Enhancer-associated RNAs as therapeutic targets

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Introduction: Regulation of gene expression involves a variety of mechanisms driven by a complex regulatory network of factors. Control of transcription is an important step in gene expression regulation, which integrates the function of cis-acting and trans-acting elements. Among cis-regulatory elements, enhancer RNA (eRNA)-producing domains recently emerged as widespread and potent regulators of transcription and cell fate decision. Thus, manipulation of eRNA levels becomes a novel and appealing avenue for the design of new therapeutic treatments.

Areas covered: In this review, we focus on eRNA-producing domains. We describe mechanisms involved in their cell-type specific selection and activation as well as their epigenetic features. In addition, we present their function and the growing evidences of their deregulation in human diseases. Finally, we discuss eRNAs as potential therapeutic targets.

Expert opinion: As key factors in the control of transcription, eRNAs appear to possess a great potential for the establishment of new therapy options. However, thorough testing as well as providing the genetic toolbox to target eRNAs will be needed to fully assess the practical and clinical possibilities.

1. Introduction

Spatiotemporal regulation of gene expression is required to carry out a variety of biological processes such as development, differentiation and apoptosis. In particular, the control of transcription initiation rates by promoters (proximal to transcription start sites [TSSs]) and enhancers (distal to TSSs) is key for proper execution of such biological programs [1-5]. The recent identification of enhancer-transcribed RNAs, and their evolving implication in gene regulation, may not only provide novel tools to manipulate endogenous enhancer activity in a specific way, but also create opportunities for using enhancers as therapeutic targets.

Distal cis-regulatory elements of transcription, such as enhancers, were first discovered in the simian virus SV40 genome in the early 1980s [6]. The inclusion of a 72bp SV40 DNA fragment upstream of the β-globin gene reporter increased its expression by 200-fold. Moreover, it was noticed that the viral enhancer remained active when placed in both sense and antisense orientation as well as upstream and downstream of the β-globin gene, thereby laying the foundation of basic enhancer features. Although it was found that the SV40 enhancer could influence the expression of β-globin over a distance of 10 kb, most enhancers in lower eukaryotes such as yeast were located within 100 – 200 bp from their target promoters [7]. Nevertheless, it seems that enhancers evolved their ability to mediate a long-range action in concomitance with the genome expansion of higher eukaryotes. Indeed, studies in drosophila and human cells have revealed that most enhancers are on average located at > 50 Kb away from their target regions [4,8]. For instance, it was shown in leukemia cells that Myc expression is regulated by a

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Article highlights.

- Cell-type specific selection of enhancer domains is mediated by cooperative binding of lineage-determining transcription factors and pioneer factors.
- Active enhancer domains are decorated with specific epigenetic features. A fraction of active enhancers are occupied by the RNA polymerase II, which leads to the production of enhancer RNAs (eRNAs).
- Some eRNA-producing domains are known to be involved in the transcriptional regulation of their neighboring genes. The regulatory function of eRNAs is mediated by the formation of DNA loops, which bring enhancers and targeted promoters to close proximity.
- Emerging evidences show that deregulation of specific enhancers is likely to occur in various human diseases.
- Recent genome-wide identification of enhancer-transcribed RNAs reveals their potential as therapeutic targets. Current efforts to target eRNAs use specific oligonucleotide-based approach and inhibitors.

This box summarizes key points contained in the article.

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group of five enhancers located 1.7 Mb downstream of the proto-oncogene [9]. Recent development of high-throughput sequencing technologies in combination with techniques to probe the three-dimensional structure of the genome allowed the precise genome-wide mapping of enhancers and the initiation of global assessment of their functions and disease-related alterations.

2. Determinants of enhancer activation

Recent large-scale studies indicated that the human genome may harbor > 400,000 putative enhancers [10-12]. This great abundance of regulatory elements suggests complex combinatorial possibilities that can spatiotemporally shape gene expression and influence cell fate. Interestingly, selection and activation of enhancers seem to mainly rely on two mechanisms. First, it was shown that cooperative binding of lineage-determining transcription factors could facilitate other factors to access condensed chromatin. For example, macrophage and B-cell lineage determination require the collaboration of the common transcription factor PU.1 with a small subset of macrophage (e.g., C/EBP and AP-1) and B-cell-specific (e.g., E2A and OCT2) factors [13]. Second, activation of enhancers can occur by sequential binding of transcription factors. In this scenario, the binding of a 'pioneer' transcription factor to condensed chromatin precedes and facilitates the recruitment of other factors by repositioning and/or epigenetically modifying nucleosomes at the bound sites [14-16]. A typical example of a pioneering transcription factor is the Forkhead box A1 (FOXA1), which is involved in fetal liver development and adult liver metabolism [17]. Alongside the classical functions of a transcription factor, FOXA1 has the ability to bind nucleosomal DNA and relax the chromatin to

enable the binding of other factors such as hormone receptors (e.g., ER, AR, PR and GR) [18].

