 75 mg/m^2 for 5 or 7 days as salvage in 10 patients with MDS (9) or AML (1) of median age 55 (range 25–67) years [86]. Seven patients achieved CR or stable disease with this regimen, 3 of whom progressed after a median of 6 cycles. The median OS (OS) for the group was 422.5 days (range 127–1,411).

Taken together these results are encouraging and a variety of studies are ongoing to determine prospectively the role of azanucleotides both before and after allo-transplant [87].

13.9 Molecular Determinants of DNMTi Response in MDS and AML

Early on in the development of azanucleosides for the treatment of myeloid disease there was considerable enthusiasm for the identification of molecular markers of disease response. Initially several authors examined gene specific methylation reversal, including *p15^{INK4B}* and ER as discussed earlier in this manuscript [10, 55, 57, 70, 71]. Disappointingly, although reversal of methylation at many loci has been documented following azanucleotide exposure, it has not been demonstrate to correlate with or predict response to treatment, but rather seems to reflect duration of exposure to hypomethylating agents [88]. Another marker of response which has been studied is p53-inducible-ribonucleotide-reductase (p53R2), a gene identified in cell line screens to be induced following decitabine exposure [89, 90]. Link and colleagues demonstrated a statistically significant concordance between response to therapy and induction of p53R2 both at the mRNA and protein levels [90]. Although these results are thought provoking, they require sampling after many cycles of therapy and it is difficult to determine how useful a biomarker of response this would be clinically.

The identification of mutations in the genes encoding *TET2* (ten–eleven translocation2) and *DNMT3A* in patients with MDS and AML have raised questions about whether response to therapy may depend upon genetic characteristics of the underlying myeloid neoplasm. Recently a number of authors have demonstrated that up

to 26% of patients with MDS demonstrate mutations in TET2, and further that MDS patients with *TET2* mutations appear to have a superior prognosis (although this is not as clear in patients with AML) [91, 92]. Since *TET2* encodes a dioxygenase which functions to convert 5-methylcytosine to 5-hydroxymethylcytosine resulting in DNA demethylation at selective loci, defects in TET2 function would be expected to result in hypermethylation. One recent study suggests that patients bearing *TET2* mutations have a superior response (CR + PR + HI) to Aza treatment 82% vs. 45% ($p = 0.007$), although OS was not different in the two groups and these results have yet to be validated [93]. By contrast with mutations involving *TET2,* mutations in *DMNT3A* have been demonstrated to predict adverse outcome in both MDS and AML, although as yet no evaluation has been made of the impact of such mutations on response to epigenetic therapies [94–96].

13.10 Conclusions

Azanucleotides have changed the landscape of treatment for patients with MDS and AML with MDS related changes. Ongoing work with these agents in patients with a variety of myeloid diseases is likely to result in advances over the next few years. Despite the considerable efficacy of these drugs, patients with underlying myelodysplasia continue to have a remarkably poor outcome and novel strategies in these diseases remain essential. As we continue to develop insight into the mechanism(s) which underlie the activity of these drugs, perhaps we will be able to understand why they work so well for some patients and what strategies will maximize the longevity of these responses. Certainly it has become clear that single agent azanucleotides given on a conventional schedule are not a panacea. Whether responses can be optimized with continuous dosing strategies, combination with other drugs, or allogeneic bone marrow transplantation remains a question yet to be answered by well designed clinical trials.

References

1. Vardiman JW, Harris NL, Brunning RD (2002) The World Health Organization (WHO) classification of the myeloid neoplasms. Blood 100:2292–2302
2. Greenberg PL (1998) Risk factors and their relationship to prognosis in myelodysplastic syndromes. Leuk Res 22(Suppl 1):S3–S6
3. Menzin J, Lang K, Earle CC, Kerney D, Mallick R (2002) The outcomes and costs of acute myeloid leukemia among the elderly. Arch Intern Med 162:1597–1603
4. Lowenberg B, Downing JR, Burnett A (1999) Acute myeloid leukemia. N Engl J Med 341:1051–1062
5. Howlader N, Noone AM, Krapcho M, Neyman N, Aminou R, Waldron W, Altekruse SF, Kosary CL, Ruhl J, Tatalovich Z, Cho H, Mariotto A, Eisner MP, Lewis DR, Chen HS, Feuer EJ, Cronin KA, Edwards BK (2011) SEER Cancer Statistics Review, 1975-2008, National Cancer Institute. Bethesda, MD, based on November 2010 SEER data submission, posted to the SEER web site

6. Kantarjian H, O'brien S, Cortes J, Giles F, Faderl S, Jabbour E, Garcia-Manero G, Wierda W, Pierce S, Shan J, Estey E (2006) Results of intensive chemotherapy in 998 patients age 65 years or older with acute myeloid leukemia or high-risk myelodysplastic syndrome: predictive prognostic models for outcome. Cancer 106:1090–1098
7. Fenaux P, Mufti GJ, Hellstrom-Lindberg E, Santini V, Finelli C, Giagounidis A, Schoch R, Gattermann N, Sanz G, List A, Gore SD, Seymour JF, Bennett JM, Byrd J, Backstrom J, Zimmerman L, McKenzie D, Beach C, Silverman LR (2009) International Vidaza High-Risk MDS Survival Study Group: Efficacy of azacitidine compared with that of conventional care regimens in the treatment of higher-risk myelodysplastic syndromes: a randomised, open-label, phase III study. Lancet Oncol 10:223–232
8. Tilly H, Castaigne S, Bordessoule D, Casassus P, Le Prise PY, Tertian G, Desablens B, Henry-Amar M, Degos L (1990) Low-dose cytarabine versus intensive chemotherapy in the treatment of acute nonlymphocytic leukemia in the elderly. J Clin Oncol 8:272–279
9. Gardin C, Turlure P, Fagot T, Thomas X, Terre C, Contentin N, Raffoux E, de Botton S, Pautas C, Reman O, Bourhis JH, Fenaux P, Castaigne S, Michallet M, Preudhomme C, de Revel T, Bordessoule D, Dombret H (2007) Postremission treatment of elderly patients with acute myeloid leukemia in first complete remission after intensive induction chemotherapy: results of the multicenter randomized Acute Leukemia French Association (ALFA) 9803 trial. Blood 109:5129–5135
10. Kantarjian H, Issa JP, Rosenfeld CS, Bennett JM, Albitar M, DiPersio J, Klimek V, Slack J, de Castro C, Ravandi F, Helmer R III, Shen L, Nimer SD, Leavitt R, Raza A, Saba H (2006) Decitabine improves patient outcomes in myelodysplastic syndromes: results of a phase III randomized study. Cancer 106:1794–1803
11. Cheson BD, Bennett JM, Kantarjian H, Pinto A, Schiffer CA, Nimer SD, Lowenberg B, Beran M, de Witte TM, Stone RM, Mittelman M, Sanz GF, Wijermans PW, Gore S, Greenberg PL (2000) World Health Organization(WHO) international working group: Report of an international working group to standardize response criteria for myelodysplastic syndromes. Blood 96:3671–3674
12. Cheson BD, Cassileth PA, Head DR, Schiffer CA, Bennett JM, Bloomfield CD, Brunning R, Gale RP, Grever MR, Keating MJ (1990) Report of the National Cancer Institute-sponsored workshop on definitions of diagnosis and response in acute myeloid leukemia. J Clin Oncol 8:813–819
13. Lubbert M, Suciu S, Baila L, Ruter BH, Platzbecker U, Giagounidis A, Selleslag D, Labar B, Germing U, Salih HR, Beeldens F, Muus P, Pfluger KH, Coens C, Hagemeijer A, Eckart Schaefer H, Ganser A, Aul C, de Witte T, Wijermans PW (2011) Low-dose decitabine versus best supportive care in elderly patients with intermediate- or high-risk myelodysplastic syndrome (MDS) ineligible for intensive chemotherapy: final results of the randomized phase III study of the European Organisation for Research and Treatment of Cancer Leukemia Group and the German MDS Study Group. J Clin Oncol 29:1987–1996
14. Kadia TM, Jabbour E, Kantarjian H (2011) Failure of hypomethylating agent-based therapy in myelodysplastic syndromes. Semin Oncol 38:682–692
15. Yang X, Lay F, Han H, Jones PA (2010) Targeting DNA methylation for epigenetic therapy. Trends Pharmacol Sci 31:536–546
16. Weiss AJ, Stambaugh JE, Mastrangelo MJ, Laucius JF, Bellet RE (1972) Phase I study of 5-azacytidine (NSC-102816). Cancer Chemother Rep 56:413–419
17. Karon M, Sieger L, Leimbrock S, Finklestein JZ, Nesbit ME, Swaney JJ (1973) 5-Azacytidine: a new active agent for the treatment of acute leukemia. Blood 42:359–365
18. McCredie KB, Bodey GP, Burgess MA, Gutterman JU, Rodriguez V, Sullivan MP, Freireich EJ (1973) Treatment of acute leukemia with 5-azacytidine (NSC-102816). Cancer Chemother Rep 57:319–323
19. Constantinides PG, Taylor SM, Jones PA (1978) Phenotypic conversion of cultured mouse embryo cells by aza pyrimidine nucleosides. Dev Biol 66:57–71
20. Jones PA, Taylor SM (1980) Cellular differentiation, cytidine analogs and DNA methylation. Cell 20:85–93

21. Jones PA, Taylor SM (1981) Hemimethylated duplex DNAs prepared from 5-azacytidine-treated cells. Nucleic Acids Res 9:2933–2947
22. Quesnel B, Guillerm G, Vereecque R, Wattel E, Preudhomme C, Bauters F, Vanrumbeke M, Fenaux P (1998) Methylation of the p15(INK4b) gene in myelodysplastic syndromes is frequent and acquired during disease progression. Blood 91:2985–2990
23. Esteller M (2003) Profiling aberrant DNA methylation in hematologic neoplasms: a view from the tip of the iceberg. Clin Immunol 109:80–88
24. Voso MT, Scardocci A, Guidi F, Zini G, Di Mario A, Pagano L, Hohaus S, Leone G (2004) Aberrant methylation of DAP-kinase in therapy-related acute myeloid leukemia and myelodysplastic syndromes. Blood 103:698–700
25. Silverman LR, Holland JF, Weinberg RS, Alter BP, Davis RB, Ellison RR, Demakos EP, Cornell CJ Jr (1993) Carey RW, Schiffer C: Effects of treatment with 5-azacytidine on the in vivo and in vitro hematopoiesis in patients with myelodysplastic syndromes. Leukemia 7(Suppl 1):21–29
26. Chitambar CR, Libnoch JA, Matthaeus WG, Ash RC, Ritch PS, Anderson T (1991) Evaluation of continuous infusion low-dose 5-azacytidine in the treatment of myelodysplastic syndromes. Am J Hematol 37:100–104
27. Silverman LR, Demakos EP, Peterson BL, Kornblith AB, Holland JC, Odchimar-Reissig R, Stone RM, Nelson D, Powell BL, DeCastro CM, Ellerton J, Larson RA, Schiffer CA, Holland JF (2002) Randomized controlled trial of azacitidine in patients with the myelodysplastic syndrome: a study of the cancer and leukemia group B. J Clin Oncol 20:2429–2440
28. Silverman LR, McKenzie DR, Peterson BL, Holland JF, Backstrom JT, Beach CL, Larson RA (2006) Cancer and Leukemia Group B: Further analysis of trials with azacitidine in patients with myelodysplastic syndrome: studies 8421, 8921, and 9221 by the Cancer and Leukemia Group B. J Clin Oncol 24:3895–3903
29. Heim S (1992) Cytogenetic findings in primary and secondary MDS. Leuk Res 16:43–46
30. Arber DA, Brunning RD, Orazi A et al (2008) Acute myeloid leukaemaia with myelodysplastic-related changes. In: Swerdlow SH, Campo E, Harris NL et al (eds) WHO classification of tumors of haematopoietic and lympohoid tissues (4th edn). Lyon, International Agency for Research on Cancer
31. Harris NL, Jaffe ES, Diebold J, Flandrin G, Muller-Hermelink HK, Vardiman J, Lister TA, Bloomfield CD (1999) World Health Organization classification of neoplastic diseases of the hematopoietic and lymphoid tissues: report of the Clinical Advisory Committee meeting-Airlie House, Virginia, November 1997. J Clin Oncol 17:3835–3849
32. Fenaux P, Mufti GJ, Hellstrom-Lindberg E, Santini V, Gattermann N, Germing U, Sanz G, List AF, Gore S, Seymour JF, Dombret H, Backstrom J, Zimmerman L, McKenzie D, Beach CL, Silverman LR (2010) Azacitidine prolongs overall survival compared with conventional care regimens in elderly patients with low bone marrow blast count acute myeloid leukemia. J Clin Oncol 28:562–569
33. Maurillo L, Venditti A, Spagnoli A, Gaidano G, Ferrero D, Oliva E, Lunghi M, D'Arco AM, Levis A, Pastore D, Di Renzo N, Santagostino A, Pavone V, Buccisano F, Musto P (2012) Azacitidine for the treatment of patients with acute myeloid leukemia: Report of 82 patients enrolled in an Italian compassionate program. Cancer 118:1014–1022
34. Al-Ali HK, Jaekel N, Junghanss C, Maschmeyer G, Krahl R, Cross M, Hoppe G, Niederwieser D (2012) Azacitidine in patients with acute myeloid leukemia medically unfit for or resistant to chemotherapy: a multicenter phase I/II study. Leuk Lymphoma 53:110–117
35. Blum W, Garzon R, Klisovic RB, Schwind S, Walker A, Geyer S, Liu S, Havelange V, Becker H, Schaaf L, Mickle J, Devine H, Kefauver C, Devine SM, Chan KK, Heerema NA, Bloomfield CD, Grever MR, Byrd JC, Villalona-Calero M, Croce CM, Marcucci G (2010) Clinical response and miR-29b predictive significance in older AML patients treated with a 10-day schedule of decitabine. Proc Natl Acad Sci USA 107:7473–7478
36. Lyons RM, Cosgriff TM, Modi SS, Gersh RH, Hainsworth JD, Cohn AL, McIntyre HJ, Fernando IJ, Backstrom JT, Beach CL (2009) Hematologic response to three alternative dosing schedules of azacitidine in patients with myelodysplastic syndromes. J Clin Oncol 27:1850–1856

37. Prebet T, Sun Z, Ketterling RP, Hicks G, Beach CL, Greenberg PL, Paietta EM, Czader M, Gabrilove J, Erba H, Tallman MS, Gore SD (2010) A 10 day schedule of azacitidine induces more complete cytogenetic remissions than the standard schedule in myelodysplasia and acute myeloid leukemia with myelodysplasia-related changes: results of the E1905 US Leukemia Intergroup Study. Blood 116(21):Abst. 4013
38. Marcucci G, Silverman L, Eller M, Lintz L, Beach CL (2005) Bioavailability of azacitidine subcutaneous versus intravenous in patients with the myelodysplastic syndromes. J Clin Pharmacol 45:597–602
39. Martin MG, Walgren RA, Procknow E, Uy GL, Stockerl-Goldstein K, Cashen AF, Westervelt P, Abboud CN, Kreisel F, Augustin K, Dipersio JF, Vij R (2009) A phase II study of 5-day intravenous azacitidine in patients with myelodysplastic syndromes. Am J Hematol 84:560–564
40. Uchida T, Ogawa Y, Kobayashi Y, Ishikawa T, Ohashi H, Hata T, Usui N, Taniwaki M, Ohnishi K, Akiyama H, Ozawa K, Ohyashiki K, Okamoto S, Tomita A, Nakao S, Tobinai K, Ogura M, Ando K, Hotta T (2011) Phase I and II study of azacitidine in Japanese patients with myelodysplastic syndromes. Cancer Sci 102:1680–1686
41. Kaminskas E, Farrell AT, Wang YC, Sridhara R, Pazdur R (2005) FDA drug approval summary: azacitidine (5-azacytidine, Vidaza) for injectable suspension. Oncologist 10:176–182
42. Ziemba A, Hayes E, Freeman BB III, Ye T, Pizzorno G (2011) Development of an oral form of azacytidine: 2'3'5'triacetyl-5-azacytidine. Chemother Res Pract 2011:965826
43. Garcia-Manero G, Stoltz ML, Ward MR, Kantarjian H, Sharma S (2008) A pilot pharmacokinetic study of oral azacitidine. Leukemia 22:1680–1684
44. Garcia-Manero G, Gore SD, Cogle C, Ward R, Shi T, Macbeth KJ, Laille E, Giordano H, Sakoian S, Jabbour E, Kantarjian H, Skikne B (2011) Phase I study of oral azacitidine in myelodysplastic syndromes, chronic myelomonocytic leukemia, and acute myeloid leukemia. J Clin Oncol 29:2521–2527
45. Hollenbach PW, Nguyen AN, Brady H, Williams M, Ning Y, Richard N, Krushel L, Aukerman SL, Heise C, MacBeth KJ (2010) A comparison of azacitidine and decitabine activities in acute myeloid leukemia cell lines. PLoS One 5:e9001
46. Pinto A, Attadia V, Fusco A, Ferrara F, Spada OA, Di Fiore PP (1984) 5-Aza-2′-deoxycytidine induces terminal differentiation of leukemic blasts from patients with acute myeloid leukemias. Blood 64:922–929
47. Petti MC, Mandelli F, Zagonel V, De Gregoris C, Merola MC, Latagliata R, Gattei V, Fazi P, Monfardini S, Pinto A (1993) Pilot study of 5-aza-2′-deoxycytidine (Decitabine) in the treatment of poor prognosis acute myelogenous leukemia patients: preliminary results. Leukemia 7(Suppl 1):36–41
48. Zagonel V, Lo Re G, Marotta G, Babare R, Sardeo G, Gattei V, De Angelis V, Monfardini S, Pinto A (1993) 5-Aza-2′-deoxycytidine (Decitabine) induces trilineage response in unfavourable myelodysplastic syndromes. Leukemia 7(Suppl 1):30–35
49. Willemze R, Archimbaud E, Muus P (1993) Preliminary results with 5-aza-2′-deoxycytidine (DAC)-containing chemotherapy in patients with relapsed or refractory acute leukemia. The EORTC Leukemia Cooperative Group. Leukemia 7(Suppl 1):49–50
50. Kantarjian HM, O'Brien SM, Estey E, Giralt S, Beran M, Rios MB, Keating M, de Vos D, Talpaz M (1997) Decitabine studies in chronic and acute myelogenous leukemia. Leukemia 11(Suppl 1):S35–S36
51. Wijermans PW, Krulder JW, Huijgens PC, Neve P (1997) Continuous infusion of low-dose 5-Aza-2′-deoxycytidine in elderly patients with high-risk myelodysplastic syndrome. Leukemia 11(Suppl 1):S19–S23
52. Wijermans P, Lubbert M, Verhoef G, Bosly A, Ravoet C, Andre M, Ferrant A (2000) Low-dose 5-aza-2′-deoxycytidine, a DNA hypomethylating agent, for the treatment of high-risk myelodysplastic syndrome: a multicenter phase II study in elderly patients. J Clin Oncol 18:956–962
53. Lubbert M, Wijermans P, Kunzmann R, Verhoef G, Bosly A, Ravoet C, Andre M, Ferrant A (2001) Cytogenetic responses in high-risk myelodysplastic syndrome following low-dose

treatment with the DNA methylation inhibitor 5-aza-2′-deoxycytidine. Br J Haematol 114:349–357
54. Issa JP, Garcia-Manero G, Giles FJ, Mannari R, Thomas D, Faderl S, Bayar E, Lyons J, Rosenfeld CS, Cortes J, Kantarjian HM (2004) Phase 1 study of low-dose prolonged exposure schedules of the hypomethylating agent 5-aza-2′-deoxycytidine (decitabine) in hematopoietic malignancies. Blood 103:1635–1640
55. Kantarjian H, Oki Y, Garcia-Manero G, Huang X, O'Brien S, Cortes J, Faderl S, Bueso-Ramos C, Ravandi F, Estrov Z, Ferrajoli A, Wierda W, Shan J, Davis J, Giles F, Saba HI, Issa JP (2007) Results of a randomized study of 3 schedules of low-dose decitabine in higher-risk myelodysplastic syndrome and chronic myelomonocytic leukemia. Blood 109:52–57
56. Steensma DP, Baer MR, Slack JL, Buckstein R, Godley LA, Garcia-Manero G, Albitar M, Larsen JS, Arora S, Cullen MT, Kantarjian H (2009) Multicenter study of decitabine administered daily for 5 days every 4 weeks to adults with myelodysplastic syndromes: the alternative dosing for outpatient treatment (ADOPT) trial. J Clin Oncol 27:3842–3848
57. Blum W, Klisovic RB, Hackanson B, Liu Z, Liu S, Devine H, Vukosavljevic T, Huynh L, Lozanski G, Kefauver C, Plass C, Devine SM, Heerema NA, Murgo A, Chan KK, Grever MR, Byrd JC, Marcucci G (2007) Phase I study of decitabine alone or in combination with valproic acid in acute myeloid leukemia. J Clin Oncol 25:3884–3891
58. Braun T, Itzykson R, Renneville A, de Renzis B, Dreyfus F, Laribi K, Bouabdallah K, Vey N, Toma A, Recher C, Royer B, Joly B, Vekhoff A, Lafon I, Sanhes L, Meurice G, Orear C, Preudhomme C, Gardin C, Ades L, Fontenay M, Fenaux P, Droin N, Solary E (2011) Groupe Francophone des Myelodysplasies: Molecular predictors of response to decitabine in advanced chronic myelomonocytic leukemia: a phase 2 trial. Blood 118:3824–3831
59. Wijermans PW, Ruter B, Baer MR, Slack JL, Saba HI, Lubbert M (2008) Efficacy of decitabine in the treatment of patients with chronic myelomonocytic leukemia (CMML). Leuk Res 32:587–591
60. Costa R, Abdulhaq H, Haq B, Shadduck RK, Latsko J, Zenati M, Atem FD, Rossetti JM, Sahovic EA, Lister J (2011) Activity of azacitidine in chronic myelomonocytic leukemia. Cancer 117:2690–2696
61. Prebet T, Gore SD, Esterni B, Gardin C, Itzykson R, Thepot S, Dreyfus F, Rauzy OB, Recher C, Ades L, Quesnel B, Beach CL, Fenaux P, Vey N (2011) Outcome of high-risk myelodysplastic syndrome after azacitidine treatment failure. J Clin Oncol 29:3322–3327
62. Jabbour E, Garcia-Manero G, Batty N, Shan J, O'Brien S, Cortes J, Ravandi F, Issa JP, Kantarjian H (2010) Outcome of patients with myelodysplastic syndrome after failure of decitabine therapy. Cancer 116:3830–3834
63. Talbert PB, Henikoff S (2010) Histone variants–ancient wrap artists of the epigenome. Nat Rev Mol Cell Biol 11:264–275
64. Lin RJ, Nagy L, Inoue S, Shao W, Miller WH Jr (1998) Evans RM: Role of the histone deacetylase complex in acute promyelocytic leukaemia. Nature 391:811–814
65. Altucci L, Gronemeyer H (2001) The promise of retinoids to fight against cancer. Nat Rev Cancer 1:181–193
66. Gelmetti V, Zhang J, Fanelli M, Minucci S, Pelicci PG, Lazar MA (1998) Aberrant recruitment of the nuclear receptor corepressor-histone deacetylase complex by the acute myeloid leukemia fusion partner ETO. Mol Cell Biol 18:7185–7191
67. Richon VM, Emiliani S, Verdin E, Webb Y, Breslow R, Rifkind RA, Marks PA (1998) A class of hybrid polar inducers of transformed cell differentiation inhibits histone deacetylases. 95:3003–3007
68. Quintas-Cardama A, Santos FP, Garcia-Manero G (2011) Histone deacetylase inhibitors for the treatment of myelodysplastic syndrome and acute myeloid leukemia. Leukemia 25:226–235
69. Cameron EE, Bachman KE, Myohanen S, Herman JG, Baylin SB (1999) Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. Nat Genet 21:103–107

70. Gore SD, Baylin S, Sugar E, Carraway H, Miller CB, Carducci M, Grever M, Galm O, Dauses T, Karp JE, Rudek MA, Zhao M, Smith BD, Manning J, Jiemjit A, Dover G, Mays A, Zwiebel J, Murgo A, Weng LJ, Herman JG (2006) Combined DNA methyltransferase and histone deacetylase inhibition in the treatment of myeloid neoplasms. Cancer Res 66:6361–6369
71. Fandy TE, Herman JG, Kerns P, Jiemjit A, Sugar EA, Choi SH, Yang AS, Aucott T, Dauses T, Odchimar-Reissig R, Licht J, McConnell MJ, Nasrallah C, Kim MK, Zhang W, Sun Y, Murgo A, Espinoza-Delgado I, Oteiza K, Owoeye I, Silverman LR, Gore SD, Carraway HE (2009) Early epigenetic changes and DNA damage do not predict clinical response in an overlapping schedule of 5-azacytidine and entinostat in patients with myeloid malignancies. Blood 114:2764–2773
72. Maslak P, Chanel S, Camacho LH, Soignet S, Pandolfi PP, Guernah I, Warrell R, Nimer S (2006) Pilot study of combination transcriptional modulation therapy with sodium phenylbutyrate and 5-azacytidine in patients with acute myeloid leukemia or myelodysplastic syndrome. Leukemia 20:212–217
73. Garcia-Manero G, Kantarjian HM, Sanchez-Gonzalez B, Yang H, Rosner G, Verstovsek S, Rytting M, Wierda WG, Ravandi F, Koller C, Xiao L, Faderl S, Estrov Z, Cortes J, O'brien S, Estey E, Bueso-Ramos C, Fiorentino J, Jabbour E, Issa JP (2006) Phase 1/2 study of the combination of 5-aza-2′-deoxycytidine with valproic acid in patients with leukemia. Blood 108:3271–3279
74. Silverman LR, Verma A, Odchimar-Reissig R, LeBlanc A, Nejfeld V, Gabrilove JL (2008) A phase I trial of the epigenetic modulators vorinostat, in combination with azacitidine (azaC) in patients with the myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML): a study of the New York Cancer Consortium. Blood 112:3656
75. Garcia-Manero G, Estey E, Jabbour E, Kadia TM, Estrov Z, Cortes J (2010) Phase II study of 5-azacitidine and vorinostat in patients (pts) with newly diagnosed myelodysplastic syndrome (MDS) or acute myelogenous leukemia (AML) not eligible for clinicalt trials because poor performance of presence of other comorbidities. Blood 116:Abstr. 604
76. Garcia-Manero G, Yang AS, Giles F, Faderl S, Ravandi F, Cortes J, Newsome WJ, Issa JP, Patterson TA, Dubay M, Li Z, Kantarjian H, Martell RE (2007) Phase I/II study of MGCD0103, an oral isotype-selective histone deacetylase (HDAC) inhibitor, in combination with 5-Azacitidine in higher-risk myelodysplastic syndrome (MDS) and acute myelogenous leukemia (AML). Blood 110
77. Grant S (2009) New agents for AML and MDS. Best Pract Res Clin Haematol 22:501–507
78. Carraway HE, Sidney Kimmel Comprehensive Cancer Center (2000–2012, Feb 11) A trial to evaluate two schedules of MS275 in combination with 5AC in elderly patients with acute myeloid leukemia (AML). In: ClinicalTrials.gov [Internet]. Bethesda (MD): National Library of Medicine (US). Available from: http://www.clinicaltrials.gov/ct2/show/NCT01305499:NCT01305499
79. Scandura JM, Roboz GJ, Moh M, Morawa E, Brenet F, Bose JR, Villegas L, Gergis US, Mayer SA, Ippoliti CM, Curcio TJ, Ritchie EK, Feldman EJ (2011) Phase 1 study of epigenetic priming with decitabine prior to standard induction chemotherapy for patients with AML. Blood 118:1472–1480
80. De Padua SL, de Lima M, Kantarjian H, Faderl S, Kebriaei P, Giralt S, Davisson J, Garcia-Manero G, Champlin R, Issa JP, Ravandi F (2009) Feasibility of allo-SCT after hypomethylating therapy with decitabine for myelodysplastic syndrome. Bone Marrow Transplant 43:839–843
81. Lubbert M, Bertz H, Ruter B, Marks R, Claus R, Wasch R, Finke J (2009) Non-intensive treatment with low-dose 5-aza-2′-deoxycytidine (DAC) prior to allogeneic blood SCT of older MDS/AML patients. Bone Marrow Transplant 44:585–588
82. Field T, Perkins J, Huang Y, Kharfan-Dabaja MA, Alsina M, Ayala E, Fernandez HF, Janssen W, Lancet J, Perez L, Sullivan D, List A, Anasetti C (2010) 5-Azacitidine for myelodysplasia before allogeneic hematopoietic cell transplantation. Bone Marrow Transplant 45:255–260