Despite the cell-type specific guidance of transcription factors in activating relevant subsets of enhancers, recent studies have underscored the involvement of histone variants in the delineation of regulatory regions such as enhancers. Interestingly, it was found in various cell lines that H3.3 and H2A.Z are enriched at both active and poised regulatory regions [19-21]. The deposition of these histone variants was suggested to create nucleosome instability, which facilitates the recruitment of transcription factors as compared to more stable canonical nucleosomes. In this respect, enhancers bound by transcription factors are less occupied by nucleosomes, which in turn is reflected by a greater sensitivity to DNA nucleases [22]. One study demonstrated that enhancers that are co-occupied by the androgen receptor (AR) and FOXA1 also harbor H2A.Z-containing nucleosomes, which are displaced upon androgen treatment [23]. More recently, Hu *et al.* demonstrated that knocking down H2A.Z in mouse embryonic stem cells increased the nucleosomal occupancy and decreased OCT4 presence at specific enhancers and promoters [20].

Collectively, the data suggests the existence of an interdependent relationship between transcription factors and chromatin states to govern the selection and activation of regulatory enhancer regions.

3. Features of enhancers

Efforts to globally identify enhancer regions led to the discovery of specific epigenetic histone marks. Using an immunoprecipitation-based microarray method (ChIP-on-chip), Heitzman *et al.* identified the preferential enrichment of histone 3 lysine 4 monomethylation (H3K4me1) at enhancers [24]. The accumulation of H3K4me1 at enhancers was accompanied by the presence of p300 and histone 3 lysine 27 acetylation (H3K27ac). Following studies in mouse and human stem cells demonstrated that the concomitant presence of H3K4me1 and H3K27ac marks active enhancers whereas enhancers decorated with H3K4me1 and histone 3 lysine 27 trimethylation (H3K27me3) were inactive or poised [25,26]. Moreover, other studies have also reported the presence of histone 3 lysine 4 trimethylation (H3K4me3), a known promoter-based epigenetic mark, at a subset of active enhancers [27,28]. Interestingly, the deposition of H3K4me1 generally precedes H3K27ac as well as nucleosomal depletion, which suggest a function for H3K4me1 in enhancer priming. This concept is also supported by the maintenance of H3K4me1 at enhancers that became functionally inactive [29,30]. Altogether, these data propose high H3K4me1 as a flag for enhancer regions, which may further help for nucleosomal depletion and/or binding of transcription factors. In addition, the presence of active (e.g., H3k27ac) or the absence of repressive (e.g., H3k27me3) epigenetic marks on histone tails appears an excellent predictor of enhancer activity.
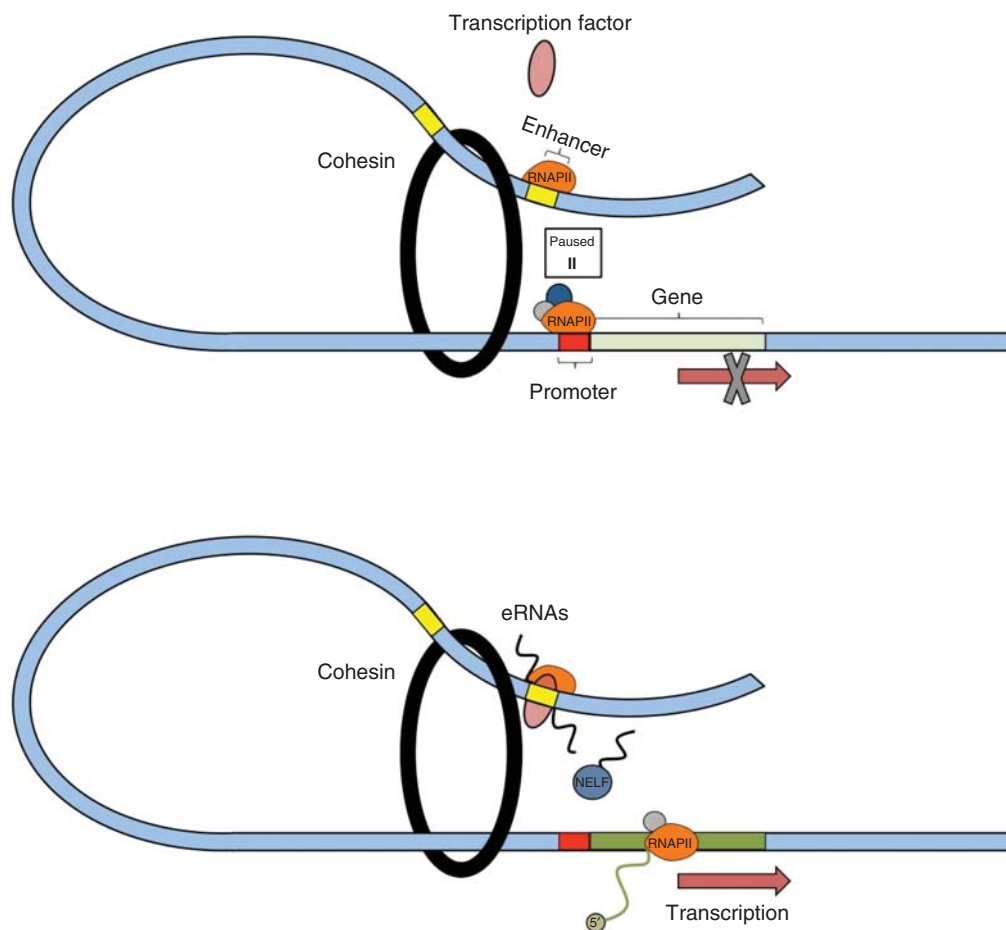

Figure 1. Schematic representation of eRNA activation and function. Transcriptional activation of enhancer domains is typically triggered by the binding of a transcription factor. eRNA-producing domains can influence the transcriptional activation of neighboring genes by acting as a decoy for NELF. The dissociation of NELF allows the release of paused RNAPII and its co-factors (gray circle). Enhancer–promoter interactions are usually stabilized by a variety of co-factors, such as cohesin (black circle), that also participate in the transcriptional activation (arrow) of targeted genes (green).
eRNA: Enhancer RNAs; NELF: Negative elongation factor.

Spatial clustering of enhancer regions, sometimes referred to as 'super-enhancers' (SEs), can also accentuate the activity of an enhancer domain. Different from typical enhancers, SEs have a greater occupancy of co-activators such as Brd4 and the Mediator complex [31-33]. Interestingly, SEs have been found to trigger higher transcriptional activity and to be more sensitive to lower levels of Mediator or drug blocking Brd4 function [31,32].

Recent reports have also demonstrated that a subset of active enhancers are occupied and transcribed by RNA polymerase II (RNAPII) [1,2,34,35]. Moreover, RNAPII occupancy correlates with the presence of H3K4me3, indicating a potential relation between H3K4me3 levels, RNAPII recruitment and transcription at enhancers [27]. Further analysis revealed that enhancer RNAs (eRNAs) were produced at RNAPII-bound enhancers and that these RNA molecules were reliable indicators of enhancer activity. Recently, the FANTOM5

project created an atlas of active transcribed enhancers in a variety of mammalian primary cell types, cancer cell lines, and tissues [36].

However, whether eRNA sequences or their secondary structure have specific functions in the transcriptional regulation of neighboring genes, is an emerging and intriguing question.

4. eRNAs as regulators of gene transcription

Two landmark studies emerged with the first examples of widespread transcription occurring at enhancer domains [1,2]. Both reports observed a positive correlation between the production of eRNAs and the expression of their neighboring protein-coding genes (Figure 1). However, no function was assigned to these new RNA molecules, which could simply represent the product of spurious transcription from an active

nearby promoter. In a subsequent study, Ørom *et al.* reported the presence of long intergenic noncoding RNAs (lincRNAs) with enhancer-like properties, named ncRNA-activating (ncRNA-a) [37]. The authors observed that depletion of these ncRNAs resulted in a decrease in expression of nearby protein-coding genes. Interestingly, both ncRNAs-a and eRNAs have been reported to interact with the Mediator complex to mediate their function [38,39]. However, the genomic regions expressing activating RNAs or eRNAs have clear and distinct epigenetic features. Thus, these two RNA species should be considered separately, despite some functional similarities.