83. Gerds AT, Gooley TA, Estey EH, Appelbaum FR, Deeg HJ, Scott BL (2012) Pre-transplant therapy with azacitidine vs induction chemotherapy and posttransplant outcome in patients with MDS. Biol Blood Marrow Transplant; in press. [Epub ahead of print]
84. Lubbert M, Bertz H, Wasch R, Marks R, Ruter B, Claus R, Finke J (2010) Efficacy of a 3-day, low-dose treatment with 5-azacytidine followed by donor lymphocyte infusions in older patients with acute myeloid leukemia or chronic myelomonocytic leukemia relapsed after allografting. Bone Marrow Transplant 45:627–632
85. Czibere A, Bruns I, Kroger N, Platzbecker U, Lind J, Zohren F, Fenk R, Germing U, Schroder T, Graf T, Haas R, Kobbe G (2010) 5-Azacytidine for the treatment of patients with acute myeloid leukemia or myelodysplastic syndrome who relapse after allo-SCT: a retrospective analysis. Bone Marrow Transplant 45:872–876
86. Bolanos-Meade J, Smith BD, Gore SD, McDevitt MA, Luznik L, Fuchs EJ, Jones RJ (2011) 5-Azacytidine as Salvage Treatment in Relapsed Myeloid Tumors After Allogeneic Bone Marrow Transplantation. Biol Blood Marrow Transplant 17:754–758
87. Loh Y (2000) Singapore General Hospital: Study of decitabine induction prior to allogeneic hematopoietic cell transplant in newly diagnosed MDS patients [cited 2012, Feb 11]. In: ClinicalTrials.gov [Internet]. Bethesda (MD): National Library of Medicine (US). http://www.clinicaltrials.gov/ct2/show/NCT01333449:NCT01333449
88. Shen L, Kantarjian H, Guo Y, Lin E, Shan J, Huang X, Berry D, Ahmed S, Zhu W, Pierce S, Kondo Y, Oki Y, Jelinek J, Saba H, Estey E, Issa JP (2010) DNA methylation predicts survival and response to therapy in patients with myelodysplastic syndromes. J Clin Oncol 28:605–613
89. Karpf AR, Lasek AW, Ririe TO, Hanks AN, Grossman D, Jones DA (2004) Limited gene activation in tumor and normal epithelial cells treated with the DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine. Mol Pharmacol 65:18–27
90. Link PA, Baer MR, James SR, Jones DA, Karpf AR (2008) p53-inducible ribonucleotide reductase (p53R2/RRM2B) is a DNA hypomethylation-independent decitabine gene target that correlates with clinical response in myelodysplastic syndrome/acute myelogenous leukemia. Cancer Res 68:9358–9366
91. Langemeijer SM, Kuiper RP, Berends M, Knops R, Aslanyan MG, Massop M, Stevens-Linders E, van Hoogen P, van Kessel AG, Raymakers RA, Kamping EJ, Verhoef GE, Verburgh E, Hagemeijer A, Vandenberghe P, de Witte T, van der Reijden BA, Jansen JH (2009) Acquired mutations in TET2 are common in myelodysplastic syndromes. Nat Genet 41:838–842
92. Kosmider O, Gelsi-Boyer V, Cheok M, Grabar S, Della-Valle V, Picard F, Viguié F, Quesnel B, Beyne-Rauzy O, Solary E, Vey N, Hunault-Berger M, Fenaux P, Mansat-De Mas V, Delabesse E, Guardiola P, Lacombe C, Vainchenker W, Preudhomme C, Dreyfus F, Bernard OA, Birnbaum D, Fontenay M, Groupe Francophone des Myélodysplasies (2009) TET2 mutation is an independent favorable prognostic factor in myelodysplastic syndromes (MDSs). Blood 114:3285–3291
93. Itzykson R, Kosmider O, Cluzeau T, Mansat-De Mas V, Dreyfus F, Beyne-Rauzy O, Quesnel B, Vey N, Gelsi-Boyer V, Raynaud S, Preudhomme C, Adès L, Fenaux P, Fontenay M, Groupe Francophone des Myelodysplasies (GFM) (2011) Impact of TET2 mutations on response rate to azacitidine in myelodysplastic syndromes and low blast count acute myeloid leukemias. Leukemia 25:1147–1152
94. Walter MJ, Ding L, Shen D, Shao J, Grillot M, McLellan M, Fulton R, Schmidt H, Kalicki-Veizer J, O'Laughlin M, Kandoth C, Baty J, Westervelt P, DiPersio JF, Mardis ER, Wilson RK, Ley TJ, Graubert TA (2011) Recurrent DNMT3A mutations in patients with myelodysplastic syndromes. Leukemia 25:1153–1158
95. Ley TJ, Ding L, Walter MJ, McLellan MD, Lamprecht T, Larson DE, Kandoth C, Payton JE, Baty J, Welch J, Harris CC, Lichti CF, Townsend RR, Fulton RS, Dooling DJ, Koboldt DC, Schmidt H, Zhang Q, Osborne JR, Lin L, O'Laughlin M, McMichael JF, Delehaunty KD, McGrath SD, Fulton LA, Magrini VJ, Vickery TL, Hundal J, Cook LL, Conyers JJ, Swift GW, Reed JP, Alldredge PA, Wylie T, Walker J, Kalicki J, Watson MA, Heath S, Shannon

WD, Varghese N, Nagarajan R, Westervelt P, Tomasson MH, Link DC, Graubert TA, DiPersio JF, Mardis ER, Wilson RK (2010) DNMT3A mutations in acute myeloid leukemia. N Engl J Med 363:2424–2433

96. Thol F, Damm F, Lüdeking A, Winschel C, Wagner K, Morgan M, Yun H, Göhring G, Schlegelberger B, Hoelzer D, Lübbert M, Kanz L, Fiedler W, Kirchner H, Heil G, Krauter J, Ganser A, Heuser M (2011) Incidence and prognostic influence of DNMT3A mutations in acute myeloid leukemia. J Clin Oncol 29:2889–2896

Chapter 14
Epigenetic Targeting Therapies to Overcome Chemotherapy Resistance

Curt Balch and Kenneth P. Nephew

Abstract It is now well established that epigenetic aberrations occur early in malignant transformation, raising the possibility of identifying chemopreventive compounds or reliable diagnostic screening using epigenetic biomarkers. Combinatorial therapies effective for the reexpression of tumor suppressors, facilitating resensitization to conventional chemotherapies, hold great promise for the future therapy of cancer. This approach may also perturb cancer stem cells and thus represent an effective means for managing a number of solid tumors. We believe that in the near future, anticancer drug regimens will routinely include epigenetic therapies, possibly in conjunction with inhibitors of "stemness" signal pathways, to effectively reduce the devastating occurrence of cancer chemotherapy resistance.

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Chemo-, radio-, and hormonal therapies have proved invaluable for the management of numerous solid and hematologic cancers. Commonly used chemotherapies include topoisomerase inhibitors, microtubule-targeting agents (for mitotic spindle disruption), and DNA-alkylating agents, while hormonal therapies include antiestrogens (such as tamoxifen) and androgen-ablating drugs [1]. Despite the success of these agents (often early during patient therapy), the majority of patients eventually develop resistance to these interventions, and it is believed that >90% of all cancer deaths result from therapy-refractory, metastatic disease [2, 3]. Resistance to therapy is believed to be multifactorial, involving reduced delivery/uptake, membrane efflux, metabolic inactivation, loss of the therapeutic target, and autocrine/paracrine signaling (involving the local tumor microenvironment). Attenuation of cancer cell death pathways, due to hyperactive growth/survival pathways and/or suppression of cell cycle arrest/apoptosis cascades, is considered a major contributor to the loss of therapeutic sensitivity in cancer [4, 5].

While tumor progression is clearly associated with DNA sequence anomalies (e.g., point mutations, DNA gains or losses within specific loci, and/or translocations), *epigenetic* aberrations are now believed to play an equivalent (or even greater) role [6–8]. Epigenetics is classically defined as the study of heritable changes in gene expression that occur without a change in the DNA sequence. Epigenetic modifications include methylation of C5 of cytosines within CG dyads, numerous posttranslational modifications of histone residues, repositioning of whole (histone octomer) nucleosomes, deposition of histone protein variants, and posttranscriptional regulation of protein translation by microRNAs [8–10].

As noted above, cancer progression is characterized by genetic and epigenetic misregulation of signal transduction cascades (often in association with altered microRNA expression) [11, 12], and it has been hypothesized that the cancer cell phenotype resembles a reversion of adult tissue cells to an embryonic-like state (i.e., loss of differentiation), with immortalization replacing age-related apoptosis and senescence [13, 14]. Analogously, one recent, increasingly accepted carcinogenesis paradigm is that a mature, heterogeneous tumor represents a "caricature" of the normal organ from which it derives, due to the abnormal differentiation of "cancer stem cells" (CSCs) [15]. Normal tissue stem cells are relatively long-lived, due to quiescence or relatively slow cell division and expression of various phenotypes that confer resistance to genotoxic or cytotoxic agents, including enhanced DNA repair, metabolic inactivation and/or expulsion of cytotoxins, oxidative stress protection, and enhanced pro-survival (i.e., antiapoptotic) signaling [16]. While not necessarily derived from normal stem cells [16], CSCs have been shown to possess numerous "stemness" phenotypes, including the aforementioned defense mechanisms against environmental insults, thus facilitating resistance to most conventional anticancer agents [15, 16]. In addition to studies of hematologic malignancies, chemoresistant stem-like cells have now been identified and characterized in several solid tumors, including hepatocellular, colon, breast, glioma, pancreatic, and ovarian cancers [16].

To reverse the multi-/pluripotent phenotypes of progenitor tumor cells, numerous well-known differentiation agents are under investigation as potential cancer therapeutics, including vitamin D, retinoids, arsenic trioxide, and phytochemicals

[18, 19]. It is feasible that successful delivery of differentiating agents to CSCs might reduce malignant stem cell populations and improve conventional therapy responses, in addition to hampering tumor regrowth [8]. Similar to normal development, which is governed by epigenetic modifications that allow tissue-specific gene expression [20], abnormal differentiation states of tumor subpopulations are also largely regulated by atypical epigenetic modifications to DNA/chromatin [21]. The existence of "epigenetic plasticity" (associated with extensive chromatin remodeling) [22] was further exemplified by the recent generation of "induced pluripotent," embryonic stem-like cells from terminally differentiated, adult tissue cells [23, 24]. By contrast, it was also demonstrated that even highly aggressive cancer cells (including melanoma and estrogen receptor-negative breast cancer cells) possess a highly "plastic" phenotype capable of reversion to their respective differentiated, normal tissue phenotypes [25, 26].

In this chapter, we discuss agents capable of reversing cancer-associated, repressive epigenetic modifications. The emphasis of this article is on the possible restoration of drug response pathways/targets that could potentially reverse chemoresistance, a destructive and usually fatal complication of numerous malignancies.

14.1 Preclinical Studies of DNA Hypomethylating and Deacetylase-Inhibiting Agents for Overcoming Drug Resistance

As noted above, cancer is often characterized by a loss of differentiated and tissue-specialized phenotypes, which are maintained by epigenetic modifications that drive lineage- and organ-specific development. Over the past 50 years, the L-1210 acute lymphoblastic and Friend erythroleukemia mouse models have been widely used to screen antileukemic compounds, several of which were found to possess differentiating activity [27, 28]. Several of those differentiating agents were later discovered to be inhibitors of repressive epigenetic modifications and more specifically, histone deacetylase and DNA methyltransferase inhibitors (HDACIs and DNMTIs, respectively) [8, 29, 30].

Preclinical cancer studies of DNA methyltransferase inhibitors (DNMTIs). The two best-characterized DNA methyltransferase inhibitors (DNMTIs) are 5-azacytidine (5-aza-C, Vidaza) and its deoxyribose analog, 5-aza-2′-deoxycytidine (5-aza-dC, decitabine), with both compounds possessing the non-methylatable pyrimidine analog azacytosine [31]. Both DNMTIs, first synthesized and shown as antileukemic in the 1960s (Fig. 14.1), are now FDA-approved for therapy of the hematologic malignancy myelodysplastic syndrome (MDS) [10] (see next section). Following cellular uptake, these cytidine analogs are triphosphorylated and incorporated into the newly synthesized DNA strand during S phase (5-aza-C is also integrated into RNA) [32]. However, a C5-to-N5 substitution in the cytosine six-member heterocyclic ring precludes methyl group acceptance, resulting in covalent and irreversible binding of the DNMT enzyme to the fraudulent base, followed by the eventual cel-

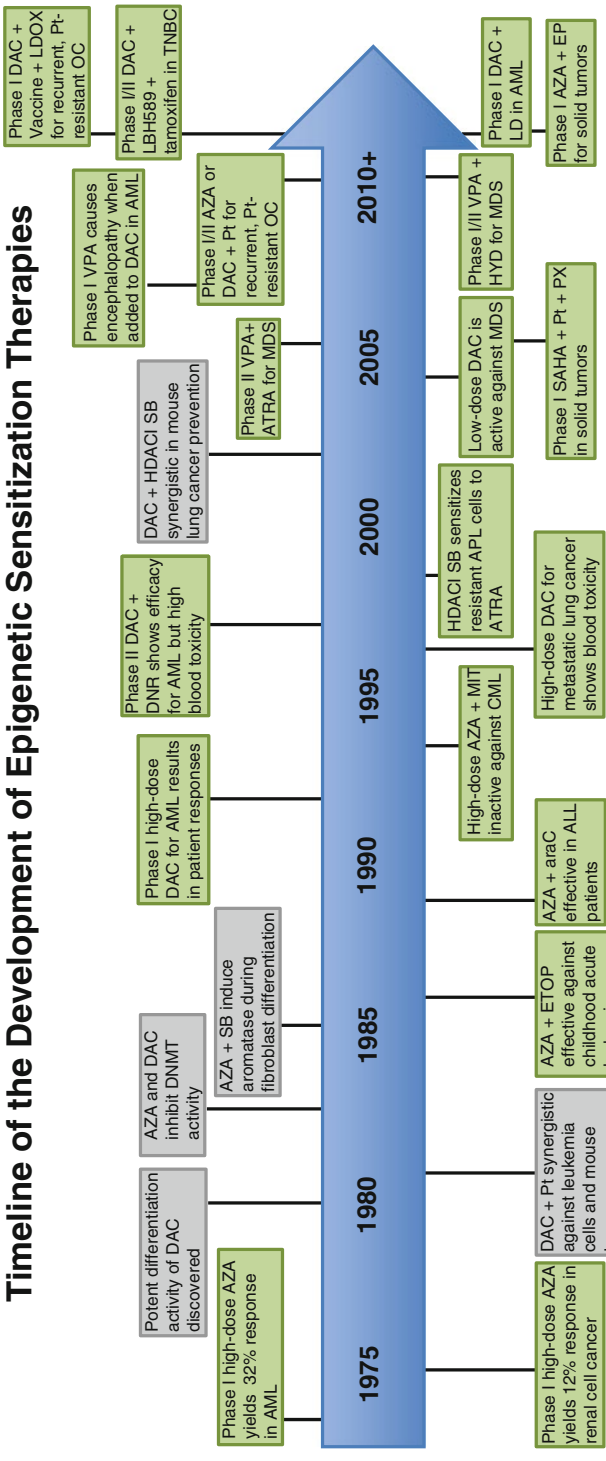

Fig. 14.1 Timeline of notable events supporting epigenetic sensitization to conventional cancer therapies. *Green boxes* indicate clinical studies, and *gray boxes* denote preclinical studies. The timeline chronology begins approximately 10 years after the discovery of DNA methyltransferase inhibitors. The cited studies are discussed in the text. See references [17, 29, 42, 43, 169, 171, 185, 211–222] for details. *AZA* 5-azacytidine or VIDAZA; *DAC* 5-aza-2′-deoxycytidine or decitabine/dacogen; *ALL* acute lymphoblastic leukemia; *AML* acute myeloid leukemia; *ara-C* arabinosylcytosine (cytarabine); *ATRA* all-trans retinoic acid; *CML* chronic myelogenous leukemia; *DNR* daunorubicin; *EP* erlotinib, Tarceva; *ETOP* etoposide; *FDA* Food and Drug Administration; *HDAC* histone deacetylase; *HDACI* histone deacetylase inhibitor; *HYD* hydralazine; *LD* lenalidomide, Revlimid; *LDOX* liposomal doxorubicin; *MDS* myelodyspastic syndrome; *MIT* mitoxatrone; *OC* epithelial ovarian cancer or primary peritoneal carcinoma; *PX* paclitaxel; *Pt* platinum; *SAHA* suberoylanilide hydroxamic acid, Vorinostat, Zolinza; *SB* sodium butyrate; *TNBC* triple negative breast cancer; *Vaccine* NY-ESO-1 protein immunization; *VPA* valproic acid (valproate)

lular depletion of DNMT, via ubiquitin-associated proteasome targeting [8, 10, 33]. Due to their requirement of nascent strand DNA incorporation, the hypomethylating activity of these cytosine analogs is replication-dependent, requiring several cell divisions to complete the demethylation of each DNA strand [34], consistent with successful patient trials typically requiring multiple treatment cycles prior to detectable response ([32, 35] and see following section).

Following their initial syntheses in 1964 [36], 5-aza-C and 5-aza-dC were later found to possess antileukemic activity in mouse disease models, elicit cancer cell differentiation, and enhance response to the chemotherapeutics etoposide and cisplatin [29, 37] (Fig. 14.1). These nucleoside analogs potently hypomethylate a number of tumor suppressor genes (TSGs), resulting in their transcriptional upregulation [6, 38, 39]. Decitabine-mediated DNA demethylation has also been reported to associate with reduced methylation at lysines 9 and 27 of histone H3 (H3K9 and H3K27, respectively), two other repressive chromatin "marks" [40, 41], in addition to enhanced acetylation at H3K9 and H3K14 (two activating chromatin marks). Such "crosstalk" between repressive chromatin modifications is believed to result from coordinated activity of histone and DNA methyltransferase enzymes associated with large, multimeric epigenetic repressive protein complexes.

Since its inception, the cytidine analog 5-aza-C has been extensively studied in cell and animal model systems. Early studies demonstrated potent antileukemic activity in the L1210 mouse model, followed by reports of 5-aza-C efficacy against solid tumors, using various preclinical cancer models (Fig. 14.1) [42, 43]. In medulloblastoma cells, 5-aza-C was also shown to inhibit proliferation, coincident with promoter demethylation and upregulation of a TSG, *KLF4* [44]. More recently, it was shown that intratracheal administration of 5-aza-C, in an orthotopic mouse lung cancer model, exhibited fivefold reduced myelosuppression and threefold enhanced survival, as compared to i.v. administration [45]. While subsequent studies further established 5-aza-C as a differentiating agent, particularly in effecting myogenesis [46–48], other work firmly established its ability to induce TSGs and initiate apoptosis in cancer cells, including those of the liver, colon, and ovary [49–51].

In contrast to 5-aza-C, its deoxyribose analog 5-aza-dC is not incorporated into RNA and is thus more stable and potent (active at submicromolar concentrations), although its activity is similarly attenuated by cytosine deaminases [8]. In a myriad of cell line studies, decitabine was shown to hypomethylate and derepress numerous TSGs, some of the most commonly studied being *p16*, *APC*, *RASSF1A*, *hMLH1*, *PTEN*, and *DAPK* [6, 38, 39]. Several of these (and other) genes encode protein constituents of apoptosis pathways, and thus (like aza-dC) in addition to differentiation, 5-aza-dC can robustly induce apoptosis [52, 53]. Preclinical studies have now firmly established 5-aza-dC activity against hematologic cancers, including acute myeloid leukemia (AML), chronic myeloid leukemia, acute lymphocytic leukemia, and MDS (Fig. 14.1) [54–57], and may also elicit senescence and autophagy [58]. Although clinical studies of 5-aza-dC have yet to demonstrate substantial activity against solid tumors (see below), preclinical studies have convincingly shown proof-of-principle for antitumor efficacy [59–62]. Moreover, in two studies, an indirect apoptotic role was found in that 5-aza-dC hypomethylated and upregulated

microRNA-181, a regulator of *NOTCH4* and *KRAS*, while in liver cancer cells, 5-aza-dC induced the tumor suppressor microRNAs 124 and 203 [63, 64]. As noted above, epigenetic alterations in cancer often hyperactivate specific oncogenic pathways; 5-aza-dC is now known to antagonize several of those pathways, while upregulating tumor suppressive signaling. Examples of oncogenic signal blockade by 5-aza-dC was demonstrated by its upregulation of the endogenous Wnt pathway inhibitor DKK, resulting in significant xenograft tumor growth inhibition [65].

In addition to 5-aza-dC and 5-aza-C, various other compounds have been shown to elicit DNA demethylation. As decitabine is subject to intracellular deamination and aqueous instability (resulting in loss of hypomethylating activity), a more stable dinucleotide, 5-aza-dC-dG (SGI-110, Astex Pharmaceuticals, Cambridge, UK), was shown to resist cytidine deaminase, while also demonstrating potent antigrowth effects against bladder cancer cells and mouse xenografts, with negligible toxicity [66, 67]. Likewise, an elaidic acid analog of 5-aza-C, CP-4200, possessed enhanced stability and much higher tumoricidal activity than the parent compound (aza-C), possibly due to its independence from nucleoside uptake transporters [68]. Using a different (genetic) approach, short inhibitory RNAs against DNMTs 1 and 3b elicited DNA demethylation and gene derepression similar to (or greater than) deoxycytosine analogs [69]. In addition to decitabine, we have also studied another cytidine analog DNMTI, zebularine, demonstrating that this agent hypomethylates TSGs and allows for the chemosensitization of platinum-resistant ovarian cancer cells lines [70]. Other zebularine studies have demonstrated its greater stability than 5-aza-dC, demethylation in tumors *in vivo*, and colon cancer chemoprevention in a widely used mouse model [71, 72]. Like 5-aza-C, however, zebularine is a ribonucleotide and thus its potency is limited by inefficient reduction prior to incorporation into DNA [73]. Toward rectifying that limitation, deoxyzebularine phosphoramidate prodrugs were recently demonstrated as more potent hypomethylating agents in vitro, while also exhibiting antineoplastic activity against pancreatic cancer cell lines [74].

Several non-nucleoside compounds have also demonstrated DNA-hypomethylating activity. These include two previously FDA-approved agents, the antihypertensive hydralazine and the antiarrhythmic procainamide [75]. However, these compounds were found much less potent than 5-aza-dC [75, 76]. A mushroom-derived antibiotic, Verticullin A, likewise displayed DNMTI activity against SW620 colon cancer cells, upregulating several genes concordant with demethylation of their respective promoters, while also resensitizing those cells to the apoptosis-inducing, "death receptor" ligand TRAIL [77]. More recently, various "rationally designed," non-nucleoside DNMT inhibitors (thus influencing enzyme activity without DNA incorporation) have also demonstrated potent downregulation of methyltransferase activity. Two of these, SGI-1027 and RG108, facilitated reexpression of silenced TSGs, while also negatively affecting growth of colon and hepatocellular carcinoma cells [78–81]. Likewise, various high-throughput screens, using various reporter assays and virtual "docking" computational approaches, are now in widespread use for the identification of non-nucleoside DNA methyltransferase [82, 83]. These approaches will almost certainly lead to the identification of novel DNA methylation inhibitors.

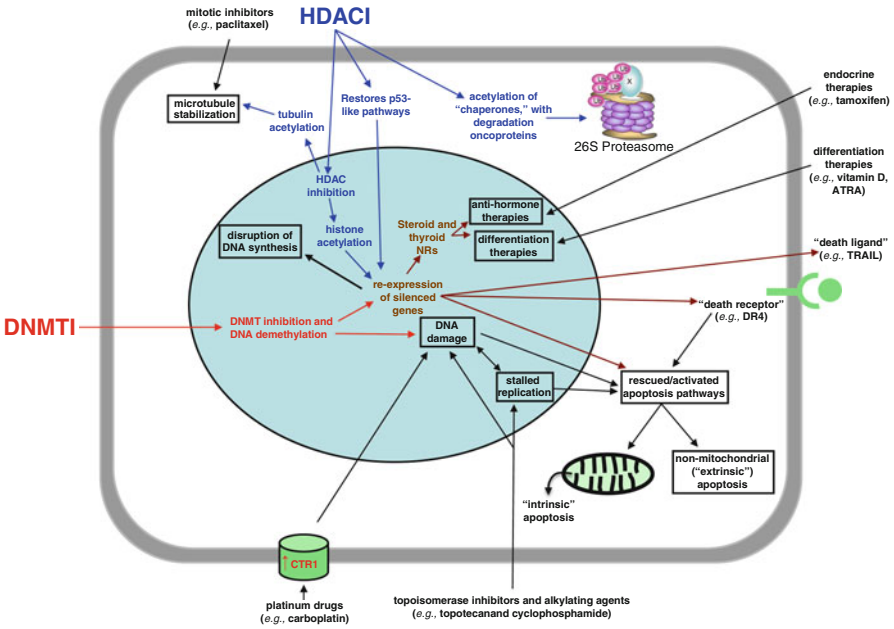

Fig. 14.2 Therapy response signals potentially affected by HDACIs and DNMTIs. Possible therapy-sensitization mechanisms by HDACIs and/or DNMTs. *Red* text denotes proteins/pathways impacted predominantly by DNMTIs, *blue* text indicates HDACI targets, and *brown* text designates possible alteration by either agent (and/or DNMTI/HDACI co-augmentation). *Black boxes* indicate pathway intersections where therapy sensitization may occur following DNMT/HDACI treatment. *ATRA* all-trans retinoic acid; *CTR1* copper transporter-1; *DNMTI* DNA methyltransferase inhibitor; *DR4* death receptor-4; *HDAC* histone deacetylase; *HDACI* histone deacetylase inhibitor; *TRAIL* TNF-related apoptosis-inducing ligand

While DNMTIs have shown success as monotherapies for hematologic cancers, accumulating evidence suggests they will be most effective when combined with conventional or targeted chemotherapies, likely via chemosensitization of resistant tumor subpopulations [8, 22, 84]. Such chemosensitization is hypothesized to result from DNMTI-mediated derepression of gene members of drug response pathways or inhibition of pro-survival pathways [8, 9, 85]. As shown in Fig. 14.2, multiple preclinical studies have now demonstrated that DNMTIs can resensitize resistant malignancies to numerous chemotherapeutics, via upregulation of pro-apoptosis pathways (both extrinsic and intrinsic), while also inhibiting oncogenic signaling cascades such as Wnt, PI3K/Akt, hedgehog, and Notch [65, 86–89]. In two early studies of the L1210 mouse leukemia models, cytoxicity of 5-aza-C was augmented by coadministration with another nucleoside analog, cytarabine; the hypothesized mechanism of action of this combination was inhibition of DNA synthesis [90]. Likewise, 5-aza-C antileukemic activity was also enhanced by a cytidine deaminase inhibitor [91]. More recently, in a study of aggressive prostate cancer, 5-aza-C caused potent but well-tolerated resensitization of tumor xenografts to docetaxel and cisplatin, concomitant with upregulation of a number of TSGs [92].

Like 5-aza-C, chemosensitization by 5-aza-dC is now well established. In one early study, 5-aza-dC combined with the topoisomerase-1 inhibitor topotecan, was synergistically cytotoxic to mouse colorectal adenocarcinomas [93]. Later, it was demonstrated that 5-aza-dC could resensitize platinum-resistant ovarian cancer cells and mouse xenografts to cisplatin, due to promoter demethylation and reexpression of the mismatch repair enzyme gene *hMLH1* [94, 95]. In two colon cancer studies, 5-aza-dC was found to be synergistically tumoricidal when combined with 5-fluorouracil (an antimetabolite) and the antineoplastic hormone irinotecan [96, 97]. Likewise, a study of colon cancer cells revealed that 5-aza-dC treatment resulted in upregulation of ten interferon pathway-associated genes, likely via induction of IFN-alpha2a and activation of STATs 1, 2, and 3 [98]. In endocrine cancers, DNMTIs have also been demonstrated to sensitize cancer cells to antihormonal therapies. For example, 5-aza-dC was shown to upregulate the DNA-methylation-repressed TSG *PTEN*, an inhibitor of the PI3K/Akt pathway, suppressing the growth of tamoxifen-resistant breast cancer cell xenografts and restoring responsiveness to antiestrogens [99]. The latter finding is further supported by a correlation of epigenetic aberrations and PI3K/Akt oncogenic signaling in breast cancer cells; those aberrations were reversible by a 5-aza-dC/PI3K inhibitor combination, which also cooperatively inhibited the growth of mouse xenografts [100]. Restoration of antiestrogen sensitivity in breast cancer is believed to be due (at least in part) to reexpression of the estrogen receptors alpha and/or beta [101, 102]. Similarly, in prostate cancer, androgen receptor silencing has been linked to both histone deacetylation and DNA methylation [103, 104]. In other prostate cancer studies, 5-aza-dC could sensitize both androgen-dependent and -independent prostate cancer cells to paclitaxel, while both DNMTIs and HDACIs cooperatively upregulated estrogen receptor-beta and delayed androgen independence in a common mouse model [105–107].

Preclinical cancer studies of histone deacetylase inhibitors (HDACIs). As histone deacetylation is another epigenetic modification repressive of TSGs, histone deacetylase inhibitors (HDACIs) also represent promising antineoplastics. Interestingly, the first HDAC inhibitor was the common organic solvent dimethylsulfoxide (DMSO), as discovered by Charlotte Friend to elicit differentiation of erythroleukemia cells [108]. Following that discovery, numerous other hybrid polar compounds were synthesized and similarly screened for differentiating activity, but whose mechanism of action (deacetylase inhibition, resulting in enhanced protein acetylation) remained unknown for over 20 years [109]. Numerous HDACIs, which antagonize the action of zinc-dependent histone deacetylases by chelation of the metal cation, have been shown to induce differentiation and apoptosis in tumor, but not normal, cells (reviewed in [110]). One proposed mechanism for cancer cell-specific HDACI toxicity is the induction of cell cycle checkpoints [111]; one such effect (G2 arrest followed by apoptosis) was also demonstrated in platinum-resistant ovarian cancer cells [112]. Of note, while HDACIs potently induce histone hyperacetylation, their effects on non-histone protein acetylation (including transcription factors, molecular chaperones, cargo transporters, and cytoskeletal proteins) may play an even greater role in their antineoplastic activity [110, 113]. In ovarian cancer in particular, several HDACIs induced cytodifferentiation and apop-

tosis of cultured cells and mouse xenografts [112, 114–117]. Newer studies suggest that HDACI repression of telomerase (*hTERT*) represents another anticancer mechanism of action (reviewed in [118]). Alternative non-epigenetic, HDACI antineoplastic effects include oncoprotein destabilization by acetylation of "chaperone" proteins (suggesting synergism with HSP inhibitors), diminished processing of "aggresomes" of misfolded proteins (suggesting synergism with proteasome inhibitors), acetylation of transcription factors, and reconstitution of p53-like tumor suppressive pathways (reviewed in [30, 110, 119], and see Fig. 14.2).

Similar to DNMTIs, preclinical studies have shown HDACIs to be most effective in combination with standard therapies, suggesting HDACI upregulation of drug response (apoptotic) or cellular differentiation pathways. In ovarian cancer preclinical studies, vorinostat alone was found effective against paclitaxel-resistant ovarian cancer cells; however, its antitumor activity was far greater in combination with paclitaxel [120–122]. Other HDACIs have similarly sensitized ovarian cancer cells to retinoids and the widely used chemotherapy cisplatin [115, 123, 124]. Similarly, our group demonstrated that a rationally designed HDACI, AR42, possessed greater cisplatin-resensitizing activity than vorinostat in chemoresistant ovarian cancer cells and mouse xenografts, enhancing both epithelial differentiation and apoptotic potential [125]. One specific example supporting HDACI-associated differentiation in therapy sensitization was that cholangiocarcinoma cells treated with the HDACI valproate upregulated numerous genes associated with differentiation, during sensitization to gemcitabine [126]. In similar studies, the HDACI Trichostatin A augmented UV-induced apoptosis over threefold in colon cancer RKO cells [127], and also sensitized osteosarcoma cells to a potentially antineoplastic, natural product geninstein [128].

Several HDACIs have also been demonstrated to upregulate "death receptor" apoptosis pathways, allowing resensitization of resistant cancer cells to death receptor ligands (Fig. 14.2). One report showed the HDACI MS-275 (entitostat) to resensitize aggressive MDA-MB-468 breast cancer cells to the death ligand TRAIL, both in cell culture and in mouse xenografts, while downregulating genes associated with the metastasis-related epithelial-to-mesenchymal transition [129]. In addition, HDACI-associated TRAIL sensitization (via reexpression of caspase-8) was markedly augmented by combination with interferon-gamma in meduloblastoma cells [130]. TRAIL sensitization by the HDACI valproate was also demonstrated in pancreatic cancer cell lines, via inhibition of HDAC2 and the restoration of extrinsic apoptosis pathways [131], while in hepatocellular carcinoma cell lines, the HDACIs valproic acid and ITF2357 both effected sensitization to TRAIL [132].

HDACIs have also shown activity against hormone-resistant neoplasms, including breast, uterine, and prostate cancers. Similar to DNMTI/antiestrogen studies HDACIs enhanced tamoxifen induction of both autophagy and apoptosis in tamoxifen-resistant breast cancer cells; that effect was further enhanced by inhibitors of autophagy [133]. In endometrial cancer studies, TSA/paclitaxel-combined treatment of mice bearing cancer cell tumor xenografts reduced tumor masses by >50% [134]. Moreover, another xenograft study showed that the HDACI apicidin reduced tumor size and repressed the angiogenesis-mediating oncoprotein VEGF [135]. Interestingly, it appears that in endometrial cancer, HDACIs may exert antigrowth

effects through repression of estrogen receptor-target genes, coincident with induction of genes targeted by the glucocorticoid receptor [136].

Preclinical studies of DNMTI/HDACI combinations. While HDACIs and DNMTIs have demonstrated clinical activity as single agent therapies for hematopoietic malignancies, DNA methylation and histone deacetylation also cooperatively inhibit gene transcription (often in multiple-repressor protein complexes), and relief of both silencing mechanisms may be necessary for maximal gene derepression [8, 137]. In ovarian cancer cells, a DNMTI/HDACI combination synergistically upregulated the pro-apoptotic gene *TMS1*/*ASC*, in contrast to either epigenetic agent alone [138], while a 5-aza-dC/vorinostat regimen induced various imprinted genes and also inhibited tumor xenograft growth [139]. Similarly, 5-aza-C combined with the HDACI Trichostatin A facilitated derepression of the progesterone receptor-B gene in endometrial cancer cells [140], while 5-aza-C plus entitostat cooperatively upregulated several pro-apoptosis genes and reduced tumor xenograft sizes by >75% in a mouse lung cancer model [141]. A newer preclinical study showed 5-aza-dC combined with the HDACI valproate was cancer-chemopreventive in a mouse medulloblastoma/rhabdosarcoma model, while each agent alone was not [142]. Interestingly, one compound, UVI5008, was found to be a "triple epigenetic inhibitor," concordantly inhibiting zinc-dependent HDACs, the DNA methyltransferase DNMT3A, and another family of HDACs that require a NAD^+ cofactor (rather than zinc), the sirtuins [143]. In that study, UV15008 potently induced apoptosis in breast cancer cells/xenografts via ROS production and activation of death receptor (i.e., extrinsic), mitochondria-independent, apoptosis [144].

It has also been reported that HDACIs and DNMTIs may actually mimic the epigenetic effects of one another. For example, it has been reported that several HDACIs can demethylate DNA, including Trichostatin A, valproate, and MS-275 (entitostat, SNDX-275) [145–148], possibly via transcriptional downregulation of DNMT-coding genes, as demonstrated in a study of human endometrial cancer cells [149]. Analogously, 5-aza-dC was also found to effect gene-specific, but not global, histone acetylation [150, 151]. However, a phase I study of AML or MDS patients examining 5-aza-C (5–14 days) followed by phenylbutyrate (5 days) demonstrated that 5-aza-C treatment alone resulted in histone acetylation in peripheral blood cells; phenylbutyrate, however, did not prevent remethylation of the cyclin-dependent kinase inhibitor gene *p15* (*CDKN2B*) [152]. Even so, these reciprocal epigenetic modifications, between HDACIs and DNMTIs, appear to be quite rare and context-dependent in nature.

While DNMTI/HDACI combinations often result in greater gene alterations than each agent in isolation, pairing of these epigenetic therapies will likely be even more effective in coordination with conventional cancer therapies [8–10]. For example, while caspase-8 gene reexpression in small cell lung cancer cells required a DNMTI/HDACI combination (thus restoring a functional apoptosis pathway), the induction of apoptosis still required the death receptor ligand TRAIL [153]. Similarly, combined treatment of decitabine and belinostat demonstrated significantly greater cisplatin sensitization of platinum-resistant ovarian cancer cell xenografts, in tumor-bearing mice, than either epigenetic therapy alone [154].

14.2 Clinical Studies of DNA Hypomethylating Agents and HDAC Inhibitors for Overcoming Drug Resistance

Four epigenetic derepressive agents are now FDA approved for two hematologic malignancies, MDS treatment with DNMTIs 5-aza-C (Vidaza) and 5-aza-dC (decitabine), and cutaneous T-cell lymphoma therapy using the HDACIs vorinostat and romidepsin [8, 9, 155]. While other hematologic malignancies will likely gain approval for monotherapy DNMTIs and HDACIs, including peripheral T-cell lymphoma and Hodgkin's disease, single-agent clinical studies of various solid tumors have proved fairly disappointing. For the latter, epigenetic drugs will likely prove most beneficial when combined with long-established approaches such as conventional cytotoxic chemotherapies, endocrine therapies, differentiation therapy, and radiotherapy [156, 157].

Studies of DNA methyltransferase inhibitors. In addition to incorporation into DNA, the ribonucleoside analog 5-aza-C is also incorporated into several RNA species, resulting in greater toxicity and lower stability than 5-aza-dC. Consequently, a more recent clinical studies have focused on 5-aza-dC (decitabine), although 5-aza-C remains widely used. An early Vidaza study of patients with acute leukemia, administered at 37–81 total mg/m^2, given over 30–60 h, resulted in some clinical benefit in 89% of patients, although substantial hematologic toxicity was observed in all patients [158]. A separate trial of 21 elderly patients with high-risk MDS, treatment with decitabine at 50 mg/m^2/day for three consecutive days, yielded a response rate of 54% (15 of 21), although significant myelotoxicity caused the death of 5/21 (17%) patients [159]. Another MDS phase I study, using an overall similar drug exposure (45 mg/m^2 b.i.d. for 3 days), yielded an overall response rate of 49%, but similarly resulted in moderate-to-severe toxicity (predominantly myelodepression), resulting in the death in 7% of the enrolled patients [160].

To possibly ameliorate the high toxicity and limited benefit of extended decitabine infusions (previously using regimens approaching its maximum tolerated dose), lower dose schedules were examined. In phase I/II sickle cell anemia studies of hydroxyurea-resistant patients, low-dose (0.3 mg/kg), repetitive doses (5 days/week for 2 weeks) of decitabine were found sufficient for demethylation and reexpression of fetal hemoglobin with little or no neutropenia [161, 162]. Such low-dose treatments were largely based on a mouse embryonic fibroblast study showing myotube differentiation and hypomethylation at low decitabine doses (1–5 μM), with cytotoxicity and increased methylation at higher (>5 μM) doses [29]. Subsequently, one MDS clinical trial examined a variety of repetitive low decitabine doses, with 1-h administration daily over longer durations (10–20 days) [163]. The results of that landmark study demonstrated that 15 mg/m^2 decitabine, administered over ten consecutive days, resulted in a response rate of 83% and was well tolerated, as compared to previous studies using >5-fold higher doses [163]. That pioneering work resulted in the widespread adoption of low-dose hypomethylating agents, both as monotherapies and in combination with other agents.

While single-agent decitabine demonstrated significant efficacy for MDS and other hematologic malignancies, solid tumor studies have been fairly disappointing, motivating studies of 5-aza-dC in combination with other conventional agents. Early combination studies, however, demonstrated minimal-to-moderate activity, with substantial toxicity. In a phase II study of non-small cell lung cancer, a maximum tolerated decitabine dose of 67 mg/m^2, given concurrently with 33 mg/m^2 cisplatin over a 2-h period for 3 consecutive days of a 21-day cycle, resulted in no objective responses and significant hematologic toxicity [164]. Similarly, a phase II trial of squamous cell cervical cancer, with decitabine administered continuously at 50 mg/m^2/day for 3 days, concurrent with 30 mg/m^2 cisplatin, resulted in eight partial and five stable disease responses; however, unacceptable toxicity was observed, resulting in one patient death [165]. However, based on the low-dose MDS efficacy study by Issa et al., newer trials have examined lower doses of decitabine in various combined regimens. One recent phase I/II combinatorial ovarian cancer study, of decitabine paired with carboplatin, demonstrated no significant improvement over carboplatin alone [166, 167]. By contrast, a separate phase IIa clinical trial of 5-aza-cytidine (Vidaza) and carboplatin resulted in one complete, three partial, and ten stable disease responses (of 29 total patients), with a 7.5-month average duration of response [168]. Likewise, our group recently completed a phase I trial of low-dose decitabine (five consecutive-day regimen), in combination with carboplatin in platinum-resistant ovarian cancer patients, revealing acceptable tolerability of the regimen [169]. Biological activity in vivo was also demonstrated, as assessed by hypomethylation of genome-wide repetitive elements (in peripheral blood cells) and specific ovarian cancer-associated genes (in plasma, ascites, or tumor) [169], resulting in one complete, six stable, and four (6-month) disease progression-free responses [169]. The successful phase II component of that study was recently described [170], and the results are promising. Other clinical studies combining 5-aza-dC with the EGFR antagonist erlotinib showed responses in 4 of 11 patients with advanced tumors [171]. However, a neuroblastoma trial of 5-aza-dC combined with cyclophosphamide or doxorubicin showed toxicity at the 5-aza-dC doses required for disease response [172]. In a 13-patient AML phase I study, decitabine combined with arsenic trioxide and/or ascorbic acid resulted in one complete remission and five patients with stable disease [173]. While chemosensitization by DNMTIs is believed to largely result from the restoration of apoptosis pathways, one recent phase II study of refractory solid tumors and lymphomas showed patient response to correlate with both DNA hypomethylation and expression of the copper transporter CTR1, a protein that facilitates platinum drug uptake [174] (Fig. 14.2).

Studies of histone deacetylase inhibitors. Like DNMTIs, despite successful studies of hematologic malignancies, solid tumor clinical trials of monotherapeutic HDACIs suggest similarly limited clinical activity. In ovarian cancer, two monotherapeutic phase I/II trials of the HDACIs vorinostat and belinostat proved tolerable but showed only moderate clinical activity [175, 176]. One recent phase II trial of the HDACI romidepsin in androgen-independent prostate cancer, although well tolerated, likewise showed minimal antineoplastic activity [177]. Another belinostat trial for metastatic renal cancer also yielded no patient responses [178]. Consequently,

it is now widely believed that these agents will be most effective in combination with conventional chemotherapies ([8, 9, 34] and see following sections).

For ovarian cancer, two recent ovarian cancer trials pairing belinostat with paclitaxel/carboplatin, and vorinostat with carboplatin, demonstrated safety and moderate clinical activity [179, 180], while planned clinical trials include HDACIs in combination with inhibitors of the DNA repair enzyme PARP or inhibitors of the embryonic signal mediator Hedgehog [181, 182]. Another phase II study of the HDACI vorinostat combined with the antiestrogen tamoxifen, in hormone-refractory breast cancer patients, yielded a clinical benefit rate (response or stable disease for over 24 weeks) of 40%, although toxicity necessitated dose adjustment in several patients [183]. Similarly, a 12-patient phase I trial combining the HDACI panobinostat with the angiogenesis inhibitor bevacizumab resulted in three partial responses and seven cases of stable disease [184].

Clinical studies of combined DNA methyltransferase and histone deacetylase inhibitors. DNMTI/HDACI combinations have also now been established to exert additive/synergistic effects on gene expression in vitro. However, success in clinical trials has been, similar to single-agent regimens, largely restricted to hematologic malignancies. For example, a phase II study of the DNMTI/HDACI combination of hydralazine and valproate for MDS showed an overall response rate of 50% [185]. Most solid tumor studies, however, have shown less efficacy. Nonetheless, one phase I clinical trial combining the HDACI valproic acid and the DNMTI azacytidine for various solid tumors demonstrated safety, in vivo biological activity, and stable disease in 25% of the enrolled patients, although no partial or complete responses were observed [186]. Likewise, a recent phase I study of 5-aza-dC/vorinostat combination resulted in 29% of non-Hodgkin's lymphoma and various solid tumors [187]. By analogy, a recent phase I/II trial of an azacytidine/entitostat combination in non-small cell lung cancer yielded major objective responses in 4 of 19 patients, and demethylation of a four-gene panel correlated with improved progression-free and overall survival [188].

Based on the above mentioned in vivo findings, it is speculated that chromatin depressive agents (singly or combined) alone may be only marginally efficient for eradicating cancer cells, thus motivating studies of their combination with conventional therapeutics [6, 8, 94]. For example, while apoptosis pathway function may be restored by epigenetic derepression, it is possible that epigenetic drugs remain inadequate as cancer cell stressors capable of provoking programmed cell death. In one phase III ovarian cancer trial (NCT00533299), the DNMTI hydralazine is being combined with the HDACI valproic acid, with or without the topoisomerase inhibitor topotecan, while a previous phase II trial of the same combination (hydralazine/valproate), coincident with four different chemotherapy regimens, yielded three partial and four stable disease responses (as assessed by the ovarian cancer marker CA-125) [189]. In various leukemias, a phase I trial of 5-aza-dC combined with valproic acid demonstrated acceptable patient tolerability and an objective response rate of 22% [190], while a melanoma trial combining 5-aza-dC and intravenous bolus interleukin-2 was well-tolerated and yielded a 31% objective response rate [191].

5-aza-C is also being examined in a phase I/II ovarian cancer trial (NCT00529022) in combination with valproic acid and carboplatin.

In addition to reactivation of TSGs (and possible chemotherapy response cascades), DNMTIs and HDACIs have also been found to induce various cancer/testis antigens (CTAs, components of the "tumor recognition complex") [34]. CTA proteins, expressed in male germ cells but normally silenced in adult tissues, are expressed in various malignancies as antigenic peptides copresented with HLA Class I/II molecules and thus may represent immunotherapy targets [192]. However, as CTA expression is often variable, due to epigenetic repression, more consistent reexpression can be achieved by DNMT and/or HDAC inhibitors [191]. Consequently, an ongoing phase I ovarian cancer trial (NCT00887796) is investigating decitabine combined with liposomal doxorubicin and peptide vaccination for the CTA NY-ESO-1, while two other trials (NCT00701298, NCT00886457, for unspecified cancers) are combining decitabine with interferon-α2b. These trials were based on the preclinical studies by Karpf et al. [98, 193] mentioned earlier. Thus, in addition to tumor suppressor reactivation, epigenetic therapies may also hold promise in immunotherapy.

14.3 Future Directions for the Use of Epigenetic Therapies for Overcoming Chemotherapy Resistance

One current focus within cancer epigenetic research is the design of specific inhibitors of enzymes facilitating other epigenetic repressive modifications, including the gene-repressive histone methyltransferases (HMTs) EZH2, which trimethylates histone H3, lysine 27 (H3K27me3), and DOT1L, which trimethylates H3K79 [194, 195]. Consistent with epigenetic gene repression in cancer, one DOT1L inhibitor, EPZ004777, showed activity against mixed lineage leukemia cells [196]. Similarly, one EZH2 inhibitor, DZNep, an *S*-adenosylmethionine (SAM) analog that also inhibits methylation of H4K20, resulting in upregulation of numerous previously silenced TSGs [194, 197]. DZNep has also shown anticancer activity against mouse prostate tumors and breast cancer, AML, and neuroblastoma cells [194, 197–199]. Similar to DNMTIs, DZNep induction of apoptosis was also augmented by HDACIs [200, 201], and recent studies of DZNep suggest possible negative effects toward CSCs [199, 202]. High-throughput approaches continue to identify various novel epigenetic therapies, including inhibitors of the Jarid family of H3K4me3 histone demethylases, the repressive HMT G9a (which trimethylates H3K9), isoform-specific HDACs, and various histone acetyltransferases [203–207]. In addition, tumor-suppressive microRNAs have been successfully delivered to tumors in mouse models of liver (miR-26a), colon (miRs-145 and -33a), and prostate cancers, using adeno-associated viruses, polyethylenimine conjugation, and rhabdomyosarcoma (miRs-1 and −206) [208–210]. Taken together it is likely that these emerging epigenetic therapeutics could be used for the much anticipated therapeutic approach of

"personalized medicine," based not only on patients' genomic/gene expression profiles, but also on their epigenetic profiles.

14.4 Summary/Conclusions

It is now well established that epigenetics is a principle mediator of mammalian development. To successfully carry out tissue/organ differentiation, genomic DNA expression is precisely regulated by a host of epigenetic modifications. It is thus not surprising that aberrant chromatin modifications result in defective differentiation states, a hallmark of cancer cells. It has also been recently shown that even highly aggressive cancer cells can revert to their original, tissue-specific differentiation state, and that epigenetic therapies may facilitate this phenomenon. Consequently, chromatin-altering agents hold promise for the treatment of numerous malignant diseases, particular when complemented with other (traditional or pathway-targeted) antineoplastic therapies.

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References

1. Gralow J, Ozols RF, Bajorin DF, Cheson BD, Sandler HM, Winer EP, Bonner J, Demetri GD, Curran W Jr, Ganz PA, Kramer BS, Kris MG, Markman M, Mayer RJ, Raghavan D, Ramsey S, Reaman GH, Sawaya R, Schuchter LM, Sweetenham JW, Vahdat LT, Davidson NE, Schilsky RL, Lichter AS (2008) Clinical cancer advances 2007: major research advances in cancer treatment, prevention, and screening—a report from the American Society of Clinical Oncology. J Clin Oncol 26(2):313–325
2. Jemal A, Siegel R, Xu J, Ward E (2010) Cancer statistics, 2010. CA Cancer J Clin 60(5):277–300
3. Longley DB, Johnston PG (2005) Molecular mechanisms of drug resistance. J Pathol 205(2):275–292
4. Raguz S, Yague E (2008) Resistance to chemotherapy: new treatments and novel insights into an old problem. Br J Cancer 99(3):387–391
5. Tredan O, Galmarini CM, Patel K, Tannock IF (2007) Drug resistance and the solid tumor microenvironment. J Natl Cancer Inst 99(19):1441–1454
6. Balch C, Huang TH, Brown R, Nephew KP (2004) The epigenetics of ovarian cancer drug resistance and resensitization. Am J Obstet Gynecol 191(5):1552–1572
7. Barton CA, Clark SJ, Hacker NF, O'Brien PM (2008) Epigenetic markers of ovarian cancer. Adv Exp Med Biol 622:35–51
8. Jones PA, Baylin SB (2007) The epigenomics of cancer. Cell 128(4):683–692
9. Esteller M (2008) Epigenetics in cancer. N Engl J Med 358(11):1148–1159

10. Rodriguez-Paredes M, Esteller M (2011) Cancer epigenetics reaches mainstream oncology. Nat Med 17(3):330–339
11. Lu L, Katsaros D, de la Longrais IA, Sochirca O, Yu H (2007) Hypermethylation of let-7a-3 in epithelial ovarian cancer is associated with low insulin-like growth factor-II expression and favorable prognosis. Cancer Res 67(21):10117–10122
12. Wiley A, Katsaros D, Chen H, Rigault de la Longrais IA, Beeghly A, Puopolo M, Singal R, Zhang Y, Amoako A, Zelterman D, Yu H (2006) Aberrant promoter methylation of multiple genes in malignant ovarian tumors and in ovarian tumors with low malignant potential. Cancer 107(2):299–308
13. Daley GQ (2008) Common themes of dedifferentiation in somatic cell reprogramming and cancer. Cold Spring Harb Symp Quant Biol 73:171–174
14. Dimri GP (2005) What has senescence got to do with cancer? Cancer Cell 7(6):505–512
15. Dalerba P, Cho RW, Clarke MF (2007) Cancer stem cells: models and concepts. Annu Rev Med 58:267–284
16. Jordan CT (2009) Cancer stem cells: controversial or just misunderstood? Cell Stem Cell 4(3):203–205
17. Von Hoff DD, Slavik M, Muggia FM (1976) 5-Azacytidine. A new anticancer drug with effectiveness in acute myelogenous leukemia. Ann Intern Med 85(2):237–245
18. Delva L, Zelent A, Naoe T, Fenaux P, Waxman S, Degos L, Chomienne C (2007) Meeting report: the 11th International Conference on Differentiation Therapy and Innovative Therapeutics in Oncology. Cancer Res 67(22):10635–10637
19. Ma WW, Adjei AA (2009) Novel agents on the horizon for cancer therapy. CA Cancer J Clin 59(2):111–137
20. Vincent A, Van Seuningen I (2009) Epigenetics, stem cells and epithelial cell fate. Differentiation 78(2–3):99–107
21. Scaffidi P, Misteli T (2010) Cancer epigenetics: from disruption of differentiation programs to the emergence of cancer stem cells. Cold Spring Harb Symp Quant Biol 75:251–258
22. Lotem J, Sachs L (2006) Epigenetics and the plasticity of differentiation in normal and cancer stem cells. Oncogene 25(59):7663–7672
23. Djuric U, Ellis J (2010) Epigenetics of induced pluripotency, the seven-headed dragon. Stem Cell Res Ther 1(1):3
24. Hochedlinger K, Plath K (2009) Epigenetic reprogramming and induced pluripotency. Development 136(4):509–523
25. Costa FF, Seftor EA, Bischof JM, Kirschmann DA, Strizzi L, Arndt K, de Fatima Bonaldo M, Soares MB, Hendrix MJ (2009) Epigenetically reprogramming metastatic tumor cells with an embryonic microenvironment. Epigenomics 1(2):387–398
26. Hendrix MJ, Seftor EA, Seftor RE, Kasemeier-Kulesa J, Kulesa PM, Postovit LM (2007) Reprogramming metastatic tumour cells with embryonic microenvironments. Nat Rev Cancer 7(4):246–255
27. Goldin A, Sandberg JS, Henderson ES, Newman JW, Frei E III, Holland JF (1971) The chemotherapy of human and animal acute leukemia. Cancer Chemother Pharmacol 55(4):309–505
28. Ney PA, D'Andrea AD (2000) Friend erythroleukemia revisited. Blood 96(12):3675–3680
29. Jones PA, Taylor SM (1980) Cellular differentiation, cytidine analogs and DNA methylation. Cell 20(1):85–93
30. Lane AA, Chabner BA (2009) Histone deacetylase inhibitors in cancer therapy. J Clin Oncol 27(32):5459–5468
31. Yang X, Lay F, Han H, Jones PA (2010) Targeting DNA methylation for epigenetic therapy. Trends Pharmacol Sci 31(11):536–546
32. Issa JP (2007) DNA methylation as a therapeutic target in cancer. Clin Cancer Res 13(6):1634–1637
33. Ewald B, Sampath D, Plunkett W (2008) Nucleoside analogs: molecular mechanisms signaling cell death. Oncogene 27(50):6522–6537
34. Yoo CB, Jones PA (2006) Epigenetic therapy of cancer: past, present and future. Nat Rev Drug Discov 5(1):37–50