Recent studies have undertaken to assess the dynamics of eRNA production as well as the function of these RNA molecules in gene regulation using different biological systems.

In one of the first examples, Wang *et al.* used the prostate cancer cell line LNCaP to demonstrate that enhancers could orchestrate a specific transcriptional program, intimately associated with the production of eRNAs [40]. Interestingly, they observed that androgen-sensitive enhancers were concomitantly bound by FoxA1 (a cell-lineage-specific transcription factor) and the AR. Using global run-on sequencing (GRO-seq), a powerful high-throughput sequencing technique to detect and measure nascent transcripts, the authors were able to observe an increase in the expression levels of eRNAs at FoxA1 and AR-bound enhancers following androgen (dihydrotestosterone or DHT) treatment. Strikingly, depletion of FoxA1, an unfavorable prognostic sign in certain prostate tumors, was shown to alter the AR-binding profile. The authors further demonstrated that eRNAs were produced at conserved and gained AR-binding sites [40], indicating that eRNA production can predict enhancer activity.

Another report lately investigated the role of the tumor suppressor p53 at enhancers [5]. They observed that p53 is able to bind with high affinity to genomic regions epigenetically characterized as enhancer domains. Using reporter assays, they observed a p53-dependent transcriptional enhancing activity from these genomic elements. Interestingly, these bound-enhancers were producing eRNAs in response to p53 induction. Furthermore, chromosome conformation capture assays revealed the contribution of p53-bound enhancers (p53BERs) to the p53 transcriptional response. Indeed, p53-bound enhancers interact with and regulate the transcription of their neighboring target genes. Additionally, the authors suggest that p53BERs act on pre-determined DNA loops, as p53 depletion did not affect the chromatin organization. Similarly, a recent report on estrogen receptor enhancers by Hah *et al.* demonstrated that inhibition of RNA-PII elongation with flavopiridol had no impact on the estradiol-induced enhancer-promoter looping, suggesting the existence of a pre-determined and transcription-independent chromatin structure [41]. However, other reports suggest that eRNAs can also contribute to the formation and/or stabilization of chromatin loops [4]. In the latter case, eRNAs were found to interact with a member of the cohesin complex

(Rad21), a protein involved in the organization of the enhancer-promoter chromatin loops [4]. Remarkably, independently of the impact on the chromatin structure, depletion of these eRNAs was able to impair the induction of their neighboring protein-coding target genes, demonstrating the potential and functionality of these RNA molecules in the enhancement of transcription [5].

Several follow-up studies revealed the functional importance of eRNAs by demonstrating their impact on the transcriptional output of nearby protein-coding genes [3,4]. For example, the transcriptional repressors Rev-Erbs were found to occupy enhancer domains. By performing GRO-seq in Rev-Erb-α and Rev-Erb-β double knockout macrophage background, the authors observed a significant de-repression of eRNA production at Rev-Erbs-bound enhancers. Interestingly, Rev-Erbs-dependent transcriptional inhibition was mediated through the recruitment of the co-repressor histone deacetylase complex (NCoR-HDAC3) at bound enhancers. When recruited to enhancers, the NCoR–HDAC3 complex reduces the levels of histone 3 lysine 9 acetylation (H3K9Ac) and the production of eRNAs. Altogether, these data demonstrated for the first time that enhancers can mediate transcriptional repression [3].

Functional eRNAs were also found with the estrogen receptor α (ER-α) in human breast cancer cells [4]. By treating human breast cancer cells with 17β-estradiol (E2), Li *et al.* observed a strong correlation between ER-α-bound enhancers, eRNA production and the ER-dependent regulation of neighboring protein-coding genes. Moreover, using a tethering reporter assay, they further demonstrated that eRNAs are required for the transcriptional activation of neighboring genes. More specifically, by fusing FOXC1 eRNA to BoxB and tethering the resulting RNA upstream of a FOXC1 promoter-luciferase reporter, they observed an increased FOXC1 promoter activity. Furthermore, depletion of the sense, but not the antisense, eRNA led to a significant decrease in promoter activity, suggesting the importance of the eRNA sequence for its function. Using a different but compelling strategy, Lam *et al.* observed that changing the eRNA sequence, without affecting the transcription factor binding site, significantly diminished the reporter enhancing activity, again pinpointing the importance of the eRNA sequence in the control of gene expression [3]. Although informative, these studies failed to address the mechanism by which eRNAs influence the transcription of their neighboring genes. However, the recent work of Schaukowitch *et al.* demonstrated that eRNAs can bind to the negative elongation factor (NELF) and transiently remove it from their target gene promoters. In this case, by removing NELF, eRNAs were able to release the paused RNAPII, thus resulting in the expression of immediate-early genes in neurons [42].

Collectively, these assays establish the notion of eRNAs as potent RNA molecules involved in transcription regulation (**Table 1**).

Table 1. Examples of eRNA-producing domains and their main features.

eRNA	Epigenetic features	Binding factors	Transcript directionality	Poly(A)	Length (kb)	Major finding	Ref.
Neuronal-activity regulated eRNAs	High H3K4me1, low H3K4me3	RNAPII, CBP, CREB, SRF, NPAS4	2D	(-)	0.5 – 2	eRNA expression positively correlates with its neighboring genes	[2]
LPS-regulated eRNAs	High H3K4me1, low H3K4me3	RNAPII, p300, PU.1	1D	(+)	0.1 – 7	eRNAs transcribed from RNAPII-associated enhancers	[1]
AR/FoxA1-regulated eRNAs	High H3K4me1, low H3K4me2, low H3K4me3, H4K5ac and H3K27Ac	RNAPII, p300, AR, FoxA1	2D	n.d	~ 1	eRNA expression positively correlates with its neighboring genes	[40]
p53-bound enhancer regions	High H3K4me1, low H3K4me3 and high H3K27Ac	RNAPII, ERα	2D	(-)	~ 0.6	eRNA acts on pre-determined long-range chromatin loops	[5]
Oestrogen-regulated eRNAs	High H3K4me1, low H3K4me3 and high H3K27Ac	RNAPII, ERα	1D and 2D	n.d	0.5 – 2	eRNAs interact with cohesin complex and stabilize the chromatin structure	[4]
Rev-Erb-regulated eRNAs	High H3K4me1, low H3K4me3	RNAPII, Rev-Erb, NCoR–HDAC3	2D	n.d	0.5 – 2	eRNA repression through Rev-Erb-dependent recruitment of NCoR-HDAC3 repressive complexes	[3]
MyoD/MyoG-regulated eRNAs	High H3K4me1, low H3K4me3 and high H3K27Ac	RNAPII, MyoD, MyoG	2D	(-)(+)	1 – 2	eRNAs facilitate chromatin accessibility	[52]
Neuronal-activity regulated eRNAs	High H3K4me1, low H3K4me3	RNAPII, NELF	2D	(-)	n.d.	eRNAs serve as decoy for the negative elongation factor (NELF) complex	[42]
LPS/NFκB-regulated eRNAs (IL1β eRNA)	High H3K4me1, low H3K4me3	NFκB	1D and 2D	n.d	n.d.	eRNAs are implicated in the innate immune response	[54]
Rosiglitazone/PPARγ-regulated eRNAs	High H3K4me1, low H3K4me3	RNAPII, p300, CBP, PPARγ	2D	(-)	0.5 – 2	eRNAs are involved in the transcriptional regulation of adipocyte genes	[53]
p53-regulated eRNAs	High H3K4me1, low H3K4me3	RNAPII, p300, p53	1D and 2D	n.d	~ 1.2	eRNAs regulated by a p53-activated lincRNA	[55]

5. Dysregulation of eRNA-producing domains in disease

Transcription at enhancers is now considered an important event intimately connected to enhancer activity. Recent years uncovered the important role of eRNAs in the tissue-specific regulation of gene expression. Although the biological relevance of eRNAs still remains poorly characterized, interesting observations have already been made in this direction. For instance, it has been shown that suppression of eRNAs, expressed at one p53-bound enhancer region, attenuated the cell cycle arrest response to p53 activation. Interestingly, knocking-down its target gene PAPPA had a similar effect [5]. This observation suggests that eRNA-producing domains affect important cellular processes and can therefore be dysregulated in human diseases. Consequently, eRNAs can be interesting therapeutic targets or even important biomarkers.