35. Jabbour E, Issa JP, Garcia-Manero G, Kantarjian H (2008) Evolution of decitabine development: accomplishments, ongoing investigations, and future strategies. Cancer 112(11):2341–2351
36. Piskala A, Sorm F (1964) Nucleic acids components and the analogues. LI. Synthesis of 1-glycosyl derivatives of 5-azauracil and 5-azacytosine. Collect Czech Chem Commun 29:2060–2076
37. Shutt RH, Krueger RG (1972) The effect of actinomycin D and 5-azacytidine on macromolecular synthesis in murine myeloma tumor cells. J Immunol 108(3):819–830
38. Takai N, Kawamata N, Walsh CS, Gery S, Desmond JC, Whittaker S, Said JW, Popoviciu LM, Jones PA, Miyakawa I, Koeffler HP (2005) Discovery of epigenetically masked tumor suppressor genes in endometrial cancer. Mol Cancer Res 3(5):261–269
39. Sasaki M, Kaneuchi M, Fujimoto S, Tanaka Y, Dahiya R (2003) Hypermethylation can selectively silence multiple promoters of steroid receptors in cancers. Mol Cell Endocrinol 202(1–2):201–207
40. Nguyen CT, Weisenberger DJ, Velicescu M, Gonzales FA, Lin JC, Liang G, Jones PA (2002) Histone H3-lysine 9 methylation is associated with aberrant gene silencing in cancer cells and is rapidly reversed by 5-aza-2′-deoxycytidine. Cancer Res 62(22):6456–6461
41. Abbosh PH, Montgomery JS, Starkey JA, Novotny M, Zuhowski EG, Egorin MJ, Moseman AP, Golas A, Brannon KM, Balch C, Huang TH, Nephew KP (2006) Dominant-negative histone H3 lysine 27 mutant derepresses silenced tumor suppressor genes and reverses the drug-resistant phenotype in cancer cells. Cancer Res 66(11):5582–5591
42. Vesely J (1982) Synergistic effect of cis-dichlorodiammineplatinum and 5-aza-2′-deoxycytidine on mouse leukemic cells in vivo and in vitro. Int J Cancer 29(1):81–85
43. Taylor SM, Jones PA (1982) Mechanism of action of eukaryotic DNA methyltransferase. Use of 5-azacytosine-containing DNA. J Mol Biol 162(3):679–692
44. Nakahara Y, Northcott PA, Li M, Kongkham PN, Smith C, Yan H, Croul S, Ra YS, Eberhart C, Huang A, Bigner D, Grajkowska W, Van Meter T, Rutka JT, Taylor MD (2010) Genetic and epigenetic inactivation of Kruppel-like factor 4 in medulloblastoma. Neoplasia 12(1):20–27
45. Mahesh S, Saxena A, Qiu X, Perez-Soler R, Zou Y (2010) Intratracheally administered 5-azacytidine is effective against orthotopic human lung cancer xenograft models and devoid of important systemic toxicity. Clin Lung Cancer 11(6):405–411
46. Walker C, Shay JW (1984) 5-Azacytidine induced myogenesis in a differentiation defective cell line. Differentiation 25(3):259–263
47. Liu L, Harrington M, Jones PA (1986) Characterization of myogenic cell lines derived by 5-azacytidine treatment. Dev Biol 117(2):331–336
48. Hustad CM, Jones PA (1991) Effect of myogenic determination on tumorigenicity of chemically transformed 10T1/2 cells. Mol Carcinog 4(2):153–161
49. Schneider-Stock R, Diab-Assef M, Rohrbeck A, Foltzer-Jourdainne C, Boltze C, Hartig R, Schonfeld P, Roessner A, Gali-Muhtasib H (2005) 5-Aza-cytidine is a potent inhibitor of DNA methyltransferase 3a and induces apoptosis in HCT-116 colon cancer cells via Gadd45- and p53-dependent mechanisms. J Pharmacol Exp Ther 312(2):525–536
50. Wang XM, Wang X, Li J, Evers BM (1998) Effects of 5-azacytidine and butyrate on differentiation and apoptosis of hepatic cancer cell lines. Ann Surg 227(6):922–931
51. Burrows JF, Chanduloy S, McIlhatton MA, Nagar H, Yeates K, Donaghy P, Price J, Godwin AK, Johnston PG, Russell SE (2003) Altered expression of the septin gene, SEPT9, in ovarian neoplasia. J Pathol 201(4):581–588
52. Balch C, Montgomery JS, Paik HI, Kim S, Huang TH, Nephew KP (2005) New anti-cancer strategies: epigenetic therapies and biomarkers. Front Biosci 10:1897–1931
53. Momparler RL (2005) Epigenetic therapy of cancer with 5-aza-2′-deoxycytidine (decitabine). Semin Oncol 32(5):443–451
54. Wilson VL, Jones PA, Momparler RL (1983) Inhibition of DNA methylation in L1210 leukemic cells by 5-aza-2′-deoxycytidine as a possible mechanism of chemotherapeutic action. Cancer Res 43(8):3493–3496

55. Momparler RL, Bouchard J, Samson J (1985) Induction of differentiation and inhibition of DNA methylation in HL-60 myeloid leukemic cells by 5-AZA-2′-deoxycytidine. Leuk Res 9(11):1361–1366
56. Limonta M, Colombo T, Damia G, Catapano CV, Conter V, Gervasoni M, Masera G, Liso V, Specchia G, Giudici G et al (1993) Cytotoxic activity and mechanism of action of 5-Aza-2′-deoxycytidine in human CML cells. Leuk Res 17(11):977–982
57. Corn PG, Kuerbitz SJ, van Noesel MM, Esteller M, Compitello N, Baylin SB, Herman JG (1999) Transcriptional silencing of the p73 gene in acute lymphoblastic leukemia and Burkitt's lymphoma is associated with 5′ CpG island methylation. Cancer Res 59(14):3352–3356
58. Schnekenburger M, Grandjenette C, Ghelfi J, Karius T, Foliguet B, Dicato M, Diederich M (2011) Sustained exposure to the DNA demethylating agent, 2′-deoxy-5-azacytidine, leads to apoptotic cell death in chronic myeloid leukemia by promoting differentiation, senescence, and autophagy. Biochem Pharmacol 81(3):364–378
59. Obata T, Toyota M, Satoh A, Sasaki Y, Ogi K, Akino K, Suzuki H, Murai M, Kikuchi T, Mita H, Itoh F, Issa JP, Tokino T, Imai K (2003) Identification of HRK as a target of epigenetic inactivation in colorectal and gastric cancer. Clin Cancer Res 9(17):6410–6418
60. Alcazar O, Achberger S, Aldrich W, Hu Z, Negrotto S, Saunthararajah Y, Triozzi P (2012) Epigenetic regulation by decitabine of melanoma differentiation in vitro and in vivo. Int J Cancer 131(1):18–29
61. Chen W, Gao N, Shen Y, Cen JN (2010) Hypermethylation downregulates Runx3 gene expression and its restoration suppresses gastric epithelial cell growth by inducing p27 and caspase3 in human gastric cancer. J Gastroenterol Hepatol 25(4):823–831
62. Tseng RC, Lee SH, Hsu HS, Chen BH, Tsai WC, Tzao C, Wang YC (2010) SLIT2 attenuation during lung cancer progression deregulates beta-catenin and E-cadherin and associates with poor prognosis. Cancer Res 70(2):543–551
63. Furuta M, Kozaki KI, Tanaka S, Arii S, Imoto I, Inazawa J (2010) miR-124 and miR-203 are epigenetically silenced tumor-suppressive microRNAs in hepatocellular carcinoma. Carcinogenesis 31(5):766–776
64. Hashimoto Y, Akiyama Y, Otsubo T, Shimada S, Yuasa Y (2010) Involvement of epigenetically silenced microRNA-181c in gastric carcinogenesis. Carcinogenesis 31(5):777–784
65. Aguilera O, Fraga MF, Ballestar E, Paz MF, Herranz M, Espada J, Garcia JM, Munoz A, Esteller M, Gonzalez-Sancho JM (2006) Epigenetic inactivation of the Wnt antagonist DICKKOPF-1 (DKK-1) gene in human colorectal cancer. Oncogene 25(29):4116–4121
66. Chuang JC, Warner SL, Vollmer D, Vankayalapati H, Redkar S, Bearss DJ, Qiu X, Yoo CB, Jones PA (2010) S110, a 5-Aza-2′-deoxycytidine-containing dinucleotide, is an effective DNA methylation inhibitor in vivo and can reduce tumor growth. Mol Cancer Ther 9(5):1443–1450
67. Yoo CB, Jeong S, Egger G, Liang G, Phiasivongsa P, Tang C, Redkar S, Jones PA (2007) Delivery of 5-aza-2′-deoxycytidine to cells using oligodeoxynucleotides. Cancer Res 67(13):6400–6408
68. Brueckner B, Rius M, Markelova MR, Fichtner I, Hals PA, Sandvold ML, Lyko F (2010) Delivery of 5-azacytidine to human cancer cells by elaidic acid esterification increases therapeutic drug efficacy. Mol Cancer Ther 9(5):1256–1264
69. Leu YW, Rahmatpanah F, Shi H, Wei SH, Liu JC, Yan PS, Huang TH (2003) Double RNA interference of DNMT3b and DNMT1 enhances DNA demethylation and gene reactivation. Cancer Res 63(19):6110–6115
70. Balch C, Yan P, Craft T, Young S, Skalnik DG, Huang TH, Nephew KP (2005) Antimitogenic and chemosensitizing effects of the methylation inhibitor zebularine in ovarian cancer. Mol Cancer Ther 4(10):1505–1514
71. Yoo CB, Chuang JC, Byun HM, Egger G, Yang AS, Dubeau L, Long T, Laird PW, Marquez VE, Jones PA (2008) Long-term epigenetic therapy with oral zebularine has minimal side effects and prevents intestinal tumors in mice. Cancer Prev Res 1(4):233–240
72. Cheng JC, Matsen CB, Gonzales FA, Ye W, Greer S, Marquez VE, Jones PA, Selker EU (2003) Inhibition of DNA methylation and reactivation of silenced genes by zebularine. J Natl Cancer Inst 95(5):399–409

73. Cheng JC, Yoo CB, Weisenberger DJ, Chuang J, Wozniak C, Liang G, Marquez VE, Greer S, Orntoft TF, Thykjaer T, Jones PA (2004) Preferential response of cancer cells to zebularine. Cancer Cell 6(2):151–158
74. Yoo CB, Valente R, Congiatu C, Gavazza F, Angel A, Siddiqui MA, Jones PA, McGuigan C, Marquez VE (2008) Activation of p16 gene silenced by DNA methylation in cancer cells by phosphoramidate derivatives of 2′-deoxyzebularine. J Med Chem 51(23):7593–7601
75. Segura-Pacheco B, Trejo-Becerril C, Perez-Cardenas E, Taja-Chayeb L, Mariscal I, Chavez A, Acuna C, Salazar AM, Lizano M, Duenas-Gonzalez A (2003) Reactivation of tumor suppressor genes by the cardiovascular drugs hydralazine and procainamide and their potential use in cancer therapy. Clin Cancer Res 9(5):1596–1603
76. Chuang JC, Yoo CB, Kwan JM, Li TW, Liang G, Yang AS, Jones PA (2005) Comparison of biological effects of non-nucleoside DNA methylation inhibitors versus 5-aza-2′-deoxycytidine. Mol Cancer Ther 4(10):1515–1520
77. Liu F, Liu Q, Yang D, Bollag WB, Robertson K, Wu P, Liu K (2011) Verticillin A overcomes apoptosis resistance in human colon carcinoma through DNA methylation-dependent upregulation of BNIP3. Cancer Res 71(21):6807–6816
78. Brueckner B, Garcia Boy R, Siedlecki P, Musch T, Kliem HC, Zielenkiewicz P, Suhai S, Wiessler M, Lyko F (2005) Epigenetic reactivation of tumor suppressor genes by a novel small-molecule inhibitor of human DNA methyltransferases. Cancer Res 65(14):6305–6311
79. Datta J, Ghoshal K, Denny WA, Gamage SA, Brooke DG, Phiasivongsa P, Redkar S, Jacob ST (2009) A new class of quinoline-based DNA hypomethylating agents reactivates tumor suppressor genes by blocking DNA methyltransferase 1 activity and inducing its degradation. Cancer Res 69(10):4277–4285
80. Medina-Franco JL, Caulfield T (2011) Advances in the computational development of DNA methyltransferase inhibitors. Drug Discov Today 16:418–425
81. Siedlecki P, Garcia Boy R, Musch T, Brueckner B, Suhai S, Lyko F, Zielenkiewicz P (2006) Discovery of two novel, small-molecule inhibitors of DNA methylation. J Med Chem 49(2):678–683
82. Medina-Franco JL, Caulfield T (2011) Advances in the computational development of DNA methyltransferase inhibitors. Drug Discov Today 16(9–10):418–425
83. Castellano S, Kuck D, Viviano M, Yoo J, Lopez-Vallejo F, Conti P, Tamborini L, Pinto A, Medina-Franco JL, Sbardella G (2011) Synthesis and biochemical evaluation of delta(2)-isoxazoline derivatives as DNA methyltransferase 1 inhibitors. J Med Chem 54(21):7663–7677
84. Balch C, Nephew KP (2010) The role of chromatin, microRNAs, and tumor stem cells in ovarian cancer. Cancer Biomark 8(4):203–221
85. Wood TE, Dalili S, Simpson CD, Sukhai MA, Hurren R, Anyiwe K, Mao X, Suarez Saiz F, Gronda M, Eberhard Y, MacLean N, Ketela T, Reed JC, Moffat J, Minden MD, Batey RA, Schimmer AD (2010) Selective inhibition of histone deacetylases sensitizes malignant cells to death receptor ligands. Mol Cancer Ther 9(1):246–256
86. Hirata H, Hinoda Y, Nakajima K, Kawamoto K, Kikuno N, Ueno K, Yamamura S, Zaman MS, Khatri G, Chen Y, Saini S, Majid S, Deng G, Ishii N, Dahiya R (2011) Wnt antagonist DKK1 acts as a tumor suppressor gene that induces apoptosis and inhibits proliferation in human renal cell carcinoma. Int J Cancer 128(8):1793–1803
87. Liu T, Zhang X, So CK, Wang S, Wang P, Yan L, Myers R, Chen Z, Patterson AP, Yang CS, Chen X (2007) Regulation of Cdx2 expression by promoter methylation, and effects of Cdx2 transfection on morphology and gene expression of human esophageal epithelial cells. Carcinogenesis 28(2):488–496
88. Xu J, Zhou JY, Tainsky MA, Wu GS (2007) Evidence that tumor necrosis factor-related apoptosis-inducing ligand induction by 5-Aza-2′-deoxycytidine sensitizes human breast cancer cells to adriamycin. Cancer Res 67(3):1203–1211
89. Chun SG, Zhou W, Yee NS (2009) Combined targeting of histone deacetylases and hedgehog signaling enhances cytoxicity in pancreatic cancer. Cancer Biol Ther 8(14):1328–1339
90. Neil GL, Berger AE, Bhuyan BK, DeSante DC (1976) Combination chemotherapy of L1210 leukemia with 1-beta-D-arabinofuranosylcytosine and 5-azacytidine. Cancer Res 36(3):1114–1120

91. Neil GL, Moxley TE, Kuentzel SL, Manak RC, Hanka LJ (1975) Enhancement by tetrahydrouridine (NSC-112907) of the oral activity of 5-azacytidine (NSC-102816) in L1210 leukemic mice. Cancer Chemother Pharmacol 59(3):459–465
92. Festuccia C, Gravina GL, D'Alessandro AM, Muzi P, Millimaggi D, Dolo V, Ricevuto E, Vicentini C, Bologna M (2009) Azacitidine improves antitumor effects of docetaxel and cisplatin in aggressive prostate cancer models. Endocr Relat Cancer 16(2):401–413
93. Anzai H, Frost P, Abbruzzese JL (1992) Synergistic cytotoxicity with 2′-deoxy-5-azacytidine and topotecan in vitro and in vivo. Cancer Res 52(8):2180–2185
94. Balch C, Montgomery JS, Paik HI, Kim S, Kim S, Huang TH, Nephew KP (2005) New anticancer strategies: epigenetic therapies and biomarkers. Front Biosci 10:1897–1931
95. Plumb JA, Strathdee G, Sludden J, Kaye SB, Brown R (2000) Reversal of drug resistance in human tumor xenografts by 2′-deoxy-5-azacytidine-induced demethylation of the hMLH1 gene promoter. Cancer Res 60(21):6039–6044
96. Morita S, Iida S, Kato K, Takagi Y, Uetake H, Sugihara K (2006) The synergistic effect of 5-aza-2′-deoxycytidine and 5-fluorouracil on drug-resistant tumors. Oncology 71(5–6):437–445
97. Ishiguro M, Iida S, Uetake H, Morita S, Makino H, Kato K, Takagi Y, Enomoto M, Sugihara K (2007) Effect of combined therapy with low-dose 5-aza-2′-deoxycytidine and irinotecan on colon cancer cell line HCT-15. Ann Surg Oncol 14(5):1752–1762
98. Karpf AR, Peterson PW, Rawlins JT, Dalley BK, Yang Q, Albertsen H, Jones DA (1999) Inhibition of DNA methyltransferase stimulates the expression of signal transducer and activator of transcription 1, 2, and 3 genes in colon tumor cells. Proc Natl Acad Sci USA 96(24):14007–14012
99. Phuong NT, Kim SK, Lim SC, Kim HS, Kim TH, Lee KY, Ahn SG, Yoon JH, Kang KW (2011) Role of PTEN promoter methylation in tamoxifen-resistant breast cancer cells. Breast Cancer Res Treat 130(1):73–83
100. Zuo T, Liu TM, Lan X, Weng YI, Shen R, Gu F, Huang YW, Liyanarachchi S, Deatherage DE, Hsu PY, Taslim C, Ramaswamy B, Shapiro CL, Lin HJ, Cheng AS, Jin VX, Huang TH (2011) Epigenetic silencing mediated through activated PI3K/AKT signaling in breast cancer. Cancer Res 71(5):1752–1762
101. Stearns V, Zhou Q, Davidson NE (2007) Epigenetic regulation as a new target for breast cancer therapy. Cancer Invest 25(8):659–665
102. Sharma D, Saxena NK, Davidson NE, Vertino PM (2006) Restoration of tamoxifen sensitivity in estrogen receptor-negative breast cancer cells: tamoxifen-bound reactivated ER recruits distinctive corepressor complexes. Cancer Res 66(12):6370–6378
103. Gao L, Alumkal J (2010) Epigenetic regulation of androgen receptor signaling in prostate cancer. Epigenetics 5(2):100–104
104. Nelson WG, Yegnasubramanian S, Agoston AT, Bastian PJ, Lee BH, Nakayama M, De Marzo AM (2007) Abnormal DNA methylation, epigenetics, and prostate cancer. Front Biosci 12:4254–4266
105. Shang D, Liu Y, Liu Q, Zhang F, Feng L, Lv W, Tian Y (2009) Synergy of 5-aza-2′-deoxycytidine (DAC) and paclitaxel in both androgen-dependent and -independent prostate cancer cell lines. Cancer Lett 278(1):82–87
106. Zorn CS, Wojno KJ, McCabe MT, Kuefer R, Gschwend JE, Day ML (2007) 5-aza-2′-deoxycytidine delays androgen-independent disease and improves survival in the transgenic adenocarcinoma of the mouse prostate mouse model of prostate cancer. Clin Cancer Res 13(7):2136–2143
107. Walton TJ, Li G, Seth R, McArdle SE, Bishop MC, Rees RC (2008) DNA demethylation and histone deacetylation inhibition co-operate to re-express estrogen receptor beta and induce apoptosis in prostate cancer cell-lines. Prostate 68(2):210–222
108. Friend C, Scher W, Holland JG, Sato T (1971) Hemoglobin synthesis in murine virus-induced leukemic cells in vitro: stimulation of erythroid differentiation by dimethyl sulfoxide. Proc Natl Acad Sci USA 68(2):378–382
109. Marks PA, Breslow R (2007) Dimethyl sulfoxide to vorinostat: development of this histone deacetylase inhibitor as an anticancer drug. Nat Biotechnol 25(1):84–90

110. Minucci S, Pelicci PG (2006) Histone deacetylase inhibitors and the promise of epigenetic (and more) treatments for cancer. Nat Rev Cancer 6(1):38–51
111. Qiu L, Burgess A, Fairlie DP, Leonard H, Parsons PG, Gabrielli BG (2000) Histone deacetylase inhibitors trigger a G2 checkpoint in normal cells that is defective in tumor cells. Mol Biol Cell 11(6):2069–2083
112. Strait KA, Warnick CT, Ford CD, Dabbas B, Hammond EH, Ilstrup SJ (2005) Histone deacetylase inhibitors induce G2-checkpoint arrest and apoptosis in cisplatinum-resistant ovarian cancer cells associated with overexpression of the Bcl-2-related protein Bad. Mol Cancer Ther 4(4):603–611
113. Choudhary C, Kumar C, Gnad F, Nielsen ML, Rehman M, Walther TC, Olsen JV, Mann M (2009) Lysine acetylation targets protein complexes and co-regulates major cellular functions. Science 325(5942):834–840
114. Plumb JA, Finn PW, Williams RJ, Bandara MJ, Romero MR, Watkins CJ, La Thangue NB, Brown R (2003) Pharmacodynamic response and inhibition of growth of human tumor xenografts by the novel histone deacetylase inhibitor PXD101. Mol Cancer Ther 2(8):721–728
115. Qian X, LaRochelle WJ, Ara G, Wu F, Petersen KD, Thougaard A, Sehested M, Lichenstein HS, Jeffers M (2006) Activity of PXD101, a histone deacetylase inhibitor, in preclinical ovarian cancer studies. Mol Cancer Ther 5(8):2086–2095
116. Uchida H, Maruyama T, Nagashima T, Asada H, Yoshimura Y (2005) Histone deacetylase inhibitors induce differentiation of human endometrial adenocarcinoma cells through upregulation of glycodelin. Endocrinology 146(12):5365–5373
117. Takai N, Desmond JC, Kumagai T, Gui D, Said JW, Whittaker S, Miyakawa I, Koeffler HP (2004) Histone deacetylase inhibitors have a profound antigrowth activity in endometrial cancer cells. Clin Cancer Res 10(3):1141–1149
118. Rahman R, Grundy R (2011) Histone deacetylase inhibition as an anticancer telomerase-targeting strategy. Int J Cancer 129(12):2765–2774
119. Xu WS, Parmigiani RB, Marks PA (2007) Histone deacetylase inhibitors: molecular mechanisms of action. Oncogene 26(37):5541–5552
120. Cooper AL, Greenberg VL, Lancaster PS, van Nagell JR Jr, Zimmer SG, Modesitt SC (2007) In vitro and in vivo histone deacetylase inhibitor therapy with suberoylanilide hydroxamic acid (SAHA) and paclitaxel in ovarian cancer. Gynecol Oncol 104:596–601
121. Dietrich CS III, Greenberg VL, DeSimone CP, Modesitt SC, van Nagell JR, Craven R, Zimmer SG (2010) Suberoylanilide hydroxamic acid (SAHA) potentiates paclitaxel-induced apoptosis in ovarian cancer cell lines. Gynecol Oncol 116(1):126–130
122. Sonnemann J, Gange J, Pilz S, Stotzer C, Ohlinger R, Belau A, Lorenz G, Beck JF (2006) Comparative evaluation of the treatment efficacy of suberoylanilide hydroxamic acid (SAHA) and paclitaxel in ovarian cancer cell lines and primary ovarian cancer cells from patients. BMC Cancer 6:183
123. Zuco V, Benedetti V, De Cesare M, Zunino F (2010) Sensitization of ovarian carcinoma cells to the atypical retinoid ST1926 by the histone deacetylase inhibitor, RC307: enhanced DNA damage response. Int J Cancer 126(5):1246–1255
124. Son DS, Wilson AJ, Parl AK, Khabele D (2010) The effects of the histone deacetylase inhibitor romidepsin (FK228) are enhanced by aspirin (ASA) in COX-1 positive ovarian cancer cells through augmentation of p21. Cancer Biol Ther 9(11):928–935
125. Yang YT, Balch C, Kulp SK, Mand MR, Nephew KP, Chen CS (2009) A rationally designed histone deacetylase inhibitor with distinct antitumor activity against ovarian cancer. Neoplasia 11(6):552–563; 553 p following 563
126. Iwahashi S, Shimada M, Utsunomiya T, Morine Y, Imura S, Ikemoto T, Mori H, Hanaoka J, Saito Y (2011) Histone deacetylase inhibitor enhances the anti-tumor effect of gemcitabine: a special reference to gene-expression microarray analysis. Oncol Rep 26(5):1057–1062
127. Kim MS, Baek JH, Chakravarty D, Sidransky D, Carrier F (2005) Sensitization to UV-induced apoptosis by the histone deacetylase inhibitor trichostatin A (TSA). Exp Cell Res 306(1):94–102
128. Roh MS, Kim CW, Park BS, Kim GC, Jeong JH, Kwon HC, Suh DJ, Cho KH, Yee SB, Yoo YH (2004) Mechanism of histone deacetylase inhibitor Trichostatin A induced apoptosis in human osteosarcoma cells. Apoptosis 9(5):583–589

129. Srivastava RK, Kurzrock R, Shankar S (2010) MS-275 sensitizes TRAIL-resistant breast cancer cells, inhibits angiogenesis and metastasis, and reverses epithelial-mesenchymal transition in vivo. Mol Cancer Ther 9(12):3254–3266
130. Hacker S, Dittrich A, Mohr A, Schweitzer T, Rutkowski S, Krauss J, Debatin KM, Fulda S (2009) Histone deacetylase inhibitors cooperate with IFN-gamma to restore caspase-8 expression and overcome TRAIL resistance in cancers with silencing of caspase-8. Oncogene 28(35):3097–3110
131. Schuler S, Fritsche P, Diersch S, Arlt A, Schmid RM, Saur D, Schneider G (2010) HDAC2 attenuates TRAIL-induced apoptosis of pancreatic cancer cells. Mol Cancer Ther 9:80
132. Pathil A, Armeanu S, Venturelli S, Mascagni P, Weiss TS, Gregor M, Lauer UM, Bitzer M (2006) HDAC inhibitor treatment of hepatoma cells induces both TRAIL-independent apoptosis and restoration of sensitivity to TRAIL. Hepatology 43(3):425–434
133. Thomas S, Thurn KT, Bicaku E, Marchion DC, Munster PN (2011) Addition of a histone deacetylase inhibitor redirects tamoxifen-treated breast cancer cells into apoptosis, which is opposed by the induction of autophagy. Breast Cancer Res Treat 130(2):437–447
134. Dowdy SC, Jiang S, Zhou XC, Hou X, Jin F, Podratz KC, Jiang SW (2006) Histone deacetylase inhibitors and paclitaxel cause synergistic effects on apoptosis and microtubule stabilization in papillary serous endometrial cancer cells. Mol Cancer Ther 5(11):2767–2776
135. Ahn MY, Chung HY, Choi WS, Lee BM, Yoon S, Kim HS (2010) Anti-tumor effect of apicidin on Ishikawa human endometrial cancer cells both in vitro and in vivo by blocking histone deacetylase 3 and 4. Int J Oncol 36(1):125–131
136. Bali P, Pranpat M, Swaby R, Fiskus W, Yamaguchi H, Balasis M, Rocha K, Wang HG, Richon V, Bhalla K (2005) Activity of suberoylanilide hydroxamic Acid against human breast cancer cells with amplification of her-2. Clin Cancer Res 11(17):6382–6389
137. Morey L, Brenner C, Fazi F, Villa R, Gutierrez A, Buschbeck M, Nervi C, Minucci S, Fuks F, Di Croce L (2008) MBD3, a component of the NuRD complex, facilitates chromatin alteration and deposition of epigenetic marks. Mol Cell Biol 28(19):5912–5923
138. Terasawa K, Sagae S, Toyota M, Tsukada K, Ogi K, Satoh A, Mita H, Imai K, Tokino T, Kudo R (2004) Epigenetic inactivation of TMS1/ASC in ovarian cancer. Clin Cancer Res 10(6):2000–2006
139. Chen MY, Liao WS, Lu Z, Bornmann WG, Hennessey V, Washington MN, Rosner GL, Yu Y, Ahmed AA, Bast RC Jr (2011) Decitabine and suberoylanilide hydroxamic acid (SAHA) inhibit growth of ovarian cancer cell lines and xenografts while inducing expression of imprinted tumor suppressor genes, apoptosis, G2/M arrest, and autophagy. Cancer 117(19):4424–4438
140. Xiong Y, Dowdy SC, Gonzalez Bosquet J, Zhao Y, Eberhardt NL, Podratz KC, Jiang SW (2005) Epigenetic-mediated upregulation of progesterone receptor B gene in endometrial cancer cell lines. Gynecol Oncol 99(1):135–141
141. Belinsky SA, Grimes MJ, Picchi MA, Mitchell HD, Stidley CA, Tesfaigzi Y, Channell MM, Liu Y, Casero RA Jr, Baylin SB, Reed MD, Tellez CS, March TH (2011) Combination therapy with vidaza and entinostat suppresses tumor growth and reprograms the epigenome in an orthotopic lung cancer model. Cancer Res 71(2):454–462
142. Ecke I, Petry F, Rosenberger A, Tauber S, Monkemeyer S, Hess I, Dullin C, Kimmina S, Pirngruber J, Johnsen SA, Uhmann A, Nitzki F, Wojnowski L, Schulz-Schaeffer W, Witt O, Hahn H (2009) Antitumor effects of a combined 5-aza-2′deoxycytidine and valproic acid treatment on rhabdomyosarcoma and medulloblastoma in Ptch mutant mice. Cancer Res 69(3):887–895
143. Herranz D, Serrano M (2010) SIRT1: recent lessons from mouse models. Nat Rev Cancer 10(12):819–823
144. Nebbioso A, Pereira R, Khanwalkar H, Matarese F, Garcia-Rodriguez J, Miceli M, Logie C, Kedinger V, Ferrara F, Stunnenberg HG, de Lera AR, Gronemeyer H, Altucci L (2011) Death receptor pathway activation and increase of ROS production by the triple epigenetic inhibitor, UVI5008. Mol Cancer Ther 10(12):2394–2404