Genome-wide association studies (GWAS) have already been used to connect alterations of cis-regulatory elements to human diseases. GWAS associates single nucleotide polymorphisms (SNPs) with a trait or disease [43]. Evidences indicate that > 90% of disease-associated SNPs might occur in the non-coding portion of the genome, suggesting that cis-regulatory elements such as enhancers can be affected [44]. Indeed, ENCODE consortium and other groups have recently mapped thousands of disease-associated SNPs to enhancer elements identified through epigenomic profiling [45]. This finding highlights the possibility that SNP-dependent disruption or creation of transcription factor binding sites at enhancers, or alteration of eRNA sequences, may affect important cellular functions.

Recently, Heinz *et al.* took advantage of the natural variation between two close mice strains (C57BL/6J and BALB/cJ) and performed an *in vivo* mutagenesis screening strategy. They observed that natural variations occurring between the two mice strains could perturb lineage-determining transcription factor binding, histone modifications and usage of specific enhancers [46]. For example, changes in the binding profile of the pioneer factors PU.1 and CCAAT/enhancer-binding protein (or C/EBPs) led to the activation of a different set of enhancers, which in turn influenced the binding of signal-dependent transcription factors and gene expression. Moreover, the authors noticed that decreasing the binding of one factor, by a natural occurring SNP, could negatively affect the recruitment of other factors and suggested a collaborative binding model for enhancer selection and function [46]. Altogether, these observations demonstrate that genomic sequence variations, such as SNPs and mutations, have the potential to influence enhancer activity and gene expression and hence may contribute or cause specific human diseases.

Interestingly, two recent studies have associated enhancer-derived transcripts with genomic instability [47,48]. Both studies focused on the antibody gene mutator activation-induced cytidine deaminase (AID). This enzyme is responsible for initiating two main antibody gene diversification processes, the somatic hypermutation and the class switch recombination (CSR), through cytosine deamination to uridine on single-stranded DNA [49]. AID hotspots occur preferentially at immunoglobulin (Ig) loci. However, AID mutational activity can also happen at non-Ig genes, often called AID off-target genes [50]. This off-target effect raises important concerns, as AID targeting to non-Ig genes can classically culminate in translocations that activate proto-oncogenes by juxtaposing them to potent Ig enhancers. This process represents a common feature to most of B cell tumors [49]. For example, Meng *et al.* were able to demonstrate that a significant portion of AID off-targets regions, in CSR-activated B cells, is located in intragenic SEs. These domains are usually occupied by RNAPII and can be transcribed bi-directionally [51]. The authors observed that high rate of convergent transcription, between a gene and its intragenic SE, increases the probability to be targeted by AID. Collectively, these reports demonstrate the implication of transcriptionally active intragenic SEs in the translocation of proto-oncogenes in B cell lymphoma.

An increasing body of evidences implicates active enhancers in key cellular processes. Moreover, because of their central and widespread role in transcription regulation, disease-associated dysregulation (e.g., translocation, SNPs, deletion and methylation) of enhancer activity is likely to gain in importance in the future (**Figure 2**).

6. Therapeutic potential of enhancer producing eRNAs

The observations described above denote eRNAs as key players in gene regulation and highlight their involvement in cellular processes such as stress response and differentiation [4,5,41,52-55]. Interestingly, a growing body of evidences suggest that dysregulation of eRNAs is associated with various human diseases and therefore raise the possibility of new therapeutic strategies. eRNA therapy is appealing as it involves control of transcription rather than acting post-transcriptionally, as observed with other non-coding RNAs (e.g., miRNAs). In addition, each eRNA-producing domain influences only a small group of neighboring genes in cis, which warrants high target specificity and minimizes potential advert effects. The tissue- and cell-type specific expression pattern of eRNAs also appear as a valuable feature in helping to reduce potential off-target impacts. However, few drawbacks can be found in association with eRNA-directed therapy. For instance, one eRNA-producing domain may contact distinct neighboring target genes in a tissue- and/or cell-type specific manner. Therefore, whereas disruption of the eRNA expression is desired in one particular set up, it may be detrimental in another. This puzzle will eventually be clarified by the refinement of the genome 3D structure using technologies such as single-cell Hi-C. However, the comprehensive resolution of
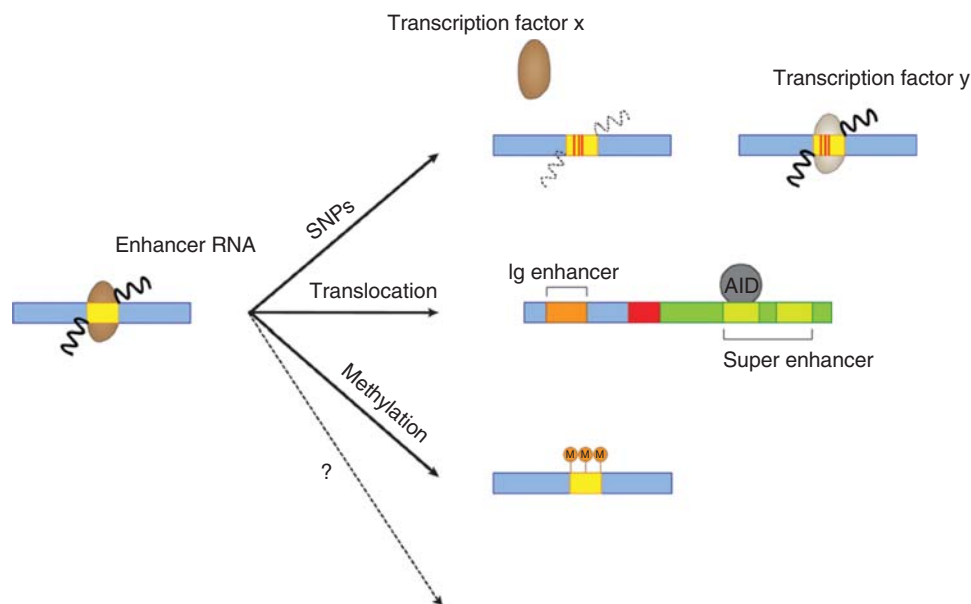
Figure 2. Dysregulation of eRNA-producing domains. Activity or function of enhancers can be altered in multiple ways. For instance, SNPs can either destroy or create transcription factor-binding motifs, which prevent or accommodate the binding of transcription factors 'x' and 'y', respectively. Transcription of intronic enhancers has been found to trigger activation-induced cytidine deaminase off-target function, which promotes the translocation of different genes (e.g., oncogenes) in the vicinity of potent Ig enhancers. Regulation of enhancers by DNA methylation has also been reported [80].
Ig: Immunoglobulin; SNPs: Single nucleotide polymorphisms.

the tissue- and cell-type specific genome structures remains a challenging aspect towards an efficient eRNA therapy. In addition, eRNAs are nuclear molecules with high turnover rate, features that are likely to make targeting a challenging enterprise. Another difficulty resides in the technical limitation of mimicking eRNA activation. Indeed, differently from other trans-acting non-coding RNAs (e.g., miRNAs), it is rather laborious to overexpress exogenous eRNAs in order to mediate a function in cis.

Despite the pros and cons associated with the manipulation of eRNA expression, the potential of eRNA therapy represent a new and exciting avenue.

6.1 Interfering with eRNA expression using oligonucleotide-based approaches

Comprehensive understanding of the function of eRNAs is still a field in its infancy. A serious effort has to be made to evaluate the potential of eRNA-directed therapy. Nonetheless, the pioneering work of Lam *et al.*, regarding the inhibition of enhancer-directed transcription by the nuclear receptors Rev-Erbs, approached the possibility to control gene expression by repressing eRNAs *in vivo* [3]. In an effort to reproduce the repressive function of Rev-Erbs on a specific macrophage lineage-associated enhancer (Mmp9-eRNA), Lam *et al.* induced sterile peritonitis in mice and subsequently delivered siRNA against the selected eRNAs. Interestingly, this experiment revealed efficient and specific inhibition of both Mmp9-eRNA and the downstream Mmp9 target gene

expression. Although it is so far the first and sole demonstration of eRNA manipulation *in vivo*, this work underscores the feasibility of eRNA therapy using oligonucleotide-based approaches.

Recent advances in antisense oligonucleotide (ASO) technology also provides interesting alternative to classical and nuclease-sensitive siRNA. For instance, ASOs which have phosphorotionate backbone and additional incorporation of modified residues such as 2′-O-methoxyethyl (2′-MOE), 2′-O-methyl (2′-OMe) or locked nucleic acids (LNAs) appears to have an increased stability and a greater affinity for their target sequences [56,57]. Interestingly, LNA molecules are already tested in clinical trials (Phase I and IIa) for the treatment of hepatitis C virus [58,59]. In this case, LNA molecules are used as anti-miRNA to block the miRNA-122's ability to increase the production of viral RNAs [60]. Mechanistically, these LNA anti-miRNAs form an RNA–ASO duplex, which leads to the RNAse-H mediated cleavage of the target RNA. Similarly, modified ASOs have been successfully used against eRNAs *in vitro* and could therefore represent interesting possibilities for therapy *in vivo* [3,4].