145. Milutinovic S, D'Alessio AC, Detich N, Szyf M (2007) Valproate induces widespread epigenetic reprogramming which involves demethylation of specific genes. Carcinogenesis 28(3):560–571
146. Dong E, Guidotti A, Grayson DR, Costa E (2007) Histone hyperacetylation induces demethylation of reelin and 67-kDa glutamic acid decarboxylase promoters. Proc Natl Acad Sci USA 104(11):4676–4681
147. Arzenani MK, Zade AE, Ming Y, Vijverberg SJ, Zhang Z, Khan Z, Sadique S, Kallenbach L, Hu L, Vukojevic V, Ekstrom TJ (2011) Genomic DNA hypomethylation by histone deacetylase inhibition implicates DNMT1 nuclear dynamics. Mol Cell Biol 31(19):4119–4128
148. Ou JN, Torrisani J, Unterberger A, Provencal N, Shikimi K, Karimi M, Ekstrom TJ, Szyf M (2007) Histone deacetylase inhibitor Trichostatin A induces global and gene-specific DNA demethylation in human cancer cell lines. Biochem Pharmacol 73(9):1297–1307
149. Xiong Y, Dowdy SC, Podratz KC, Jin F, Attewell JR, Eberhardt NL, Jiang SW (2005) Histone deacetylase inhibitors decrease DNA methyltransferase-3B messenger RNA stability and down-regulate de novo DNA methyltransferase activity in human endometrial cells. Cancer Res 65(7):2684–2689
150. Scott SA, Dong WF, Ichinohasama R, Hirsch C, Sheridan D, Sanche SE, Geyer CR, Decoteau JF (2006) 5-Aza-2′-deoxycytidine (decitabine) can relieve p21WAF1 repression in human acute myeloid leukemia by a mechanism involving release of histone deacetylase 1 (HDAC1) without requiring p21WAF1 promoter demethylation. Leuk Res 30(1):69–76
151. Egger G, Aparicio AM, Escobar SG, Jones PA (2007) Inhibition of histone deacetylation does not block resilencing of p16 after 5-aza-2′-deoxycytidine treatment. Cancer Res 67(1):346–353
152. Gore SD, Baylin S, Sugar E, Carraway H, Miller CB, Carducci M, Grever M, Galm O, Dauses T, Karp JE, Rudek MA, Zhao M, Smith BD, Manning J, Jiemjit A, Dover G, Mays A, Zwiebel J, Murgo A, Weng LJ, Herman JG (2006) Combined DNA methyltransferase and histone deacetylase inhibition in the treatment of myeloid neoplasms. Cancer Res 66(12):6361–6369
153. Kaminskyy VO, Surova OV, Vaculova A, Zhivotovsky B (2011) Combined inhibition of DNA methyltransferase and histone deacetylase restores caspase-8 expression and sensitizes SCLC cells to TRAIL. Carcinogenesis 32(10):1450–1458
154. Steele N, Finn P, Brown R, Plumb JA (2009) Combined inhibition of DNA methylation and histone acetylation enhances gene re-expression and drug sensitivity in vivo. Br J Cancer 100(5):758–763
155. Matei DE, Nephew KP (2010) Epigenetic therapies for chemoresensitization of epithelial ovarian cancer. Gynecol Oncol 116(2):195–201
156. Kristensen LS, Nielsen HM, Hansen LL (2009) Epigenetics and cancer treatment. Eur J Pharmacol 625(1–3):131–142
157. Sigalotti L, Fratta E, Coral S, Cortini E, Covre A, Nicolay HJ, Anzalone L, Pezzani L, Di Giacomo AM, Fonsatti E, Colizzi F, Altomonte M, Calabro L, Maio M (2007) Epigenetic drugs as pleiotropic agents in cancer treatment: biomolecular aspects and clinical applications. J Cell Physiol 212(2):330–344
158. Momparler RL, Rivard GE, Gyger M (1985) Clinical trial on 5-aza-2′-deoxycytidine in patients with acute leukemia. Pharmacol Ther 30(3):277–286
159. Wijermans PW, Krulder JW, Huijgens PC, Neve P (1997) Continuous infusion of low-dose 5-Aza-2′-deoxycytidine in elderly patients with high-risk myelodysplastic syndrome. Leukemia 11(suppl 1):S19–S23
160. Wijermans P, Lubbert M, Verhoef G, Bosly A, Ravoet C, Andre M, Ferrant A (2000) Low-dose 5-aza-2′-deoxycytidine, a DNA hypomethylating agent, for the treatment of high-risk myelodysplastic syndrome: a multicenter phase II study in elderly patients. J Clin Oncol 18(5):956–962
161. DeSimone J, Koshy M, Dorn L, Lavelle D, Bressler L, Molokie R, Talischy N (2002) Maintenance of elevated fetal hemoglobin levels by decitabine during dose interval treatment of sickle cell anemia. Blood 99(11):3905–3908

162. Koshy M, Dorn L, Bressler L, Molokie R, Lavelle D, Talischy N, Hoffman R, van Overveld W, DeSimone J (2000) 2-deoxy 5-azacytidine and fetal hemoglobin induction in sickle cell anemia. Blood 96(7):2379–2384
163. Issa JP, Garcia-Manero G, Giles FJ, Mannari R, Thomas D, Faderl S, Bayar E, Lyons J, Rosenfeld CS, Cortes J, Kantarjian HM (2004) Phase 1 study of low-dose prolonged exposure schedules of the hypomethylating agent 5-aza-2′-deoxycytidine (decitabine) in hematopoietic malignancies. Blood 103(5):1635–1640
164. Invest New DrugsSchwartsmann G, Schunemann H, Gorini CN, Filho AF, Garbino C, Sabini G, Muse I, DiLeone L, Mans DR (2000) A phase I trial of cisplatin plus decitabine, a new DNA-hypomethylating agent, in patients with advanced solid tumors and a follow-up early phase II evaluation in patients with inoperable non-small cell lung cancer. Invest New Drugs 18(1):83–91
165. Pohlmann P, DiLeone LP, Cancella AI, Caldas AP, Dal Lago L, Campos O Jr, Monego E, Rivoire W, Schwartsmann G (2002) Phase II trial of cisplatin plus decitabine, a new DNA hypomethylating agent, in patients with advanced squamous cell carcinoma of the cervix. Am J Clin Oncol 25(5):496–501
166. Appleton K, Mackay HJ, Judson I, Plumb JA, McCormick C, Strathdee G, Lee C, Barrett S, Reade S, Jadayel D, Tang A, Bellenger K, Mackay L, Setanoians A, Schatzlein A, Twelves C, Kaye SB, Brown R (2007) Phase I and pharmacodynamic trial of the DNA methyltransferase inhibitor decitabine and carboplatin in solid tumors. J Clin Oncol 25(29):4603–4609
167. Glasspool RM, Gore M, Rustin G, McNeish I, Wilson R, Pledge S, Paul J, Mackean M, Halford S, Kaye S (2009) Randomized phase II study of in combination with carboplatin compared with carboplatin alone in patients with recurrent advanced ovarian cancer. J Clin Oncol 26(15S (May 20 suppl)):Abstract 5562
168. Fu S, Hu W, Iyer R, Kavanagh JJ, Coleman RL, Levenback CF, Sood AK, Wolf JK, Gershenson DM, Markman M, Hennessy BT, Kurzrock R, Bast RC Jr (2011) Phase 1b-2a study to reverse platinum resistance through use of a hypomethylating agent, azacitidine, in patients with platinum-resistant or platinum-refractory epithelial ovarian cancer. Cancer 117(8):1661–1669
169. Fang F, Balch C, Schilder J, Breen T, Zhang S, Shen C, Li L, Kulesavage C, Snyder AJ, Nephew KP, Matei DE (2010) A phase 1 and pharmacodynamic study of decitabine in combination with carboplatin in patients with recurrent, platinum-resistant, epithelial ovarian cancer. Cancer 116(17):4043–4053
170. Matei D, Fang F, Shen C, Schilder J, Arnold A, Zeng Y, Berry WA, Huang T, Nephew KP (2012). Epigenetic resensitization to platinum in ovarian cancer. Cancer Res 72(9): 2197–2205
171. Bauman J, Verschraegen C, Belinsky S, Muller C, Rutledge T, Fekrazad M, Ravindranathan M, Lee SJ, Jones D (2012) A phase I study of 5-azacytidine and erlotinib in advanced solid tumor malignancies. Cancer Chemother Pharmacol 69(2):547–554
172. George RE, Lahti JM, Adamson PC, Zhu K, Finkelstein D, Ingle AM, Reid JM, Krailo M, Neuberg D, Blaney SM, Diller L (2010) Phase I study of decitabine with doxorubicin and cyclophosphamide in children with neuroblastoma and other solid tumors: a Children's Oncology Group study. Pediatr Blood Cancer 55(4):629–638
173. Welch JS, Klco JM, Gao F, Procknow E, Uy GL, Stockerl-Goldstein KE, Abboud CN, Westervelt P, DiPersio JF, Hassan A, Cashen AF, Vij R (2011) Combination decitabine, arsenic trioxide, and ascorbic acid for the treatment of myelodysplastic syndrome and acute myeloid leukemia: a phase I study. Am J Hematol 86(9):796–800
174. Stewart DJ, Issa JP, Kurzrock R, Nunez MI, Jelinek J, Hong D, Oki Y, Guo Z, Gupta S, Wistuba II (2009) Decitabine effect on tumor global DNA methylation and other parameters in a phase I trial in refractory solid tumors and lymphomas. Clin Cancer Res 15(11): 3881–3888
175. Modesitt SC, Sill M, Hoffman JS, Bender DP (2008) A phase II study of vorinostat in the treatment of persistent or recurrent epithelial ovarian or primary peritoneal carcinoma: a Gynecologic Oncology Group study. Gynecol Oncol 109(2):182–186

176. Mackay HJ, Hirte H, Colgan T, Covens A, MacAlpine K, Grenci P, Wang L, Mason J, Pham PA, Tsao MS, Pan J, Zwiebel J, Oza AM (2010) Phase II trial of the histone deacetylase inhibitor belinostat in women with platinum resistant epithelial ovarian cancer and micropapillary (LMP) ovarian tumours. Eur J Cancer 46(9):1573–1579
177. Molife LR, Attard G, Fong PC, Karavasilis V, Reid AH, Patterson S, Riggs CE Jr, Higano C, Stadler WM, McCulloch W, Dearnaley D, Parker C, de Bono JS (2010) Phase II, two-stage, single-arm trial of the histone deacetylase inhibitor (HDACi) romidepsin in metastatic castration-resistant prostate cancer (CRPC). Ann Oncol 21(1):109–113
178. Hainsworth JD, Infante JR, Spigel DR, Arrowsmith ER, Boccia RV, Burris HA (2011) A phase II trial of panobinostat, a histone deacetylase inhibitor, in the treatment of patients with refractory metastatic renal cell carcinoma. Cancer Invest 29(7):451–455
179. Takai N, Narahara H (2010) Histone deacetylase inhibitor therapy in epithelial ovarian cancer. J Oncol 2010:458431
180. Thurn KT, Thomas S, Moore A, Munster PN (2011) Rational therapeutic combinations with histone deacetylase inhibitors for the treatment of cancer. Future Oncol 7(2):263–283
181. Rodon J, Iniesta MD, Papadopoulos K (2009) Development of PARP inhibitors in oncology. Expert Opin Investig Drugs 18(1):31–43
182. Teicher BA (2010) Combinations of PARP, hedgehog and HDAC inhibitors with standard drugs. Curr Opin Pharmacol 10(4):397–404
183. Munster PN, Thurn KT, Thomas S, Raha P, Lacevic M, Miller A, Melisko M, Ismail-Khan R, Rugo H, Moasser M, Minton SE (2011) A phase II study of the histone deacetylase inhibitor vorinostat combined with tamoxifen for the treatment of patients with hormone therapy-resistant breast cancer. Br J Cancer 104(12):1828–1835
184. Drappatz J, Lee EQ, Hammond S, Grimm SA, Norden AD, Beroukhim R, Gerard M, Schiff D, Chi AS, Batchelor TT, Doherty LM, Ciampa AS, Lafrankie DC, Ruland S, Snodgrass SM, Raizer JJ, Wen PY (2012) Phase I study of panobinostat in combination with bevacizumab for recurrent high-grade glioma. J Neurooncol 107(1):133–138
185. Candelaria M, Herrera A, Labardini J, Gonzalez-Fierro A, Trejo-Becerril C, Taja-Chayeb L, Perez-Cardenas E, de la Cruz-Hernandez E, Arias-Bofill D, Vidal S, Cervera E, Duenas-Gonzalez A (2011) Hydralazine and magnesium valproate as epigenetic treatment for myelodysplastic syndrome. Preliminary results of a phase-II trial. Ann Hematol 90(4):379–387
186. Braiteh F, Soriano AO, Garcia-Manero G, Hong D, Johnson MM, Silva Lde P, Yang H, Alexander S, Wolff J, Kurzrock R (2008) Phase I study of epigenetic modulation with 5-azacytidine and valproic acid in patients with advanced cancers. Clin Cancer Res 14(19):6296–6301
187. Stathis A, Hotte SJ, Chen EX, Hirte HW, Oza AM, Moretto P, Webster S, Laughlin A, Stayner LA, McGill S, Wang L, Zhang WJ, Espinoza-Delgado I, Holleran JL, Egorin MJ, Siu LL (2011) Phase I study of decitabine in combination with vorinostat in patients with advanced solid tumors and non-Hodgkin's lymphomas. Clin Cancer Res 17(6):1582–1590
188. Juergens RA, Wrangle J, Vendetti FP, Murphy SC, Zhao M, Coleman B, Sebree R, Rodgers K, Hooker CM, Franco N, Lee BH, Tsai S, Delgado IE, Rudek MA, Belinsky SA, Herman JG, Baylin SB, Brock MV, Rudin CM (2011) Combination epigenetic therapy has efficacy in patients with refractory advanced non-small cell lung cancer. Cancer Discov 1:598–607
189. Candelaria M, Gallardo-Rincon D, Arce C, Cetina L, Aguilar-Ponce JL, Arrieta O, Gonzalez-Fierro A, Chavez-Blanco A, de la Cruz-Hernandez E, Camargo MF, Trejo-Becerril C, Perez-Cardenas E, Perez-Plasencia C, Taja-Chayeb L, Wegman-Ostrosky T, Revilla-Vazquez A, Duenas-Gonzalez A (2007) A phase II study of epigenetic therapy with hydralazine and magnesium valproate to overcome chemotherapy resistance in refractory solid tumors. Ann Oncol 18(9):1529–1538
190. Garcia-Manero G, Kantarjian HM, Sanchez-Gonzalez B, Yang H, Rosner G, Verstovsek S, Rytting M, Wierda WG, Ravandi F, Koller C, Xiao L, Faderl S, Estrov Z, Cortes J, O'Brien S, Estey E, Bueso-Ramos C, Fiorentino J, Jabbour E, Issa JP (2006) Phase 1/2 study of the combination of 5-aza-2′-deoxycytidine with valproic acid in patients with leukemia. Blood 108(10):3271–3279
191. Gollob JA, Sciambi CJ, Peterson BL, Richmond T, Thoreson M, Moran K, Dressman HK, Jelinek J, Issa JP (2006) Phase I trial of sequential low-dose 5-aza-2′-deoxycytidine plus

high-dose intravenous bolus interleukin-2 in patients with melanoma or renal cell carcinoma. Clin Cancer Res 12(15):4619–4627
192. Simpson AJ, Caballero OL, Jungbluth A, Chen YT, Old LJ (2005) Cancer/testis antigens, gametogenesis and cancer. Nat Rev Cancer 5(8):615–625
193. Karpf AR (2006) A potential role for epigenetic modulatory drugs in the enhancement of cancer/germ-line antigen vaccine efficacy. Epigenetics 1(3):116–120
194. Tan J, Yang X, Zhuang L, Jiang X, Chen W, Lee PL, Karuturi RK, Tan PB, Liu ET, Yu Q (2007) Pharmacologic disruption of Polycomb-repressive complex 2-mediated gene repression selectively induces apoptosis in cancer cells. Genes Dev 21(9):1050–1063
195. Yao Y, Chen P, Diao J, Cheng G, Deng L, Anglin JL, Prasad BV, Song Y (2011) Selective inhibitors of histone methyltransferase DOT1L: design, synthesis, and crystallographic studies. J Am Chem Soc 133(42):16746–16749
196. Daigle SR, Olhava EJ, Therkelsen CA, Majer CR, Sneeringer CJ, Song J, Johnston LD, Scott MP, Smith JJ, Xiao Y, Jin L, Kuntz KW, Chesworth R, Moyer MP, Bernt KM, Tseng JC, Kung AL, Armstrong SA, Copeland RA, Richon VM, Pollock RM (2011) Selective killing of mixed lineage leukemia cells by a potent small-molecule DOT1L inhibitor. Cancer Cell 20(1):53–65
197. Wang C, Liu Z, Woo CW, Li Z, Wang L, Wei JS, Marquez VE, Bates SE, Jin Q, Khan J, Ge K, Thiele CJ (2012) EZH2 mediates epigenetic silencing of neuroblastoma suppressor genes CASZ1, CLU, RUNX3 and NGFR. Cancer Res 72(1):315–324
198. Zhou J, Bi C, Cheong LL, Mahara S, Liu SC, Tay KG, Koh TL, Yu Q, Chng WJ (2011) The histone methyltransferase inhibitor, DZNep, up-regulates TXNIP, increases ROS production, and targets leukemia cells in AML. Blood 118(10):2830–2839
199. Crea F, Hurt EM, Mathews LA, Cabarcas SM, Sun L, Marquez VE, Danesi R, Farrar WL (2011) Pharmacologic disruption of Polycomb Repressive Complex 2 inhibits tumorigenicity and tumor progression in prostate cancer. Mol Cancer 10:40
200. Fiskus W, Wang Y, Sreekumar A, Buckley KM, Shi H, Jillella A, Ustun C, Rao R, Fernandez P, Chen J, Balusu R, Koul S, Atadja P, Marquez VE, Bhalla KN (2009) Combined epigenetic therapy with the histone methyltransferase EZH2 inhibitor 3-deazaneplanocin A and the histone deacetylase inhibitor panobinostat against human AML cells. Blood 114(13):2733–2743
201. Hayden A, Johnson PW, Packham G, Crabb SJ (2011) S-adenosylhomocysteine hydrolase inhibition by 3-deazaneplanocin A analogues induces anti-cancer effects in breast cancer cell lines and synergy with both histone deacetylase and HER2 inhibition. Breast Cancer Res Treat 127(1):109–119
202. Suva ML, Riggi N, Janiszewska M, Radovanovic I, Provero P, Stehle JC, Baumer K, Le Bitoux MA, Marino D, Cironi L, Marquez VE, Clement V, Stamenkovic I (2009) EZH2 is essential for glioblastoma cancer stem cell maintenance. Cancer Res 69(24):9211–9218
203. Quinn AM, Allali-Hassani A, Vedadi M, Simeonov A (2010) A chemiluminescence-based method for identification of histone lysine methyltransferase inhibitors. Mol Biosyst 6(5):782–788
204. King ON, Li XS, Sakurai M, Kawamura A, Rose NR, Ng SS, Quinn AM, Rai G, Mott BT, Beswick P, Klose RJ, Oppermann U, Jadhav A, Heightman TD, Maloney DJ, Schofield CJ, Simeonov A (2010) Quantitative high-throughput screening identifies 8-hydroxyquinolines as cell-active histone demethylase inhibitors. PLoS One 5(11):e15535
205. Tang W, Luo T, Greenberg EF, Bradner JE, Schreiber SL (2011) Discovery of histone deacetylase 8 selective inhibitors. Bioorg Med Chem Lett 21(9):2601–2605
206. Vedadi M, Barsyte-Lovejoy D, Liu F, Rival-Gervier S, Allali-Hassani A, Labrie V, Wigle TJ, Dimaggio PA, Wasney GA, Siarheyeva A, Dong A, Tempel W, Wang SC, Chen X, Chau I, Mangano TJ, Huang XP, Simpson CD, Pattenden SG, Norris JL, Kireev DB, Tripathy A, Edwards A, Roth BL, Janzen WP, Garcia BA, Petronis A, Ellis J, Brown PJ, Frye SV, Arrowsmith CH, Jin J (2011) A chemical probe selectively inhibits G9a and GLP methyltransferase activity in cells. Nat Chem Biol 7(8):566–574
207. Cole PA (2008) Chemical probes for histone-modifying enzymes. Nat Chem Biol 4(10): 590–597

208. Kota J, Chivukula RR, O'Donnell KA, Wentzel EA, Montgomery CL, Hwang HW, Chang TC, Vivekanandan P, Torbenson M, Clark KR, Mendell JR, Mendell JT (2009) Therapeutic microRNA delivery suppresses tumorigenesis in a murine liver cancer model. Cell 137(6):1005–1017
209. Ibrahim AF, Weirauch U, Thomas M, Grunweller A, Hartmann RK, Aigner A (2011) MicroRNA replacement therapy for miR-145 and miR-33a is efficacious in a model of colon carcinoma. Cancer Res 71(15):5214–5224
210. Taulli R, Bersani F, Foglizzo V, Linari A, Vigna E, Ladanyi M, Tuschl T, Ponzetto C (2009) The muscle-specific microRNA miR-206 blocks human rhabdomyosarcoma growth in xenotransplanted mice by promoting myogenic differentiation. J Clin Invest 119(8):2366–2378
211. Avramis VI, Mecum RA, Nyce J, Steele DA, Holcenberg JS (1989) Pharmacodynamic and DNA methylation studies of high-dose 1-beta-D-arabinofuranosyl cytosine before and after in vivo 5-azacytidine treatment in pediatric patients with refractory acute lymphocytic leukemia. Cancer Chemother Pharmacol 24(4):203–210
212. Belinsky SA, Klinge DM, Stidley CA, Issa JP, Herman JG, March TH, Baylin SB (2003) Inhibition of DNA methylation and histone deacetylation prevents murine lung cancer. Cancer Res 63(21):7089–7093
213. Goldberg J, Gryn J, Raza A, Bennett J, Browman G, Bryant J, Grunwald H, Larson R, Vogler R, Preisler H (1993) Mitoxantrone and 5-azacytidine for refractory/relapsed ANLL or CML in blast crisis: a leukemia intergroup study. Am J Hematol 43(4):286–290
214. Hakami N, Look AT, Steuber PC, Krischer J, Castleberry R, Harris R, Ravindranath Y, Vietti TJ (1987) Combined etoposide and 5-azacitidine in children and adolescents with refractory or relapsed acute nonlymphocytic leukemia: a Pediatric Oncology Group Study. J Clin Oncol 5(7):1022–1025
215. Huang Y, Nayak S, Jankowitz R, Davidson NE, Oesterreich S (2011) Epigenetics in breast cancer: what's new? Breast Cancer Res 13(6):225
216. Kim MS, Blake M, Baek JH, Kohlhagen G, Pommier Y, Carrier F (2003) Inhibition of histone deacetylase increases cytotoxicity to anticancer drugs targeting DNA. Cancer Res 63(21):7291–7300
217. Leshin M (1985) 5-Azacytidine and sodium butyrate induce expression of aromatase in fibroblasts from chickens carrying the henny feathering trait but not from wild-type chickens. Proc Natl Acad Sci USA 82(9):3005–3009
218. Liu WH, Yung BY (1998) Mortalization of human promyelocytic leukemia HL-60 cells to be more susceptible to sodium butyrate-induced apoptosis and inhibition of telomerase activity by down-regulation of nucleophosmin/B23. Oncogene 17(23):3055–3064
219. Momparler RL, Bouffard DY, Momparler LF, Dionne J, Belanger K, Ayoub J (1997) Pilot phase I-II study on 5-aza-2′-deoxycytidine (Decitabine) in patients with metastatic lung cancer. Anticancer Drugs 8(4):358–368
220. Pollyea DA, Kohrt HE, Gallegos L, Figueroa ME, Abdel-Wahab O, Zhang B, Bhattacharya S, Zehnder J, Liedtke M, Gotlib JR, Coutre S, Berube C, Melnick A, Levine R, Mitchell BS, Medeiros BC (2012) Safety, efficacy and biological predictors of response to sequential azacitidine and lenalidomide for elderly patients with acute myeloid leukemia. Leukemia 26(5):893–901
221. Schwartsmann G, Fernandes MS, Schaan MD, Moschen M, Gerhardt LM, Di Leone L, Loitzembauer B, Kalakun L (1997) Decitabine (5-Aza-2′-deoxycytidine; DAC) plus daunorubicin as a first line treatment in patients with acute myeloid leukemia: preliminary observations. Leukemia 11(suppl 1):S28–S31
222. Willemze R, Archimbaud E, Muus P (1993) Preliminary results with 5-aza-2′-deoxycytidine (DAC)-containing chemotherapy in patients with relapsed or refractory acute leukemia. The EORTC Leukemia Cooperative Group. Leukemia 7(suppl 1):49–50

Chapter 15
Methods for Cancer Epigenome Analysis

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Abstract Accurate detection of epimutations in tumor cells is crucial for understanding the molecular pathogenesis of cancer. Alterations in DNA methylation in cancer are functionally important and clinically relevant, but even this well-studied area is continually re-evaluated in light of unanticipated results, such as the strong association between aberrant DNA methylation in adult tumors and polycomb group profiles in embryonic stem cells, cancer-associated genetic mutations in epigenetic regulators such as *DNMT3A* and *TET* family genes, and the discovery of altered 5-hydroxymethylcytosine, a product of TET proteins acting on 5-methylcytosine, in human tumors with TET mutations. The abundance and distribution of covalent histone modifications in primary cancer tissues relative to normal cells is an important but largely uncharted area, although there is good evidence for a mechanistic role of cancer-specific alterations in histone modifications in tumor etiology, drug response, and tumor progression. Meanwhile, the discovery of new epigenetic marks continues, and there are many useful methods for epigenome analysis applicable to primary tumor samples, in addition to cancer cell lines. For DNA methylation and hydroxymethylation, next-generation sequencing allows increasingly inexpensive and quantitative whole-genome profiling. Similarly, the refinement and maturation of chromatin immunoprecipitation with next-generation sequencing (ChIP-seq) has made possible genome-wide mapping of histone modifications, open chromatin, and transcription factor binding sites. Computational tools have been developed apace with these epigenome methods to better enable accurate interpretation of the profiling data.

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Abbreviations

5MC	5-methylcytosine
5HMC	5-hydroxymethylcytosine
ChIP-seq	Chromatin immunoprecipitation-sequencing
MBD	Methyl binding domain
MeDIP	Methyl DNA immunoprecipitation
MRE	Methyl-sensitive restriction enzyme
RRBS	Reduced representation bisulfite sequencing

15.1 Introduction

DNA methylation is required for genome function through its roles in maintenance of chromatin structure, chromosome stability, and transcription [1–4]. 5-methylcytosine (5MC) is found at a subset of 5′-CpG-3′ dinucleotides and is also sometimes observed at CpNpG, notably in embryonic stem cells [5–7] but also in adult tissues [8]. The modified DNA base 5-hydroxymethylcytosine (5HMC) is also present in mammalian genomes, albeit at a much lower levels compared to 5MC [9, 10]. TET proteins catalyze the hydroxylation of 5MC to generate 5HMC, and can act further on 5HMC to yield 5-formylcytosine and carboxylcytosine [10–12].

The N-terminal tails of histone proteins are modified by acetylation, methylation, phosphorylation, ubiquitylation, crotonylation [13], and other covalent modifications. At some histone residues, such as histone H3 lysine 4 (H3K4), methylation can be mono-, di-, or tri-methyl. Furthermore, multiple types of modifications can exist on a single histone molecule. In addition to DNA methylation and histone modifications, there are other interrelated, potentially epigenetic mechanisms including specific deposition of histone variants, noncoding RNAs, chromatin remodeling, and nuclear organization, which are not discussed here. Current epigenomic methods, especially those making use of next-generation sequencing, provide powerful tools to map 5MC, 5HMC, and histone modifications at high resolution across the genome. However, there are many considerations for selecting the most suitable method, including ease of use, cost, resolution, specificity, quantitation, and availability of computational methods to analyze the data. We describe current epigenomic methods below, focusing primarily on genome-scale mapping methods that use next-generation sequencing.

15.2 Methods for Measurement of DNA Methylation and Hydroxymethylation

There are three main approaches to detect 5MC and 5HMC. Methyl-sensitive restriction enzymes (MRE) cut DNA based on methylation status of cytosines within their recognition sequences (Fig. 15.1a). A second approach includes

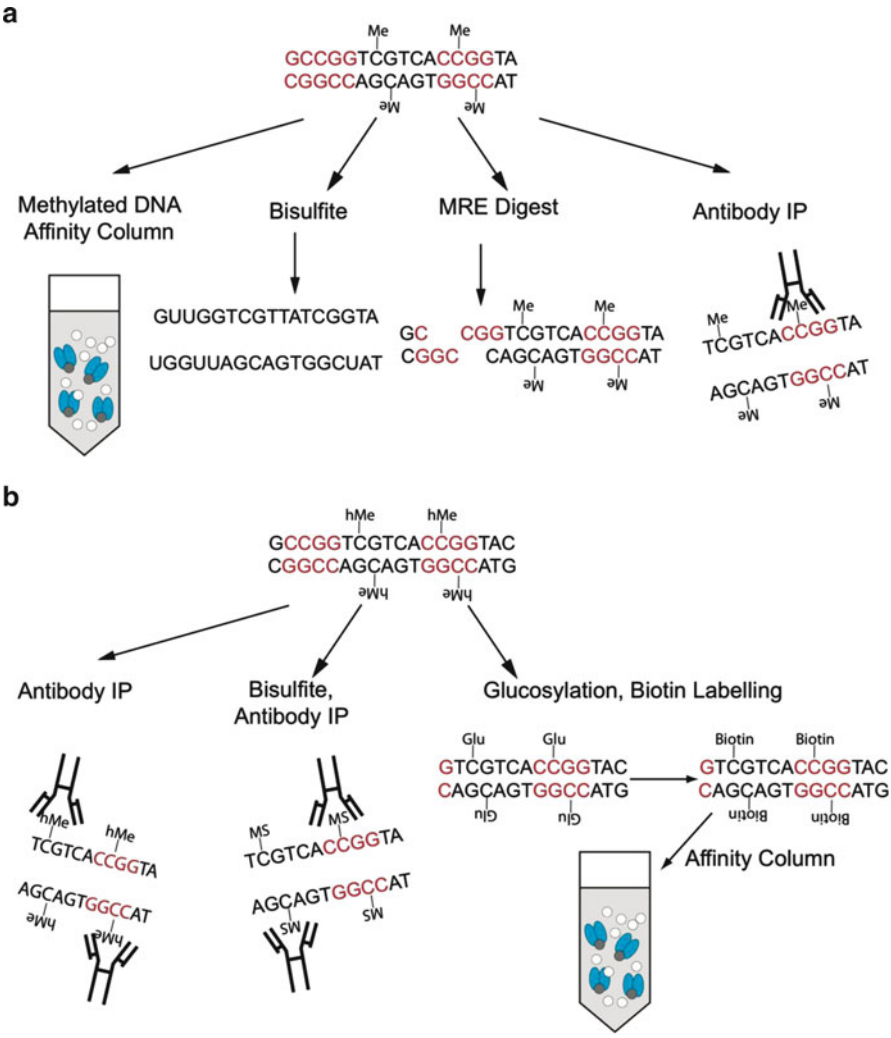

Fig. 15.1 A summary of methods for direct detection of cytosine methylation and hydroxymethylation. (**a**) Methylated DNA can be detected with methyl-sensitive restriction enzymes (MRE), the use of antibodies specific for 5-methylcytosine (5MC), by binding to affinity columns that contain methylated DNA binding domains or by the conversion of DNA with sodium bisulfite. It is important to note that some MRE are also sensitive to hydroxymethylation. (**b**) Several methods have been developed to detect 5 hydroxymethylcytosine (5HMC). These include the addition of a biotin tag to 5HMC through glucosylation and subsequent chemical steps which is followed by an affinity pulldown of the biotin tag, the use of antibodies specific for 5HMC and conversion of 5HMC to 5-cytosine methylenesulfonate (MS) which is then immunoprecipitated with an antibody specific to 5CMS. *Me* methylated cytosine; *hMe* hydroxymethylated cytosine; *Glu* glucosylated cytosine

differential chemical conversion or enzymatic modification of cytosine according to methylation/hydroxymethylation status, such as sodium bisulfite conversion and 5HMC-specific glucosylation. Third, enrichment methods include methyl DNA immunoprecipitation (MeDIP), hydroxyMeDIP (hMeDIP), and methyl binding domain (MBD) affinity purification that are used to enrich for methylated or hydroxymethylated regions. These approaches can be applied to investigate a single locus, hundreds of thousands of loci, or to all mappable sites genome-wide.

15.2.1 Overview of DNA Methylation Reagents

MRE have been used widely for precise, reliable, and inexpensive methylation detection. MRE only assay CpGs within their recognition sites but when multiple non-redundant and frequent-cutting MRE are used in parallel, this limitation is less problematic. There are approximately 50 unique MRE, though only a few have a methylation-insensitive isoschizomer. MRE can resolve the methylation status regionally or at individual CpGs, depending on the platform used following MRE digestion. Some MRE are inhibited by methylation or hydroxymethylation, for example, *Hpa*II [10]. The reliability of MRE enables their straightforward application to next-generation sequencing (MRE-seq) allowing analysis of greater than one million CpGs.

Antibodies against 5MC and 5HMC, and columns containing methylated DNA-binding proteins (domains of MBD2 or MeCP2 alone, or MBD2b combination with MBD3L) allow enrichment for 5MC/5HMC independent of DNA sequence (Fig. 15.1a, b) [14–17]. Enrichment is greater for regions with higher methylated CpG content relative to fully methylated regions with lower CpG content. These reagents are simple to use and many are commercially available. The lower-limit of resolution is determined initially by the size range of DNA prior to enrichment, generally 100–300 bp, and subsequently by the platform used to assess the enrichment, commonly oligonucleotide arrays and next-generation sequencing.

Chemicals including sodium bisulfite and hydrazine react differentially with unmethylated vs. methylated cytosine and allow DNA methylation mapping at single base resolution (Fig. 15.1a) [18–20]. Of these, sodium bisulfite is the most commonly used as it results in a positive display of methylation, among other advantages. Sodium bisulfite initiates conversion of cytosine to uracil, which is replaced by thymine during PCR amplification. In contrast, methylated cytosines are non-reactive, and remain as cytosine after bisulfite treatment. Sequencing of individual clones of the PCR product allows assessment of methylation status of contiguous CpGs derived from a single genomic DNA fragment. Bisulfite has many advantages, including single CpG resolution, detection of strand and allele-specific methylation, and detection of non-CpG cytosine methylation. Unlike other methylation-detection reagents, bisulfite provides estimates of absolute rather than relative DNA methylation levels, depending on the platform used. The reduced sequence complexity of the genome following bisulfite treatment complicates its

application to oligonucleotide arrays [21], but is not a major issue when a sequencing platform is used. Hydroxymethylated cytosines are resistant to conversion to uracil and are indistinguishable from 5MC in bisulfite sequencing. The reaction of 5HMC with bisulfite yields cytosine methylenesulfonate, which can be specifically detected with an affinity method [22]. Alternatively, the hydroxyl group of 5HMC can be enzymatically glucosylated and biotin labeled to detect 5HMC [22, 23].

15.2.2 Methyl-Sensitive Restriction Enzyme Methods

The HTF (*Hpa*II tiny fragments) enrichment by ligation-mediated PCR, or HELP assay, uses the methyl-sensitive *Hpa*II along with its methylation-insensitive isoschizomer *Msp*I to identify unmethylated CpG sites within the sequence 5′-CCGG-3′ [24]. Genomic DNA digested separately with each enzyme is size-selected to capture small DNA fragments. Custom adaptors complementary to digest ends are ligated and the adaptor-ligated molecules are amplified by PCR. The amplification products can be analyzed using a variety of platforms, including next-generation sequencing on the Illumina platform (HELP-seq) [25]. Methyl-seq is a second Illumina sequencing-based assay that uses *Hpa*II/*Msp*I [26]. Similar to HELP, the protocol involves separate *Hpa*II and *Msp*I digests, adaptor ligation, and Illumina sequencing. Approximately 65% of the CpG islands (CGIs) in the human genome are sampled using Methyl-seq. MRE methods are generally biased to CGIs, which constitute 1–2% of the genome and 7% of all CpGs in the genome. Methyl-seq is similarly biased, though non-CGI sites account for ~61% of the regions assayed, including a variety of genomic sequences such as promoters, exons, introns, and intergenic regions.

Ball et al. reported a third variation of MRE-seq, using *Hpa*II/*Msp*I digestion with Illumina sequencing to analyze DNA methylation in the PGP1 EBV-transformed B-lymphocyte cell line [27]. This approach, termed methyl-sensitive cut counting (MSCC), assayed ~1.4 million unique *Hpa*II sites. Using MSCC and a complementary method, bisulfite padlock probe sequencing (BSPP) to assay the methylation status of approximately 10,000 CpGs, highly expressed genes were found to be associated with high gene-body methylation and low promoter methylation. MSCC read counts were linearly related to BSPP percent methylation at 381 CpG sites that were assayed with both methods, suggesting that MSCC allows relative quantification of methylation levels.

DNA methylation has also been assessed through traditional Sanger sequencing combined with MRE in digital karyotyping [28, 29]. Using a combination of MRE that recognize 6–8 bp sites and methylation insensitive restriction enzymes, a library of short sequence tags is generated. The number of tags sequenced reflects the level of methylation at each recognition site, with lower tag counts representing greater methylation levels. In this method, the number of sites analyzed depends on the MRE used—use of *Asc*I, for example, can generate over 5,000 unique tags that correspond to >4,000 genes.

These sequencing-based methods demonstrate the utility of MRE for analysis of DNA methylation. The single CpG resolution and ability to assay a significant portion of the methylome with next-generation sequencing, including most CGIs, makes this a powerful, accurate, and straightforward way to assess methylation across the genome. When used alone, the MRE-seq methods enable relative rather than absolute methylation levels to be estimated. An integrative method [30, 31] that combines MRE-seq in parallel with MeDIP-seq to increase resolution, CpG coverage, and accuracy in quantitation is discussed below.

15.2.3 *Mcr*BC and CHARM

The methylation-dependent restriction enzyme *Mcr*BC recognizes methylated DNA and cuts near its recognition sequence. *Mcr*BC recognizes $R^mC(N)_{55–103}R^mC$ and cuts once between each pair of half-sites, close to one half-site or the other. The cuts can be distributed over several base pairs and approximately 30 base pairs distant from the methylated base, generating a distribution of DNA ends rather than precisely defined DNA ends. *Mcr*BC is useful to size-separate methylated DNA from unmethylated DNA, since the unmethylated DNA remains high-molecular weight after digestion. *Mcr*BC was initially applied to microarrays [32].

The "comprehensive high-throughput arrays for relative methylation" (CHARM) method is an array-based technique for methylation profiling using *Mcr*BC [33]. To improve specificity and sensitivity, probes were optimized based on location and CpG density on custom arrays. Because neighboring CpG sites tend to have a highly correlated methylation status, neighboring probe signals are averaged to reduce background noise without loss of sensitivity or specificity, though modestly reducing resolution. By comparing CHARM to MeDIP or *Hpa*II on arrays, Irizarry et al. showed that *Mcr*BC yields better methylome coverage than *Hpa*II and less bias for CpG density than MeDIP. Using CHARM, aberrant DNA methylation was found in colon cancer at sequences up to 2 kb flanking CGIs, referred to as CGI shores [34]. These data demonstrate the utility of *Mcr*BC-based methylation detection, and the new biological insights afforded by the CHARM method.

15.2.4 Methyl DNA Immunoprecipitation

In addition to MRE and *Mcr*BC, methylation can be assessed by immunoprecipitation of methylated DNA with a monoclonal antibody against 5-methylcytidine (MeDIP) [14]. This antibody does not recognize 5HMC [35], which can be specifically immunoprecipitated with an anti-5HMC antibody [36–39]. A major advantage of MeDIP-based detection is that it is not limited to a specific restriction site and theoretically any fragment with a methylated cytosine is immunoprecipitated. One approach involves the coupling of MeDIP with DNA

microarrays to obtain relative methylation levels at the loci represented on the array [14, 40–44].

MeDIP combined with next-generation sequencing (MeDIP-seq) can be used to interrogate the majority of mappable CpG and non-CpG cytosines in the genome. In a step forward from array-based methods, MeDIP-seq allows analysis of monoallelic methylation and methylation in a significant number of repeat sequences. Most protocols generate a MeDIP sequencing library by sonicating DNA followed by end-repair, adaptor ligation, immunoprecipitation with the anti-methylcytidine antibody and PCR amplification. The methylation-enriched library is sequenced and the reads are mapped back to a reference genome. A specific genomic region shows higher read density when methylated in one sample compared to when the same region is unmethylated in another sample, although read density between different regions is affected by the density of methylated CpGs, DNA copy number, and potentially other factors (discussed in Robinson et al. [45, 46]). These considerations are also important for MBD affinity-based approaches. MeDIP-seq has been applied to a variety of sample types from multiple organisms including human cancer [30, 31, 47–53].

Several computational methods have been specifically designed for analyzing MeDIP data while addressing local density of methylated CpGs. MEDME (modeling experimental data with MeDIP enrichment) is a combination of analytical and experimental methodologies that improve the interpretation of MeDIP-chip data, and addresses the non-linear relationship between enrichment signal and CpG density that is particular to MeDIP-chip [54]. A second analytical method for MeDIP-chip and also MeDIP–seq data called Bayesian tool for methylation analysis (BATMAN) uses a CpG density-derived coupling factor to quantify methylation levels across a range of CpG densities [47]. MEDIPS is a third approach that, like BATMAN, uses a CpG density coupling factor and in addition provides a framework for evaluating quality control parameters, estimating absolute methylation and comparing samples to detect regions of statistically significant differential methylation [51]. MeDIP-chip and MeDIP-seq are lower resolution compared to bisulfite-based methods. On the other hand, MeDIP-seq provides comprehensive methylome coverage at a fraction of the cost of shotgun bisulfite sequencing. Experimental and computational advances should enable increased resolution and quantitation of methylation levels using MeDIP-seq alone or in combination with MRE-seq.

15.2.5 Affinity-Based Enrichment Using Methyl Binding Domains

The Methylated CpG Island Recovery Assay (MIRA) is an alternative to MeDIP for selecting/enriching for methylated DNA, particularly at CGIs [15–17]. MIRA involves size fractionation of DNA, either by sonication or with *Mse*I which recognizes 5′-TTAA, a site that is typically found outside of CGIs. After digestion, adaptors are ligated to the DNA followed by selective binding of methylated fragments

on a column with full-length MBD2b and MBD3L1 proteins. MBD2b is a methyl-binding protein that exhibits a high affinity for methylated DNA relative to unmethylated DNA [15]. MBD3L1 lacks a methyl-CpG binding domain but can interact with MBD2b and improves enrichment of methylated DNA [15]. The methylated DNA eluted from the column is amplified by PCR, fluorescently labeled and hybridized to a microarray.

There are several similar approaches that combine affinity enrichment with Illumina sequencing. In MethylCap-seq, the MBD of MeCP2 is used to capture methylated DNA fragments after sonication [52, 55]. Binding occurs at low salt concentration and then a step-wise elution of captured DNA is performed by increasing the salt concentration, allowing collection of fractions with differing methylated CpG density, with highly methylated, CpG-dense fragments eluting at the higher salt concentrations. The eluates can be sequenced separately or pooled. The MBD2 MBD alone can be used for enrichment followed by Illumina sequencing, called MBD-isolated Genome Sequencing (MiGS) [56]. In this protocol, a single elution is performed. MBD2 enrichment with serial elution in increasing salt has been called MBD-seq [31, 57] or MBDCap-seq [45].

Several studies have directly compared MeDIP-seq with MBD affinity-based sequencing. Harris et al. found that MeDIP-seq and MBD-seq were 99% concordant using binary methylation calls in 200 bp windows or 1,000 bp windows [31]. MeDIP-seq enriched more at regions of low methylated CpG density compared to MBD-seq. Also, MeDIP-seq appeared to detect non-CpG methylation (i.e., at CpNpG) but MBD-seq did not, as predicted. Bock et al. compared MeDIP-seq with MethylCap-seq and observed similar levels of accuracy in quantifying methylation when comparing each to Infinium 27 K data. In both of these studies, MeDIP-seq and MBD affinity-based sequencing performed well in comparison with bisulfite next-generation sequencing.

15.2.6 Integrative MeDIP- and MRE-seq

MeDIP-seq and other affinity-based methods provide a positive display of methylated loci, and the absence of signal usually represents unmethylated loci, but also could be a result of regions that are difficult to PCR amplify or sequence, or insufficient sequencing depth. A method that combines MeDIP-seq with MRE-seq leverages their complementarity [30, 31, 58]. Independent MeDIP-seq and MRE-seq libraries are generated from the same DNA sample and sequenced separately. For MRE-seq, three to five parallel digests are performed using the MRE *Hpa*II, *Aci*I, *Hin*61, *Bsh*1236I, and *Hpy*CH4IV; the digests are size-selected and combined into a single library. Because the restriction sites from these enzymes are non-overlapping, each additional enzyme greatly increases coverage of unique CpG sites. At a moderate sequencing depth integrated MeDIP- and 3 enzyme MRE-seq together interrogate either uniquely or as multimapping sites ~22 million of the ~29 million

CpGs in the haploid human genome [31]. The integrative method is useful for detecting intermediate methylation, including regions of allelic methylation that overlap with monoallelic histone modifications and monoallelic gene expression [31]. This illustrates another significant advantage of sequencing-based epigenome analyses—the ability to assign an epigenetic state to a given genetic allele. For extensive DNA methylation profiles of human cells and tissues, see http://vizhub.wustl.edu/.

15.2.7 Indirect Methylation Detection with Demethylating Agents and Expression Arrays

Genetic or chemical inhibition of DNA methylation followed by expression array analysis can identify genes that may have been silenced by DNA methylation [59–63]. siRNA or shRNA can be used to knock down the DNA methyltransferases, or cell lines can be treated with demethylating agents such as 5-aza-2′deoxycytidine (5-aza) alone, or 5-aza in combination with histone deacetylase inhibitors. 5-aza is a cytidine analog that is incorporated into DNA and covalently binds and inhibits DNA methyltransferase, resulting in passive demethylation. 5-aza treatment results in activation of genes that were silenced by DNA methylation, provided that the appropriate transcription factors are present. However, interpretation of this indirect assessment of methylation is complicated by the fact that genes lacking promoter methylation may also exhibit an increase in expression following 5-aza treatment [64]. Presumably this results from demethylation at other loci within the same gene or in genes upstream that are required for its expression, though direct effects on unmethylated regulatory elements cannot be ruled out. Furthermore, this approach is best applied to cells grown in culture such as cell lines or early passage primary cells [65], as 5-aza requires replication to induce passive demethylation. The application of this approach to cultured tumor cells is complicated by epigenetic silencing that results from long-term culturing, rather than cancer or cell type-specificity.

15.2.8 Reduced Representation Bisulfite Sequencing

Bisulfite treatment converts unmethylated cytosines to uracil but methylcytosine and hydroxymethylcytosine are resistant to conversion. When followed by cloning and Sanger sequencing, this approach yields quantitative, allelic, contiguous, and base resolution of cytosine methylation. However, the shotgun bisulfite approach has been quite expensive for mammalian methylomes. It is important to note that hydroxymethylcytosine and methylcytosine cannot be distinguished by bisulfite sequencing as both block conversion.

To retain the advantages of methylation detection by bisulfite while reducing the cost of shotgun bisulfite sequencing, Meissner et al. developed a technique that interrogates DNA fragments from a reduced representation of the bisulfite-treated genome [66–68]. The reduction comes from DNA digestion with methylation-insensitive restriction enzyme *Msp*I and fragment size selection. After digestion, the ends of the DNA are filled-in with dGTP and methylated dCTP, followed by the addition of an A overhang to enable adaptor ligation. The adaptors used for this assay are methylated at cytosine residues to prevent conversion during bisulfite treatment. The adaptor-ligated DNA is then size selected on a gel and two fractions are excised—the sizes of which depend on the organism. For mouse DNA, approximately 300,000 *Msp*I fragments that span 40–220 bp are analyzed, which corresponds to nearly 1.4 million CpG sites analyzed at the nucleotide level [67]. These fragments are then bisulfite treated, PCR amplified, and size selected again to generate a sequencing library. Several factors must be considered with this approach. First, the choice of a restriction enzyme to fractionate the DNA will bias the portion of the genome that is represented. A second consideration is the process of mapping reads of bisulfite-converted DNA to the genome. Several mapping algorithms for "bisulfite genomes" have been developed [67, 69–71]. Compared to other sequencing methods, reduced representation bisulfite sequencing (RRBS) provides an efficient way to generate absolute quantification of methylation of more than one million CpG sites at single base pair resolution. Methylation at non-CpG cytosines can also be assessed by RRBS [8]. RRBS has been successfully applied to nanogram quantities of genomic DNA [72] and to large numbers of human cell and tissue types (http://vizhub.wustl.edu/).

15.2.9 Shotgun Bisulfite Sequencing

Shotgun sequencing of bisulfite-treated DNA has been successfully applied to several organisms, including humans [7, 69, 70, 73–78] and provides comprehensive, single cytosine quantification of methylation level when sequence coverage is sufficiently deep. A single-CpG-resolution shotgun bisulfite experiment on human DNA requires hundreds of millions of sequencing reads, with the exact number varying depending on the desired sequencing depth and on read lengths [78]. Many regions >200 bp in the mammalian genome do not contain CpGs and thus a large number of sequence reads may be uninformative, at least for CpG methylation. Prior selection of sequences, for example, through sequence capture methodology, or enrichment of methylated DNA or unmethylated DNA followed by shotgun sequencing could increase the efficiency and decrease the cost of this approach. Bisulfite sequencing that first employs selective "reduction" of the genome (e.g., RRBS) is far less expensive. Nevertheless, the cost of sequencing full DNA methylomes has decreased 20-fold since the first human methylome [7]. Shotgun bisulfite methylomes have been generated for a breast cancer cell line and primary human

mammary epithelial cells [79] and primary colorectal cancer and adjacent normal colon tissue [80].

RRBS and shotgun bisulfite sequencing require algorithms that are tailored to mapping the sequence reads from bisulfite-treated DNA back onto the genome. Several algorithms have been developed for this computationally intensive problem [67, 69–71, 81, 82]. The reduction in base complexity from the bisulfite conversion and the fact that a CpG can be methylated or unmethylated are issues that are addressable though complex when aligning bisulfite reads. Due to the bisulfite conversion process, the forward and reverse strands of DNA are no longer complementary and the sequence reads therefore are aligned to four different bisulfite-converted genomes: forward BS, forward BS reverse complement, reverse BS, reverse BS reverse complement). Thus, for this mapping there is increased search space along with a reduction of sequence complexity, requiring significant computation time for the read mapping [31].

15.2.10 Other Bisulfite Methods

Illumina Infinium methylation assays are mid-range platforms using bisulfite conversion and bead arrays to quantify methylation levels at individual CpGs. The HumanMethylation27 and HumanMethylation450 formats interrogate 27,578 and >450,000 CpGs, respectively. Bead-bound oligonucleotides corresponding to the methylated and unmethylated states of a single CpG site are hybridized to bisulfite-converted DNA and differentially labeled with Cy3 or Cy5. The methylation level is determined by the ratio of Cy3 and Cy5 fluorescence on the bead array. The HumanMethylation27 BeadChip interrogates 12 samples at a time and includes probes from 1,000 cancer-related genes and from putative promoters of 110 miRNA, among others. While there are on average 2 CpG sites assayed per gene for the majority of genes, 150 genes known to exhibit aberrant tumor-specific methylation are assayed at 5–10 CpGs each. The vast majority of 27 K probes are located in promoters. The 450 K platform expands the genomic regions that are assayed by Infinium. Genes are broadly profiled, with probes in the promoter, 5′ UTR, first exon, gene body, and 3′ UTR. Ninety nine percent of CGIs have probes, and the CGI shores, 2 kb regions flanking CGIs, and regions flanking shores, called "shelves," are also examined for most CGIs. Like the 27 K assay, a single 450 BeadChip can assay 12 samples. Both versions require 500 ng of DNA prior to bisulfite conversion. These methods do not assess multiple closely apposed CpGs individually, and such regions are generally avoided in the assay development. This bias is likely to impact biological insights drawn from this data.

Another bisulfite-based method, the Sequenom EpiTyper assay, utilizes MALDI-TOF mass spectrometry to analyze RNA cleavage fragments derived from post-bisulfite PCR products that contain a promoter to drive transcription [83, 84]. This unique assay allows high-throughput quantitative methylation analysis at hundreds

of loci, usually at single CpG resolution, and is quite useful for candidate loci in hundreds of samples, or as a follow-up to genome-wide profiling.

BSPPs are molecular inversion probes designed to target and capture specific CpG sites from bisulfite-converted DNA [27, 85]. The strategy is similar to RRBS in that a subset of CpG sites are analyzed by bisulfite sequencing to reduce the genomic space that must be covered, but with the advantage that particular CpGs can be assayed, instead of only those located within a set of restriction fragments. Tens of thousands of BSPPs can be amplified in single reaction and sequenced on the Illumina platform. Deng et al. were able to assay ~66,000 CpG sites, primarily in CGIs [85]. A prominent advantage of this technology is that it is customizable and can target a specific set of CpG sites of interest to the investigator.

15.3 Detection of 5-Hydroxymethylcytosine

5HMC is abundant in mammalian genomes. The tissue-specificity, genomic distribution, and functional significance of 5HMC are under investigation. Pre-existing 5MC is hydroxylated by the TET family of dioxygenases (TET1, TET2, and TET3) to yield 5HMC [10, 86]. TET proteins can further modify 5HMC resulting in formylmethylcytosine, carboxymethylcytosine, and possibly through steps mediated by base excision repair, unmodified cytosine [11, 12]. *TET1* is an *MLL* translocation partner in acute myeloid leukemia [87, 88] and *TET2* mutations occur in myeloid malignancies associated with decreased 5HMC [89], suggesting that dysregulation of 5HMC plays a role in cancer.

Detecting and quantifying 5HMC is challenging because many reagents used for detecting 5MC do not distinguish 5HMC from 5MC. Like 5MC, 5HMC is resistant to C-to-U transition following bisulfite treatment [90], and these bases are indistinguishable by bisulfite cloning and sequencing or other bisulfite-based methods. In addition, 5HMC reacts with bisulfite to yield cytosine 5-methylenesulfonate (CMS) and DNA with dense CMS is inefficiently amplified during PCR due to *Taq* polymerase stalling at CMS sites [90]. As a result, quantification of hydroxymethylation in regions of dense 5HMC, if they exist in some biological contexts, may be underestimated with bisulfite-based methods. MRE-based methods also do not distinguish 5MC from 5HMC, depending on the enzymes used, such as *Hpa*II, which is inhibited by 5MC or 5HMC in its recognition sequence [10]. Finally, affinity-based 5MC methods (MeDIP-seq, MBD-seq, etc.) are specific to 5MC and do not detect 5HMC directly, but could indirectly enrich for regions with 5HMC when it occurs on the same DNA fragment as 5MC [35].

Global quantification of 5HMC levels (measuring the relative or absolute amount of 5HMC present within a DNA sample) can be assayed by thin layer chromatography (TLC) [9, 10] and high-performance liquid chromatography-mass spectrometry (HPLC-MS) [9, 91]. Recently, a profusion of 5HMC mapping techniques have also been developed, many of which can be employed for genome-wide analysis.

15.3.1 5HMC Glucosylation Methods

There are several methods based on in vitro glucosylation of 5HMC in DNA that can be used for global quantification or mapping of 5HMC. These methods use bacteriophage T4 beta-glucosyltransferase (BGT) to catalyze the addition of a glucose moiety to the hydroxyl group of 5HMC. For global quantification, a radiolabeled substrate (uridine 5′-diphosphate-[^{3}H]-glucose) is used in the BGT-catalyzed reaction. The amount of labeled substrate incorporated is compared to standards, allowing absolute quantification [92]. A mapping method called GLIB (glucosylation, periodate oxidation, biotinylation) combines glucosylation by BGT with subsequent chemical reactions, resulting in the addition of two biotin molecules to each 5HMC [22]. The biotin-tagged 5HMC DNA is then pulled down with streptavidin and sequenced on the Helicos single molecule platform. GLIB has high sensitivity, with 90% recovery of DNA fragments containing a single 5HMC molecule. Song et al. present a second mapping method, in which a chemically engineered glucose containing an azide group is transferred to 5HMC by BGT [23]. The azide group is then chemically tagged with biotin and affinity enriched, with global quantification performed using avidin-horseradish peroxidase and genome-wide mapping through Illumina sequencing. Finally, a method has been developed utilizing the restriction endonuclease *Msp*I, which cuts C^{m}CGG and C^{hm}CGG, but not C^{gluc}CGG sites. Locus-specific 5HMC can be estimated using *Msp*I digestion on BGT-modified DNA followed by quantitative PCR across the cleavage site [36, 93].

15.3.2 5HMC Affinity Enrichment Methods

There are two enrichment methods for 5HMC based on antibodies that detect 5HMC itself or 5-cytosine methylenesulfonate (CMS), the product of reacting 5HMC with sodium bisulfite. The 5HMC antibody with sequencing approach, hMeDIP-seq [36–39], is similar to MeDIP-seq, and informatic tools originally developed for MeDIP-seq data have been employed in hMeDIP-seq. Monoclonal and polyclonal anti-5HMC antibodies are commercially available, but their 5HMC-density dependence [22, 89], along with the relatively low genomic abundance of 5HMC in some tissues, might result in inefficient pulldown of 5HMC-sparse regions. The anti-CMS antibody sequencing approach was developed as a more sensitive, less density-dependent alternative to hMeDIP-seq [22]. CMS pulldown had lower background and decreased density dependence compared to commercial anti-5HMC antibodies. CMS-enriched libraries were sequenced on the Illumina platform. Since Illumina library construction protocols usually require at least one PCR step, the tendency of *Taq* polymerase to stall at regions of dense CMS could be problematic.

The rapid development of methods for the detection and quantification of 5HMC has paralleled the exciting pace of discovery of the distribution and potential

functional roles of this "sixth base." Computational tools that are specific for hMeDIP-seq and CMS-pulldown have not been reported yet. For hMeDIP-seq, tools developed for MeDIP-seq, such as MEDIPS [51] have been adapted [38]. Stroud et al. used SICER, which was originally developed for analyzing chromatin immunoprecipitation-sequencing (ChIP-seq) data for diffusely distributed histone modifications, to define regions of 5HMC enrichment [39]. The next generation of genome-wide mapping methods for 5HMC may involve direct detection of the modified base by single molecule sequencing [23, 94].

15.4 Chromatin Immunoprecipitation-Sequencing

Alterations in histone modification patterns and transcription factor binding impact gene expression and have been implicated in tumorigenesis, cancer cell stemness, metastasis, and drug resistance [95–98]. Chromatin immunoprecipitation coupled with next-generation sequencing (ChIP-seq) has become the gold standard to study histone modifications and transcription factor binding genome-wide. It provides higher resolution, improved signal-to-noise ratios, and when using indexed libraries, it is less expensive than coupling ChIP with microarrays (ChIP-chip) [99]. Fresh or fresh frozen tissue or cells are either kept native (N-ChIP) [100] or formaldehyde cross-linked to preserve weaker DNA–protein interactions (X-ChIP) [101], followed by cell lysis (Fig. 15.2). N-ChIP is primarily used for histone modifications, where the DNA histone interactions are inherently strong [99]. Antibody specificity and immunoprecipitation are more efficient with N-ChIP as epitopes can be disrupted by formaldehyde [100], however, N-ChIP cannot be applied to proteins with lower DNA binding affinities such as transcription factors. Cross-linking ameliorates this problem, and minimizes stochastic nucleosome movement that can occur during N-ChIP [100], however, it also may fix transient non-functional interactions and reacts at lysines which may create biases. Native or cross-linked chromatin is then fragmented by sonication or micrococcal nuclease (MNase) digestion. Both methods impart bias in downstream sequencing [102]. MNase creates higher resolution, primarily mononucleosome (~146 bp) fragments, but is less efficient at cutting between G and C bases, creating greater fragmentation bias [103, 104]. In contrast, sonication provides decreased resolution (200–600 bp) but is more uniform [99]. Fragmented chromatin is immunoprecipitated with an antibody that specifically recognizes the epitope of interest. The success of ChIP reactions is dependent on antibody quality. Polyclonal antibodies are advantageous for X-ChIP experiments, as they reduce the chance of cross-linking destroying antibody interactions [101], but may have increased cross-reactivity. Relative enrichment of ChIP DNA is assayed via qPCR. Enrichment varies greatly with the protein of interest, antibody quality, and positive and negative control regions of the genome that are used. To minimize the number of reads contributing to background noise, it is common to require greater enrichment in ChIP-seq (5–50-fold) when compared to single locus ChIP-PCR [102]. Purified ChIP DNA sequencing libraries are constructed by end

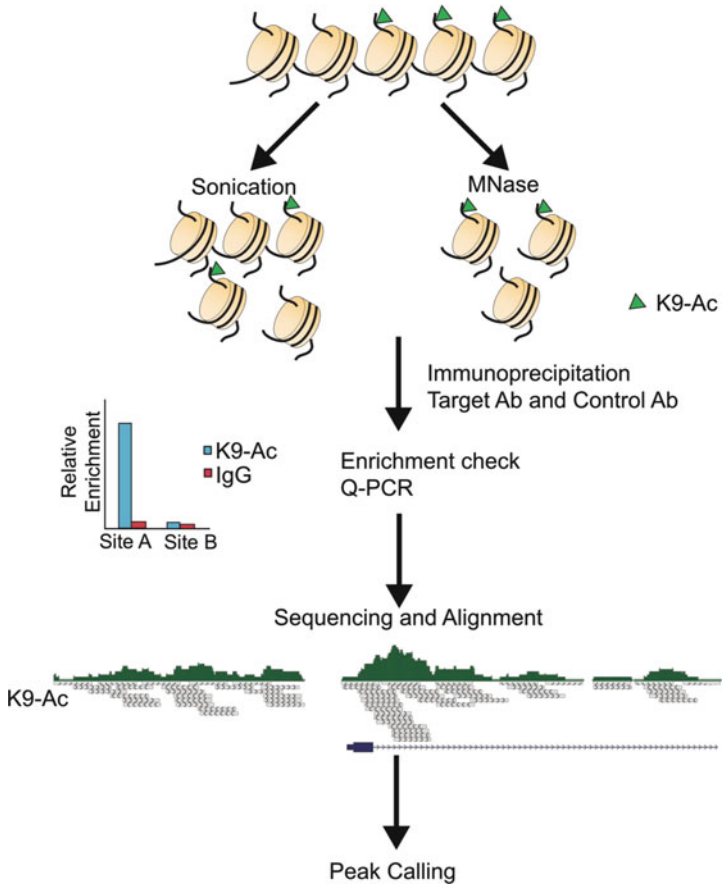

Fig. 15.2 Overview of chromatin immunoprecipitation-sequencing. DNA is fractionated via sonication (~200–600 bp) or with micrococcal nuclease (~146 bp). The fractionated DNA is then immunoprecipitated (IP) with a target antibody and an isotype control antibody. The efficiency of the immunoprecipitation is assayed by quantitative PCR, testing regions that are known to be bound (site A, positive control) or not bound (site B, negative control). The enriched DNA is then used to generate a DNA sequencing library, which is sequenced and reads are aligned to the appropriate genome. Each read is depicted as a *grey line*, the read densities are displayed above in *green* and a gene is shown in *blue*. Finally, the aligned reads are used to generate peaks that mark regions of statistically significant enrichment of reads for the IP of the histone mark or chromatin protein of interest

repair, A base addition, adapter ligation, PCR amplification and size selection. Additional bias may occur during library construction and PCR amplification, as both GC-rich and GC-poor regions are underrepresented [99, 102]. The total number of sequence reads required depends on the quality of ChIP enrichment, the expected number of peaks and peak size, but sequencing multiple-indexed ChIP libraries in a single lane is common practice.

15.4.1 ChIP-seq Data Analysis

Transforming the millions of sequencing reads generated by ChIP-seq into biologically interpretable data is a computationally demanding, multi-step process for which a variety of tools have been developed. While many tools address the same problem, each tool is different and can impact the final result. The first and most resource-intensive step is aligning the sequence reads to the genome. Most sequencing platforms come with alignment pipelines, however, third-party aligners are commonly used, such as MAQ [105], Bowtie [106], BWA [107], SOAP [108, 109], and PASH [110]. These packages differ by alignment algorithm, as well as how multi-aligning reads and gapped vs. un-gapped alignments are handled, resulting in differences in sensitivity and specificity. For most cancer samples a gapped aligner is preferred to allow for the variety of genetic aberrations accumulated in the tumor. Aligned reads are then analyzed to find enriched areas or "peaks" in the genome, for which a number of "peak calling" algorithms have been created [99, 111]. Though the exact method varies between programs, most shift tags based on chromatin fragment size to accumulate tags near the true binding site and increase peak resolution [111]. Regions of statistical enrichment of IP tags relative to a background control are calculated. The most commonly used control is input DNA isolated from the same chromatin batch as the ChIP [99]. This reduces false positives introduced from fragmentation and mappability biases, and controls for genetic differences such as copy number alterations that affect read density. Finally, peaks are filtered based on uneven distributions of sense and antisense tag accumulation [111]. Most current peak callers identify focal enrichments such as transcription factor binding sites, however, some have been developed for broader marks like histone modifications associated with heterochromatin [112–114]. Many groups are actively researching ways to reduce noise and increase true positives.

15.4.2 Application of ChIP-seq to Cancer Epigenomes

The network of transcription regulatory factor interactions and their effects on gene expression in cancer are under investigation. ChIP-seq was initially used to profile T-cells, and since then a main focus has been on embryonic stem cells and cell lines [115–117]. Recently, distinct chromatin states or "signatures" comprised of combinatorial histone marks have been linked to specific functional genomic elements by integrating multiple ChIP-seq data across human cell lines [118–120]. The combinatorial histone signatures identified in these studies have not been investigated in the context of tumor progression. Multidimensional epigenomic profiles of tumors also provide a novel means of sub-type classification, identifying prognostic markers, and insight into tumor cell of origin. ChIP-seq will also help the annotation and functional characterization of non-genic susceptibility loci, as has been recently performed in prostate cancer [121] and in GWAS studies [120]. New techniques are being developed to perform ChIP-seq on a small number of cells, creating an

opportunity to better analyze intratumoral heterogeneity of epigenomic patterns [122, 123]. Finally, chromosome conformation capture (3C) technology [124] and its high-throughput derivatives (4C [125], 5C [126], Hi-C [127], ChIP-Loop [128, 129], ChIA-PET [130]) detect distal DNA–DNA interactions (e.g., promoter-enhancer), but can also be used to identify complex genomic rearrangements in cancers [131]. Coupling ChIP with 3C technologies followed by sequencing will likely be a powerful way to study how both epigenetic patterns and associated structural interactions change during the process of tumorigenesis.

15.5 Future Directions

Recent unanticipated data offer new understanding of, and stimulate new investigations into aberrant epigenetic patterns in cancer. First, promoters with polycomb-mediated histone modifications in ES cells are among those commonly aberrantly hypermethylated in adult tumors [132–134]. Second, cancer-associated mutations occur in the DNA methyltransferase *DNMT3A* [135, 136], suggesting another possible origin of DNA methylation abnormalities, though this remains to be determined. Similarly, the occurrence of *TET1* translocation [87, 88] and *TET2* mutations in cancer points to an etiologic role for these epigenetic regulators and their marks. Finally, human tissues harbor abundant 5HMC, a product of TET proteins acting on 5MC, while cancers with TET mutations tend to have reduced 5HMC.

The future of cancer epigenomic methods will be shaped by two technological trends. First, the rapid pace of advances in next-generation sequencing will continue to improve 5MC/5HMC, histone modification, and chromatin conformation mapping. Genome-wide epigenomic experiments will become increasingly inexpensive and accessible, though paralleled with needs for increased computational power and data storage. Second, direct single molecule sequencing that distinguishes between modified bases without bisulfite conversion could revolutionize mapping of 5MC and 5HMC. For example, in single molecule real-time (SMRT) sequencing, fluorescently labeled nucleotides are incorporated by DNA polymerase on complementary DNA strands. Real-time monitoring of the kinetics of this process can identify both unmodified and modified bases, including N6-methyladenine, 5MC, and 5HMC [94]. SMRT sequencing has also been combined with selective glucosylation and cleavable biotin labeling of 5HMC to improve detection kinetics [23]. Similarly, the direct detection of modified bases via inexpensively produced nanopores, if they become amenable to high-throughput, could be technologically transformative [137].

References

1. Trasler JM, Trasler DG, Bestor TH, Li E, Ghibu F (1996) DNA methyltransferase in normal and Dnmtn/Dnmtn mouse embryos. Dev Dyn 206(3):239–247. doi:10.1002/(SICI)1097-0177(199607)206:3<239::AID-AJA2>3.0.CO;2-J [pii] 10.1002/(SICI)1097-0177(199607)206:3<239::AID-AJA2>3.0.CO;2-J

2. Chen RZ, Pettersson U, Beard C, Jackson-Grusby L, Jaenisch R (1998) DNA hypomethylation leads to elevated mutation rates. Nature 395(6697):89–93
3. Maraschio P, Zuffardi O, Dalla Fior T, Tiepolo L (1988) Immunodeficiency, centromeric heterochromatin instability of chromosomes 1, 9, and 16, and facial anomalies: the ICF syndrome. J Med Genet 25(3):173–180
4. Xu GL, Bestor TH, Bourc'his D, Hsieh CL, Tommerup N, Bugge M, Hulten M, Qu X, Russo JJ, Viegas-Pequignot E (1999) Chromosome instability and immunodeficiency syndrome caused by mutations in a DNA methyltransferase gene. Nature 402(6758):187–191. doi:10.1038/46052
5. Clark SJ, Harrison J, Frommer M (1995) CpNpG methylation in mammalian cells. Nat Genet 10(1):20–27. doi:10.1038/ng0595-20
6. Ramsahoye BH, Biniszkiewicz D, Lyko F, Clark V, Bird AP, Jaenisch R (2000) Non-CpG methylation is prevalent in embryonic stem cells and may be mediated by DNA methyltransferase 3a. Proc Natl Acad Sci USA 97(10):5237–5242. doi:97/10/5237 [pii]
7. Lister R, Pelizzola M, Dowen RH, Hawkins RD, Hon G, Tonti-Filippini J, Nery JR, Lee L, Ye Z, Ngo QM, Edsall L, Antosiewicz-Bourget J, Stewart R, Ruotti V, Millar AH, Thomson JA, Ren B, Ecker JR (2009) Human DNA methylomes at base resolution show widespread epigenomic differences. Nature 462(7271):315–322. doi:nature08514 [pii] 10.1038/nature08514
8. Ziller MJ, Muller F, Liao J, Zhang Y, Gu H, Bock C, Boyle P, Epstein CB, Bernstein BE, Lengauer T, Gnirke A, Meissner A (2011) Genomic distribution and inter-sample variation of non-CpG methylation across human cell types. PLoS Genet 7(12):e1002389. doi:10.1371/journal.pgen.1002389 PGENETICS-D-11-00694 [pii]
9. Kriaucionis S, Heintz N (2009) The nuclear DNA base 5-hydroxymethylcytosine is present in Purkinje neurons and the brain. Science 324(5929):929–930. doi:1169786 [pii] 10.1126/science.1169786
10. Tahiliani M, Koh KP, Shen Y, Pastor WA, Bandukwala H, Brudno Y, Agarwal S, Iyer LM, Liu DR, Aravind L, Rao A (2009) Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. Science 324(5929):930–935. doi:1170116 [pii] 10.1126/science.1170116
11. Ito S, Shen L, Dai Q, Wu SC, Collins LB, Swenberg JA, He C, Zhang Y (2011) Tet proteins can convert 5-methylcytosine to 5-formylcytosine and 5-carboxylcytosine. Science 333(6047):1300–1303. doi:science.1210597 [pii] 10.1126/science.1210597
12. He YF, Li BZ, Li Z, Liu P, Wang Y, Tang Q, Ding J, Jia Y, Chen Z, Li L, Sun Y, Li X, Dai Q, Song CX, Zhang K, He C, Xu GL (2011) Tet-mediated formation of 5-carboxylcytosine and its excision by TDG in mammalian DNA. Science 333(6047):1303–1307. doi:science.1210944 [pii] 10.1126/science.1210944
13. Tan M, Luo H, Lee S, Jin F, Yang JS, Montellier E, Buchou T, Cheng Z, Rousseaux S, Rajagopal N, Lu Z, Ye Z, Zhu Q, Wysocka J, Ye Y, Khochbin S, Ren B, Zhao Y (2011) Identification of 67 histone marks and histone lysine crotonylation as a new type of histone modification. Cell 146(6):1016–1028. doi:S0092-8674(11)00891-9 [pii] 10.1016/j.cell.2011.08.008
14. Weber M, Davies JJ, Wittig D, Oakeley EJ, Haase M, Lam WL, Schubeler D (2005) Chromosome-wide and promoter-specific analyses identify sites of differential DNA methylation in normal and transformed human cells. Nat Genet 37(8):853–862. doi:ng1598 [pii] 10.1038/ng1598
15. Rauch T, Li H, Wu X, Pfeifer GP (2006) MIRA-assisted microarray analysis, a new technology for the determination of DNA methylation patterns, identifies frequent methylation of homeodomain-containing genes in lung cancer cells. Cancer Res 66(16):7939–7947. doi:66/16/7939 [pii] 10.1158/0008-5472.CAN-06-1888
16. Rauch T, Wang Z, Zhang X, Zhong X, Wu X, Lau SK, Kernstine KH, Riggs AD, Pfeifer GP (2007) Homeobox gene methylation in lung cancer studied by genome-wide analysis with a microarray-based methylated CpG island recovery assay. Proc Natl Acad Sci USA 104(13):5527–5532. doi:0701059104 [pii] 10.1073/pnas.0701059104

17. Rauch TA, Wu X, Zhong X, Riggs AD, Pfeifer GP (2009) A human B cell methylome at 100-base pair resolution. Proc Natl Acad Sci USA 106(3):671–678. doi:0812399106 [pii] 10.1073/pnas.0812399106
18. Frommer M, McDonald LE, Millar DS, Collis CM, Watt F, Grigg GW, Molloy PL, Paul CL (1992) A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. Proc Natl Acad Sci USA 89(5):1827–1831
19. Pfeifer GP, Riggs AD (1996) Genomic sequencing by ligation-mediated PCR. Mol Biotechnol 5(3):281–288
20. Clark SJ, Statham A, Stirzaker C, Molloy PL, Frommer M (2006) DNA methylation: bisulphite modification and analysis. Nat Protoc 1(5):2353–2364. doi:nprot.2006.324 [pii] 10.1038/nprot.2006.324
21. Gitan RS, Shi H, Chen CM, Yan PS, Huang TH (2002) Methylation-specific oligonucleotide microarray: a new potential for high-throughput methylation analysis. Genome Res 12(1):158–164. doi:10.1101/gr.202801
22. Pastor WA, Pape UJ, Huang Y, Henderson HR, Lister R, Ko M, McLoughlin EM, Brudno Y, Mahapatra S, Kapranov P, Tahiliani M, Daley GQ, Liu XS, Ecker JR, Milos PM, Agarwal S, Rao A (2011) Genome-wide mapping of 5-hydroxymethylcytosine in embryonic stem cells. Nature 473(7347):394–397. doi:nature10102 [pii] 10.1038/nature10102
23. Song CX, Szulwach KE, Fu Y, Dai Q, Yi C, Li X, Li Y, Chen CH, Zhang W, Jian X, Wang J, Zhang L, Looney TJ, Zhang B, Godley LA, Hicks LM, Lahn BT, Jin P, He C (2011) Selective chemical labeling reveals the genome-wide distribution of 5-hydroxymethylcytosine. Nat Biotechnol 29(1):68–72. doi:nbt.1732 [pii] 10.1038/nbt.1732
24. Khulan B, Thompson RF, Ye K, Fazzari MJ, Suzuki M, Stasiek E, Figueroa ME, Glass JL, Chen Q, Montagna C, Hatchwell E, Selzer RR, Richmond TA, Green RD, Melnick A, Greally JM (2006) Comparative isoschizomer profiling of cytosine methylation: the HELP assay. Genome Res 16(8):1046–1055. doi:gr.5273806 [pii] 10.1101/gr.5273806
25. Oda M, Glass JL, Thompson RF, Mo Y, Olivier EN, Figueroa ME, Selzer RR, Richmond TA, Zhang X, Dannenberg L, Green RD, Melnick A, Hatchwell E, Bouhassira EE, Verma A, Suzuki M, Greally JM (2009) High-resolution genome-wide cytosine methylation profiling with simultaneous copy number analysis and optimization for limited cell numbers. Nucleic Acids Res 37(12):3829–3839. doi:gkp260 [pii] 10.1093/nar/gkp260
26. Brunner AL, Johnson DS, Kim SW, Valouev A, Reddy TE, Neff NF, Anton E, Medina C, Nguyen L, Chiao E, Oyolu CB, Schroth GP, Absher DM, Baker JC, Myers RM (2009) Distinct DNA methylation patterns characterize differentiated human embryonic stem cells and developing human fetal liver. Genome Res 19(6):1044–1056. doi:gr.088773.108 [pii] 10.1101/gr.088773.108
27. Ball MP, Li JB, Gao Y, Lee JH, LeProust EM, Park IH, Xie B, Daley GQ, Church GM (2009) Targeted and genome-scale strategies reveal gene-body methylation signatures in human cells. Nat Biotechnol 27(4):361–368. doi:nbt.1533 [pii] 10.1038/nbt.1533
28. Hu M, Yao J, Cai L, Bachman KE, van den Brule F, Velculescu V, Polyak K (2005) Distinct epigenetic changes in the stromal cells of breast cancers. Nat Genet 37(8):899–905. doi:ng1596 [pii] 10.1038/ng1596
29. Bloushtain-Qimron N, Yao J, Snyder EL, Shipitsin M, Campbell LL, Mani SA, Hu M, Chen H, Ustyansky V, Antosiewicz JE, Argani P, Halushka MK, Thomson JA, Pharoah P, Porgador A, Sukumar S, Parsons R, Richardson AL, Stampfer MR, Gelman RS, Nikolskaya T, Nikolsky Y, Polyak K (2008) Cell type-specific DNA methylation patterns in the human breast. Proc Natl Acad Sci USA 105(37):14076–14081. doi:0805206105 [pii] 10.1073/pnas.0805206105
30. Maunakea AK, Nagarajan RP, Bilenky M, Ballinger TJ, D'Souza C, Fouse SD, Johnson BE, Hong C, Nielsen C, Zhao Y, Turecki G, Delaney A, Varhol R, Thiessen N, Shchors K, Heine VM, Rowitch DH, Xing X, Fiore C, Schillebeeckx M, Jones SJ, Haussler D, Marra MA, Hirst M, Wang T, Costello JF (2010) Conserved role of intragenic DNA methylation in regulating alternative promoters. Nature 466(7303):253–257. doi:nature09165 [pii] 10.1038/nature09165

31. Harris RA, Wang T, Coarfa C, Nagarajan RP, Hong C, Downey SL, Johnson BE, Fouse SD, Delaney A, Zhao Y, Olshen A, Ballinger T, Zhou X, Forsberg KJ, Gu J, Echipare L, O'Geen H, Lister R, Pelizzola M, Xi Y, Epstein CB, Bernstein BE, Hawkins RD, Ren B, Chung WY, Gu H, Bock C, Gnirke A, Zhang MQ, Haussler D, Ecker JR, Li W, Farnham PJ, Waterland RA, Meissner A, Marra MA, Hirst M, Milosavljevic A, Costello JF (2010) Comparison of sequencing-based methods to profile DNA methylation and identification of monoallelic epigenetic modifications. Nat Biotechnol 28(10):1097–1105. doi:nbt.1682 [pii] 10.1038/nbt.1682
32. Rabinowicz PD, Schutz K, Dedhia N, Yordan C, Parnell LD, Stein L, McCombie WR, Martienssen RA (1999) Differential methylation of genes and retrotransposons facilitates shotgun sequencing of the maize genome. Nat Genet 23(3):305–308. doi:10.1038/15479
33. Irizarry RA, Ladd-Acosta C, Carvalho B, Wu H, Brandenburg SA, Jeddeloh JA, Wen B, Feinberg AP (2008) Comprehensive high-throughput arrays for relative methylation (CHARM). Genome Res 18(5):780–790. doi:gr.7301508 [pii] 10.1101/gr.7301508
34. Irizarry RA, Ladd-Acosta C, Wen B, Wu Z, Montano C, Onyango P, Cui H, Gabo K, Rongione M, Webster M, Ji H, Potash JB, Sabunciyan S, Feinberg AP (2009) The human colon cancer methylome shows similar hypo- and hypermethylation at conserved tissue-specific CpG island shores. Nat Genet 41(2):178–186. doi:ng.298 [pii] 10.1038/ng.298
35. Jin SG, Kadam S, Pfeifer GP (2010) Examination of the specificity of DNA methylation profiling techniques towards 5-methylcytosine and 5-hydroxymethylcytosine. Nucleic Acids Res 38(11):e125. doi:gkq223 [pii] 10.1093/nar/gkq223
36. Ficz G, Branco MR, Seisenberger S, Santos F, Krueger F, Hore TA, Marques CJ, Andrews S, Reik W (2011) Dynamic regulation of 5-hydroxymethylcytosine in mouse ES cells and during differentiation. Nature 473(7347):398–402. doi:nature10008 [pii] 10.1038/nature10008
37. Xu Y, Wu F, Tan L, Kong L, Xiong L, Deng J, Barbera AJ, Zheng L, Zhang H, Huang S, Min J, Nicholson T, Chen T, Xu G, Shi Y, Zhang K, Shi YG (2011) Genome-wide regulation of 5hmC, 5mC, and gene expression by Tet1 hydroxylase in mouse embryonic stem cells. Mol Cell 42(4):451–464. doi:S1097-2765(11)00283-8 [pii] 10.1016/j.molcel.2011.04.005
38. Williams K, Christensen J, Pedersen MT, Johansen JV, Cloos PA, Rappsilber J, Helin K (2011) TET1 and hydroxymethylcytosine in transcription and DNA methylation fidelity. Nature 473(7347):343–348. doi:nature10066 [pii] 10.1038/nature10066
39. Stroud H, Feng S, Morey Kinney S, Pradhan S, Jacobsen SE (2011) 5-Hydroxymethylcytosine is associated with enhancers and gene bodies in human embryonic stem cells. Genome Biol 12(6):R54. doi:gb-2011-12-6-r54 [pii] 10.1186/gb-2011-12-6-r54
40. Keshet I, Schlesinger Y, Farkash S, Rand E, Hecht M, Segal E, Pikarski E, Young RA, Niveleau A, Cedar H, Simon I (2006) Evidence for an instructive mechanism of de novo methylation in cancer cells. Nat Genet 38(2):149–153. doi:ng1719 [pii] 10.1038/ng1719
41. Fouse SD, Shen Y, Pellegrini M, Cole S, Meissner A, Van Neste L, Jaenisch R, Fan G (2008) Promoter CpG methylation contributes to ES cell gene regulation in parallel with Oct4/Nanog, PcG complex, and histone H3K4/K27 trimethylation. Cell Stem Cell 2(2):160–169. doi:S1934-5909(07)00327-X [pii] 10.1016/j.stem.2007.12.011
42. Shen Y, Matsuno Y, Fouse SD, Rao N, Root S, Xu R, Pellegrini M, Riggs AD, Fan G (2008) X-inactivation in female human embryonic stem cells is in a nonrandom pattern and prone to epigenetic alterations. Proc Natl Acad Sci USA 105(12):4709–4714. doi:0712018105 [pii] 10.1073/pnas.0712018105
43. Zhang X, Yazaki J, Sundaresan A, Cokus S, Chan SW, Chen H, Henderson IR, Shinn P, Pellegrini M, Jacobsen SE, Ecker JR (2006) Genome-wide high-resolution mapping and functional analysis of DNA methylation in arabidopsis. Cell 126(6):1189–1201. doi:S0092-8674(06)01018-X [pii] 10.1016/j.cell.2006.08.003
44. Zilberman D, Gehring M, Tran RK, Ballinger T, Henikoff S (2007) Genome-wide analysis of Arabidopsis thaliana DNA methylation uncovers an interdependence between methylation and transcription. Nat Genet 39(1):61–69. doi:ng1929 [pii] 10.1038/ng1929
45. Robinson MD, Stirzaker C, Statham AL, Coolen MW, Song JZ, Nair SS, Strbenac D, Speed TP, Clark SJ (2010) Evaluation of affinity-based genome-wide DNA methylation data: effects

of CpG density, amplification bias, and copy number variation. Genome Res 20(12):1719–1729. doi:gr.110601.110 [pii] 10.1101/gr.110601.110
46. Robinson MD, Statham AL, Speed TP, Clark SJ (2010) Protocol matters: which methylome are you actually studying? Epigenomics 2(4):587–598. doi:10.2217/epi.10.36
47. Down TA, Rakyan VK, Turner DJ, Flicek P, Li H, Kulesha E, Graf S, Johnson N, Herrero J, Tomazou EM, Thorne NP, Backdahl L, Herberth M, Howe KL, Jackson DK, Miretti MM, Marioni JC, Birney E, Hubbard TJ, Durbin R, Tavare S, Beck S (2008) A Bayesian deconvolution strategy for immunoprecipitation-based DNA methylome analysis. Nat Biotechnol 26(7):779–785. doi:nbt1414 [pii] 10.1038/nbt1414
48. Pomraning KR, Smith KM, Freitag M (2009) Genome-wide high throughput analysis of DNA methylation in eukaryotes. Methods 47(3):142–150. doi:S1046-2023(08)00182-5 [pii] 10.1016/j.ymeth.2008.09.022
49. Ruike Y, Imanaka Y, Sato F, Shimizu K, Tsujimoto G (2010) Genome-wide analysis of aberrant methylation in human breast cancer cells using methyl-DNA immunoprecipitation combined with high-throughput sequencing. BMC Genomics 11:137. doi:1471-2164-11-137 [pii] 10.1186/1471-2164-11-137
50. Li N, Ye M, Li Y, Yan Z, Butcher LM, Sun J, Han X, Chen Q, Zhang X, Wang J (2010) Whole genome DNA methylation analysis based on high throughput sequencing technology. Methods 52(3):203–212. doi:S1046-2023(10)00127-1 [pii] 10.1016/j.ymeth.2010.04.009
51. Chavez L, Jozefczuk J, Grimm C, Dietrich J, Timmermann B, Lehrach H, Herwig R, Adjaye J (2010) Computational analysis of genome-wide DNA methylation during the differentiation of human embryonic stem cells along the endodermal lineage. Genome Res 20(10):1441–1450. doi:gr.110114.110 [pii] 10.1101/gr.110114.110
52. Bock C, Tomazou EM, Brinkman AB, Muller F, Simmer F, Gu H, Jager N, Gnirke A, Stunnenberg HG, Meissner A (2010) Quantitative comparison of genome-wide DNA methylation mapping technologies. Nat Biotechnol 28(10):1106–1114. doi:nbt.1681 [pii] 10.1038/nbt.1681
53. Feber A, Wilson GA, Zhang L, Presneau N, Idowu B, Down TA, Rakyan VK, Noon LA, Lloyd AC, Stupka E, Schiza V, Teschendorff AE, Schroth GP, Flanagan A, Beck S (2011) Comparative methylome analysis of benign and malignant peripheral nerve sheath tumors. Genome Res 21(4):515–524. doi:gr.109678.110 [pii] 10.1101/gr.109678.110
54. Pelizzola M, Koga Y, Urban AE, Krauthammer M, Weissman S, Halaban R, Molinaro AM (2008) MEDME: an experimental and analytical methodology for the estimation of DNA methylation levels based on microarray derived MeDIP-enrichment. Genome Res 18(10):1652–1659. doi:gr.080721.108 [pii] 10.1101/gr.080721.108
55. Brinkman AB, Simmer F, Ma K, Kaan A, Zhu J, Stunnenberg HG (2010) Whole-genome DNA methylation profiling using MethylCap-seq. Methods 52(3):232–236. doi:S1046-2023(10)00166-0 [pii] 10.1016/j.ymeth.2010.06.012
56. Serre D, Lee BH, Ting AH (2010) MBD-isolated genome sequencing provides a high-throughput and comprehensive survey of DNA methylation in the human genome. Nucleic Acids Res 38(2):391–399. doi:gkp992 [pii] 10.1093/nar/gkp992
57. Lan X, Adams C, Landers M, Dudas M, Krissinger D, Marnellos G, Bonneville R, Xu M, Wang J, Huang TH, Meredith G, Jin VX (2011) High resolution detection and analysis of CpG dinucleotides methylation using MBD-Seq technology. PLoS One 6(7):e22226. doi:10.1371/journal.pone.0022226 PONE-D-11-02256 [pii]
58. Zhou X, Maricque B, Xie M, Li D, Sundaram V, Martin EA, Koebbe BC, Nielsen C, Hirst M, Farnham P, Kuhn RM, Zhu J, Smirnov I, Kent WJ, Haussler D, Madden PA, Costello JF, Wang T (2011) The human epigenome browser at washington university. Nat Methods 8(12):989–990. doi:10.1038/nmeth.1772 nmeth.1772 [pii]
59. Karpf AR, Peterson PW, Rawlins JT, Dalley BK, Yang Q, Albertsen H, Jones DA (1999) Inhibition of DNA methyltransferase stimulates the expression of signal transducer and activator of transcription 1, 2, and 3 genes in colon tumor cells. Proc Natl Acad Sci USA 96(24):14007–14012
60. Yamashita K, Upadhyay S, Osada M, Hoque MO, Xiao Y, Mori M, Sato F, Meltzer SJ, Sidransky D (2002) Pharmacologic unmasking of epigenetically silenced tumor suppressor

genes in esophageal squamous cell carcinoma. Cancer Cell 2(6):485–495. doi:S1535610802002155 [pii]
61. Foltz G, Yoon JG, Lee H, Ryken TC, Sibenaller Z, Ehrich M, Hood L, Madan A (2009) DNA methyltransferase-mediated transcriptional silencing in malignant glioma: a combined whole-genome microarray and promoter array analysis. Oncogene. doi:onc2009122 [pii] 10.1038/onc.2009.122
62. Cameron EE, Bachman KE, Myohanen S, Herman JG, Baylin SB (1999) Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. Nat Genet 21(1):103–107
63. Shames DS, Girard L, Gao B, Sato M, Lewis CM, Shivapurkar N, Jiang A, Perou CM, Kim YH, Pollack JR, Fong KM, Lam CL, Wong M, Shyr Y, Nanda R, Olopade OI, Gerald W, Euhus DM, Shay JW, Gazdar AF, Minna JD (2006) A genome-wide screen for promoter methylation in lung cancer identifies novel methylation markers for multiple malignancies. PLoS Med 3(12):e486. doi:06-PLME-RA-0315R2 [pii] 10.1371/journal.pmed.0030486
64. Gius D, Cui H, Bradbury CM, Cook J, Smart DK, Zhao S, Young L, Brandenburg SA, Hu Y, Bisht KS, Ho AS, Mattson D, Sun L, Munson PJ, Chuang EY, Mitchell JB, Feinberg AP (2004) Distinct effects on gene expression of chemical and genetic manipulation of the cancer epigenome revealed by a multimodality approach. Cancer Cell 6(4):361–371
65. Mueller W, Nutt CL, Ehrich M, Riemenschneider MJ, von Deimling A, van den Boom D, Louis DN (2007) Downregulation of RUNX3 and TES by hypermethylation in glioblastoma. Oncogene 26(4):583–593. doi:1209805 [pii] 10.1038/sj.onc.1209805
66. Meissner A, Gnirke A, Bell GW, Ramsahoye B, Lander ES, Jaenisch R (2005) Reduced representation bisulfite sequencing for comparative high-resolution DNA methylation analysis. Nucleic Acids Res 33(18):5868–5877. doi:33/18/5868 [pii] 10.1093/nar/gki901
67. Meissner A, Mikkelsen TS, Gu H, Wernig M, Hanna J, Sivachenko A, Zhang X, Bernstein BE, Nusbaum C, Jaffe DB, Gnirke A, Jaenisch R, Lander ES (2008) Genome-scale DNA methylation maps of pluripotent and differentiated cells. Nature 454(7205):766–770. doi:nature07107 [pii] 10.1038/nature07107
68. Smith ZD, Gu H, Bock C, Gnirke A, Meissner A (2009) High-throughput bisulfite sequencing in mammalian genomes. Methods 48(3):226–232. doi:S1046-2023(09)00111-X [pii] 10.1016/j.ymeth.2009.05.003
69. Cokus SJ, Feng S, Zhang X, Chen Z, Merriman B, Haudenschild CD, Pradhan S, Nelson SF, Pellegrini M, Jacobsen SE (2008) Shotgun bisulphite sequencing of the Arabidopsis genome reveals DNA methylation patterning. Nature 452(7184):215–219
70. Lister R, O'Malley RC, Tonti-Filippini J, Gregory BD, Berry CC, Millar AH, Ecker JR (2008) Highly integrated single-base resolution maps of the epigenome in Arabidopsis. Cell 133(3):523–536. doi:S0092-8674(08)00448-0 [pii] 10.1016/j.cell.2008.03.029
71. Xi Y, Li W (2009) BSMAP: whole genome bisulfite sequence MAPping program. BMC Bioinformatics 10:232. doi:1471-2105-10-232 [pii] 10.1186/1471-2105-10-232
72. Gu H, Smith ZD, Bock C, Boyle P, Gnirke A, Meissner A (2011) Preparation of reduced representation bisulfite sequencing libraries for genome-scale DNA methylation profiling. Nat Protoc 6(4):468–481. doi:nprot.2010.190 [pii] 10.1038/nprot.2010.190
73. Xiang H, Zhu J, Chen Q, Dai F, Li X, Li M, Zhang H, Zhang G, Li D, Dong Y, Zhao L, Lin Y, Cheng D, Yu J, Sun J, Zhou X, Ma K, He Y, Zhao Y, Guo S, Ye M, Guo G, Li Y, Li R, Zhang X, Ma L, Kristiansen K, Guo Q, Jiang J, Beck S, Xia Q, Wang W, Wang J (2010) Single base-resolution methylome of the silkworm reveals a sparse epigenomic map. Nat Biotechnol 28(5):516–520. doi:nbt.1626 [pii] 10.1038/nbt.1626
74. Schroeder DI, Lott P, Korf I, LaSalle JM (2011) Large-scale methylation domains mark a functional subset of neuronally expressed genes. Genome Res 21(10):1583–1591. doi:gr.119131.110 [pii] 10.1101/gr.119131.110
75. Feng S, Cokus SJ, Zhang X, Chen PY, Bostick M, Goll MG, Hetzel J, Jain J, Strauss SH, Halpern ME, Ukomadu C, Sadler KC, Pradhan S, Pellegrini M, Jacobsen SE (2010) Conservation and divergence of methylation patterning in plants and animals. Proc Natl Acad Sci USA 107(19):8689–8694. doi:1002720107 [pii] 10.1073/pnas.1002720107

76. Zemach A, McDaniel IE, Silva P, Zilberman D (2010) Genome-wide evolutionary analysis of eukaryotic DNA methylation. Science 328(5980):916–919. doi:science.1186366 [pii] 10.1126/science.1186366
77. Laurent L, Wong E, Li G, Huynh T, Tsirigos A, Ong CT, Low HM, Kin Sung KW, Rigoutsos I, Loring J, Wei CL (2010) Dynamic changes in the human methylome during differentiation. Genome Res 20(3):320–331. doi:gr.101907.109 [pii] 10.1101/gr.101907.109
78. Lister R, Pelizzola M, Kida YS, Hawkins RD, Nery JR, Hon G, Antosiewicz-Bourget J, O'Malley R, Castanon R, Klugman S, Downes M, Yu R, Stewart R, Ren B, Thomson JA, Evans RM, Ecker JR (2011) Hotspots of aberrant epigenomic reprogramming in human induced pluripotent stem cells. Nature 471(7336):68–73. doi:nature09798 [pii] 10.1038/nature09798
79. Hon GC, Hawkins RD, Caballero OL, Lo C, Lister R, Pelizzola M, Valsesia A, Ye Z, Kuan S, Edsall LE, Camargo AA, Stevenson BJ, Ecker JR, Bafna V, Strausberg RL, Simpson AJ, Ren B (2011) Global DNA hypomethylation coupled to repressive chromatin domain formation and gene silencing in breast cancer. Genome Res. doi:gr.125872.111 [pii] 10.1101/gr.125872.111
80. Berman BP, Weisenberger DJ, Aman JF, Hinoue T, Ramjan Z, Liu Y, Noushmehr H, Lange CP, van Dijk CM, Tollenaar RA, Van Den Berg D, Laird PW (2011) Regions of focal DNA hypermethylation and long-range hypomethylation in colorectal cancer coincide with nuclear lamina-associated domains. Nat Genet 44(1):40–46. doi:10.1038/ng.969 ng.969 [pii]
81. Krueger F, Andrews SR (2011) Bismark: a flexible aligner and methylation caller for Bisulfite-Seq applications. Bioinformatics 27(11):1571–1572. doi:btr167 [pii] 10.1093/bioinformatics/btr167
82. Xi Y, Bock C, Muller F, Sun D, Meissner A, Li W (2011) RRBSMAP: a fast, accurate and user-friendly alignment tool for reduced representation bisulfite sequencing. Bioinformatics. doi:btr668 [pii] 10.1093/bioinformatics/btr668
83. Ehrich M, Nelson MR, Stanssens P, Zabeau M, Liloglou T, Xinarianos G, Cantor CR, Field JK, van den Boom D (2005) Quantitative high-throughput analysis of DNA methylation patterns by base-specific cleavage and mass spectrometry. Proc Natl Acad Sci USA 102(44):15785–15790. doi:0507816102 [pii] 10.1073/pnas.0507816102
84. Raval A, Tanner SM, Byrd JC, Angerman EB, Perko JD, Chen SS, Hackanson B, Grever MR, Lucas DM, Matkovic JJ, Lin TS, Kipps TJ, Murray F, Weisenburger D, Sanger W, Lynch J, Watson P, Jansen M, Yoshinaga Y, Rosenquist R, de Jong PJ, Coggill P, Beck S, Lynch H, de la Chapelle A, Plass C (2007) Downregulation of death-associated protein kinase 1 (DAPK1) in chronic lymphocytic leukemia. Cell 129(5):879–890. doi:S0092-8674(07)00512-0 [pii] 10.1016/j.cell.2007.03.043
85. Deng J, Shoemaker R, Xie B, Gore A, LeProust EM, Antosiewicz-Bourget J, Egli D, Maherali N, Park IH, Yu J, Daley GQ, Eggan K, Hochedlinger K, Thomson J, Wang W, Gao Y, Zhang K (2009) Targeted bisulfite sequencing reveals changes in DNA methylation associated with nuclear reprogramming. Nat Biotechnol 27(4):353–360. doi:nbt.1530 [pii] 10.1038/nbt.1530
86. Ito S, D'Alessio AC, Taranova OV, Hong K, Sowers LC, Zhang Y (2010) Role of Tet proteins in 5mC to 5hmC conversion, ES-cell self-renewal and inner cell mass specification. Nature 466(7310):1129–1133. doi:nature09303 [pii] 10.1038/nature09303
87. Ono R, Taki T, Taketani T, Taniwaki M, Kobayashi H, Hayashi Y (2002) LCX, leukemia-associated protein with a CXXC domain, is fused to MLL in acute myeloid leukemia with trilineage dysplasia having t(10;11)(q22;q23). Cancer Res 62(14):4075–4080
88. Lorsbach RB, Moore J, Mathew S, Raimondi SC, Mukatira ST, Downing JR (2003) TET1, a member of a novel protein family, is fused to MLL in acute myeloid leukemia containing the t(10;11)(q22;q23). Leukemia 17(3):637–641. doi:10.1038/sj.leu.2402834 [pii]
89. Ko M, Huang Y, Jankowska AM, Pape UJ, Tahiliani M, Bandukwala HS, An J, Lamperti ED, Koh KP, Ganetzky R, Liu XS, Aravind L, Agarwal S, Maciejewski JP, Rao A (2010) Impaired hydroxylation of 5-methylcytosine in myeloid cancers with mutant TET2. Nature 468(7325):839–843. doi:nature09586 [pii] 10.1038/nature09586

90. Huang Y, Pastor WA, Shen Y, Tahiliani M, Liu DR, Rao A (2010) The behaviour of 5-hydroxymethylcytosine in bisulfite sequencing. PLoS One 5(1):e8888. doi:10.1371/journal.pone.0008888
91. Globisch D, Munzel M, Muller M, Michalakis S, Wagner M, Koch S, Bruckl T, Biel M, Carell T (2010) Tissue distribution of 5-hydroxymethylcytosine and search for active demethylation intermediates. PLoS One 5(12):e15367. doi:10.1371/journal.pone.0015367
92. Szwagierczak A, Bultmann S, Schmidt CS, Spada F, Leonhardt H (2010) Sensitive enzymatic quantification of 5-hydroxymethylcytosine in genomic DNA. Nucleic Acids Res 38(19):e181. doi:gkq684 [pii] 10.1093/nar/gkq684
93. Kinney SM, Chin HG, Vaisvila R, Bitinaite J, Zheng Y, Esteve PO, Feng S, Stroud H, Jacobsen SE, Pradhan S (2011) Tissue-specific distribution and dynamic changes of 5-hydroxymethylcytosine in mammalian genomes. J Biol Chem 286(28):24685–24693. doi:M110.217083 [pii] 10.1074/jbc.M110.217083
94. Flusberg BA, Webster DR, Lee JH, Travers KJ, Olivares EC, Clark TA, Korlach J, Turner SW (2010) Direct detection of DNA methylation during single-molecule, real-time sequencing. Nat Methods 7(6):461–465. doi:nmeth.1459 [pii] 10.1038/nmeth.1459
95. Ke X-S, Qu Y, Rostad K, Li W-C, Lin B, Halvorsen OJ, Haukaas SA, Jonassen I, Petersen K, Goldfinger N, Rotter V, Akslen LA, Oyan AM, Kalland K-H (2009) Genome-wide profiling of histone h3 lysine 4 and lysine 27 trimethylation reveals an epigenetic signature in prostate carcinogenesis. PLoS One 4:e4687
96. Fraga MF, Ballestar E, Villar-Garea A, Boix-Chornet M, Espada J, Schotta G, Bonaldi T, Haydon C, Ropero S, Petrie K, Iyer NG, Pérez-Rosado A, Calvo E, Lopez JA, Cano A, Calasanz MJ, Colomer D, Piris MA, Ahn N, Imhof A, Caldas C, Jenuwein T, Esteller M (2005) Loss of acetylation at Lys16 and trimethylation at Lys20 of histone H4 is a common hallmark of human cancer. Nat Genet 37:391–400
97. Varambally S, Dhanasekaran SM, Zhou M, Barrette TR, Kumar-Sinha C, Sanda MG, Ghosh D, Pienta KJ, Sewalt RGAB, Otte AP, Rubin MA, Chinnaiyan AM (2002) The polycomb group protein EZH2 is involved in progression of prostate cancer. Nature 419:624–629
98. Sharma SV, Lee DY, Li B, Quinlan MP, Takahashi F, Maheswaran S, McDermott U, Azizian N, Zou L, Fischbach MA (2010) A chromatin-mediated reversible drug-tolerant state in cancer cell subpopulations. Cell 141:69–80
99. Park PJ (2009) ChIP-seq: advantages and challenges of a maturing technology. Nat Rev Genet 10:669–680
100. O'Neill LP, Turner BM, Turner B (2003) ChIP with native chromatin: advantages and problems relative to methods using cross-linked material. Methods 31(1):76–82
101. Orlando V (2000) Mapping chromosomal proteins in vivo by formaldehyde-crosslinked-chromatin immunoprecipitation. Trends Biochem Sci 25:99–104
102. Barski A, Zhao K (2009) Genomic location analysis by ChIP-Seq. J Cell Biochem 107(107):11–18
103. Dingwall C, Lomonossoff GP, Laskey RA (1981) High sequence specificity of micrococcal nuclease. Nucleic Acids Res 9:2659–2673
104. Hörz W, Altenburger W (1981) Sequence specific cleavage of DNA by micrococcal nuclease. Nucleic Acids Res 9:2643–2658
105. Li H, Ruan J, Durbin R (2008) Mapping short DNA sequencing reads and calling variants using mapping quality scores. Genome Res 18:1851–1858
106. Langmead B, Trapnell C, Pop M, Salzberg SL (2009) Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol 10:R25
107. Li H, Durbin R (2010) Fast and accurate long-read alignment with Burrows-Wheeler transform. Bioinformatics 26:589–595
108. Li R, Yu C, Li Y, Lam T-W, Yiu S-M, Kristiansen K, Wang J (2009) SOAP2: an improved ultrafast tool for short read alignment. Bioinformatics 25:1966–1967
109. Li R, Li Y, Kristiansen K, Wang J (2008) SOAP: short oligonucleotide alignment program. Bioinformatics 24:713–714

110. Coarfa C, Yu F, Miller CA, Chen Z, Harris RA, Milosavljevic A (2010) Pash 3.0: a versatile software package for read mapping and integrative analysis of genomic and epigenomic variation using massively parallel DNA sequencing. BMC Bioinformatics 11:572. doi:1471-2105-11-572 [pii] 10.1186/1471-2105-11-572
111. Pepke S, Wold B, Mortazavi A (2009) Computation for ChIP-seq and RNA-seq studies. Nat Methods 6:S22–S32
112. Xu H, Handoko L, Wei X, Ye C, Sheng J, Wei C-L, Lin F, Sung W-K (2010) A signal-noise model for significance analysis of ChIP-seq with negative control. Bioinformatics 26:1199–1204
113. Xu H, Wei C-L, Lin F, Sung W-K (2008) An HMM approach to genome-wide identification of differential histone modification sites from ChIP-seq data. Bioinformatics 24:2344–2349
114. Zang C, Schones DE, Zeng C, Cui K, Zhao K, Peng W (2009) A clustering approach for identification of enriched domains from histone modification ChIP-Seq data. Bioinformatics 25:1952–1958
115. Barski A, Cuddapah S, Cui K, Roh T-Y, Schones DE, Wang Z, Wei G, Chepelev I, Zhao K (2007) High-resolution profiling of histone methylations in the human genome. Cell 129:823–837
116. Johnson DS, Mortazavi A, Myers RM, Wold B (2007) Genome-wide mapping of in vivo protein-DNA interactions. Science 316:1497–1502
117. Mikkelsen TS, Ku M, Jaffe DB, Issac B, Lieberman E, Giannoukos G, Alvarez P, Brockman W, Kim T-K, Koche RP, Lee W, Mendenhall E, O'Donovan A, Presser A, Russ C, Xie X, Meissner A, Wernig M, Jaenisch R, Nusbaum C, Lander ES, Bernstein BE (2007) Genome-wide maps of chromatin state in pluripotent and lineage-committed cells. Nature 448:553–560
118. Heintzman ND, Stuart RK, Hon G, Fu Y, Ching CW, Hawkins RD, Barrera LO, Van Calcar S, Qu C, Ching KA, Wang W, Weng Z, Green RD, Crawford GE, Ren B (2007) Distinct and predictive chromatin signatures of transcriptional promoters and enhancers in the human genome. Nat Genet 39:311–318
119. Heintzman ND, Hon GC, Hawkins RD, Kheradpour P, Stark A, Harp LF, Ye Z, Lee LK, Stuart RK, Ching CW, Ching KA, Antosiewicz-Bourget JE, Liu H, Zhang X, Green RD, Lobanenkov VV, Stewart R, Thomson JA, Crawford GE, Kellis M, Ren B (2009) Histone modifications at human enhancers reflect global cell-type-specific gene expression. Nature 459:108–112
120. Ernst J, Kheradpour P, Mikkelsen TS, Shoresh N, Ward LD, Epstein CB, Zhang X, Wang L, Issner R, Coyne M, Ku M, Durham T, Kellis M, Bernstein BE (2011) Mapping and analysis of chromatin state dynamics in nine human cell types. Nature 473:43–49
121. Wasserman NF, Aneas I, Nobrega MA (2010) An 8q24 gene desert variant associated with prostate cancer risk confers differential in vivo activity to a MYC enhancer. Genome Res 20:1191–1197
122. Adli M, Bernstein BE (2011) Whole-genome chromatin profiling from limited numbers of cells using nano-ChIP-seq. Nat Protoc 6:1656–1668
123. Goren A, Ozsolak F, Shoresh N, Ku M, Adli M, Hart C, Gymrek M, Zuk O, Regev A, Milos PM, Bernstein BE (2010) Chromatin profiling by directly sequencing small quantities of immunoprecipitated DNA. Nat Methods 7:47–49
124. Dekker J, Rippe K, Dekker M, Kleckner N (2002) Capturing chromosome conformation. Science 295:1306–1311
125. Zhao Z, Tavoosidana G, Sjölinder M, Göndör A, Mariano P, Wang S, Kanduri C, Lezcano M, Sandhu KS, Singh U, Pant V, Tiwari V, Kurukuti S, Ohlsson R (2006) Circular chromosome conformation capture (4C) uncovers extensive networks of epigenetically regulated intra- and interchromosomal interactions. Nat Genet 38:1341–1347
126. Dostie J, Richmond TA, Arnaout RA, Selzer RR, Lee WL, Honan TA, Rubio ED, Krumm A, Lamb J, Nusbaum C, Green RD, Dekker J (2006) Chromosome Conformation Capture Carbon Copy (5C): a massively parallel solution for mapping interactions between genomic elements. Genome Res 16:1299–1309

127. Lieberman-Aiden E, van Berkum NL, Williams L, Imakaev M, Ragoczy T, Telling A, Amit I, Lajoie BR, Sabo PJ, Dorschner MO, Sandstrom R, Bernstein B, Bender MA, Groudine M, Gnirke A, Stamatoyannopoulos J, Mirny LA, Lander ES, Dekker J (2009) Comprehensive mapping of long-range interactions reveals folding principles of the human genome. Science 326:289–293
128. Cai S, Lee CC, Kohwi-Shigematsu T (2006) SATB1 packages densely looped, transcriptionally active chromatin for coordinated expression of cytokine genes. Nat Genet 38:1278–1288
129. Simonis M, Kooren J, de Laat W (2007) An evaluation of 3C-based methods to capture DNA interactions. Nat Methods 4:895–901
130. Fullwood MJ, Wei C-L, Liu ET, Ruan Y (2009) Next-generation DNA sequencing of paired-end tags (PET) for transcriptome and genome analyses. Genome Res 19:521–532
131. Simonis M, Klous P, Homminga I, Galjaard R-J, Rijkers E-J, Grosveld F, Meijerink JPP, de Laat W (2009) High-resolution identification of balanced and complex chromosomal rearrangements by 4C technology. Nat Methods 6:837–842
132. Ohm JE, McGarvey KM, Yu X, Cheng L, Schuebel KE, Cope L, Mohammad HP, Chen W, Daniel VC, Yu W, Berman DM, Jenuwein T, Pruitt K, Sharkis SJ, Watkins DN, Herman JG, Baylin SB (2007) A stem cell-like chromatin pattern may predispose tumor suppressor genes to DNA hypermethylation and heritable silencing. Nat Genet 39(2):237–242. doi:ng1972 [pii] 10.1038/ng1972
133. Widschwendter M, Fiegl H, Egle D, Mueller-Holzner E, Spizzo G, Marth C, Weisenberger DJ, Campan M, Young J, Jacobs I, Laird PW (2007) Epigenetic stem cell signature in cancer. Nat Genet 39(2):157–158. doi:ng1941 [pii] 10.1038/ng1941
134. Schlesinger Y, Straussman R, Keshet I, Farkash S, Hecht M, Zimmerman J, Eden E, Yakhini Z, Ben-Shushan E, Reubinoff BE, Bergman Y, Simon I, Cedar H (2007) Polycomb-mediated methylation on Lys27 of histone H3 pre-marks genes for de novo methylation in cancer. Nat Genet 39(2):232–236. doi:ng1950 [pii] 10.1038/ng1950
135. Ley TJ, Ding L, Walter MJ, McLellan MD, Lamprecht T, Larson DE, Kandoth C, Payton JE, Baty J, Welch J, Harris CC, Lichti CF, Townsend RR, Fulton RS, Dooling DJ, Koboldt DC, Schmidt H, Zhang Q, Osborne JR, Lin L, O'Laughlin M, McMichael JF, Delehaunty KD, McGrath SD, Fulton LA, Magrini VJ, Vickery TL, Hundal J, Cook LL, Conyers JJ, Swift GW, Reed JP, Alldredge PA, Wylie T, Walker J, Kalicki J, Watson MA, Heath S, Shannon WD, Varghese N, Nagarajan R, Westervelt P, Tomasson MH, Link DC, Graubert TA, DiPersio JF, Mardis ER, Wilson RK (2010) DNMT3A mutations in acute myeloid leukemia. N Engl J Med 363(25):2424–2433. doi:10.1056/NEJMoa1005143
136. Yamashita Y, Yuan J, Suetake I, Suzuki H, Ishikawa Y, Choi YL, Ueno T, Soda M, Hamada T, Haruta H, Takada S, Miyazaki Y, Kiyoi H, Ito E, Naoe T, Tomonaga M, Toyota M, Tajima S, Iwama A, Mano H (2010) Array-based genomic resequencing of human leukemia. Oncogene 29(25):3723–3731. doi:onc2010117 [pii] 10.1038/onc.2010.117
137. Clarke J, Wu HC, Jayasinghe L, Patel A, Reid S, Bayley H (2009) Continuous base identification for single-molecule nanopore DNA sequencing. Nat Nanotechnol 4(4):265–270. doi:nnano.2009.12 [pii] 10.1038/nnano.2009.12

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