Alternatively, the eRNA function could potentially be inactivated by steric-blocking. In this specific situation, the function of the eRNA would be prevented by modified targeting ASOs such as phosphorodiamidate morpholino oligomers (PMOs) [61]. Differently from the above-mentioned modified ASOs, PMOs do not induce RNase H-mediated cleavage of the targeted RNA, but instead disrupt the structure or

physically prevent the binding of proteins [62]. A recent publication demonstrated that at least some ER-regulated eRNAs have the ability to interact with the cohesin complex (e.g., rad21 and smc3 subunits) and modulate the enhancer–promoter interaction [4]. Therefore, specific disruption of enhancer–promoter contacts through PMO-mediated blocking of rad21 and/or SMC3 interacting domains could be of great interest in particular cases.

6.2 BET bromodomain inhibition

The bromodomain and extraterminal domain (BET) family members are adaptor proteins involved in the regulation of transcription. BET proteins, including Brd2, Brd3, Brd4 and Brdt, have two conserved bromodomains, which are utilized to interact with acetylated lysines present on various polypeptides and histone tails, thereby affecting chromatin function [63]. Interestingly, genome-wide analysis of Brd4 chromatin occupancy by chromatin immunoprecipitation followed by sequencing (ChIP-seq) revealed its association with active promoters and enhancers [31,64]. Moreover, genomic sites bound by Brd4 were also enriched with acetylated lysines such as H3K27ac [31]. Through its association with the positive transcription elongation factor p-TEFb, Brd4 can promote the phosphorylation of negative regulators (e.g., NELF and DSIF), which alleviates their functions and resumes paused transcription [65]. In addition, p-TEFb can phosphorylate RNAPII carboxy-terminal domain at serine-2 to promote active elongation [66]. Because of their involvement in transcription regulation, BET bromodomains were considered as potential therapeutic targets and efforts to block their function resulted in the design of two small inhibitory molecules named JQ1 and I-BET [67,68]. Whereas JQ1 was initially shown to increase the survival of mice bearing Brd4-NUT fusion-induced midline carcinomas, I-BET was found to impair the inflammatory response induced by lipopolysaccharide (LPS) in mice. In both cases, BET inhibitors reduce the ability of BET proteins to interact with chromatin by competing for the bromodomain pocket occupancy.

In line with the function of BET proteins as general transcriptional regulators, expression analysis demonstrated that a small but global reduction of mRNA levels was occurring upon BET inhibitors treatment [31,64]. However, the analysis also revealed that transcription suppression was more accentuated in certain specific loci and in a cell-type specific manner, reminiscent of enhancer activity. Indeed, it was shown that highly sensitive regions were composed of enhancer clusters or SEs, which are heavily occupied by Brd4. For instance, several Brd4-bound SEs have been found in association with important multiple myeloma genes, including MYC, CCND2, PIM1 and XBP1 [31]. Interestingly, whereas JQ1-mediated inhibition of MYC expression is dependent on upstream enhancers (IgH enhancers) in multiple myeloma, its repression depends on another group of downstream enhancers (E1-E5 enhancers) in leukemia, once again highlighting the cell-type specific influence of BET

inhibitors [9,69]. More recently, Kanno *et al.* demonstrated that Brd4 is required for eRNA synthesis at enhancers and that JQ1 can efficiently inhibit this process [70]. Although the way BET inhibitors mediate their action at enhancers has not yet been fully addressed, it is possible that Brd4 inhibition prevents RNAPII pause-release and thus the transcription of eRNAs. Altogether, these data suggest that Brd4-directed therapy may be an interesting way to block enhancer function or eRNA production at cell-type specific enhancers.

Although eRNA-directed therapies remain to be rigorously tested, preliminary results are positive and encouraging. Pioneer works already promote alternative ways to efficiently manipulate eRNA levels (Figure 3). Whether the usage of a specific (RNAi-based), a more global (e.g., BET inhibitors) or a combine approach will yield any therapeutic benefits is still unknown.

7. Expert opinion

eRNAs were recently identified as key players in transcriptional regulation of gene expression [1-5]. In parallel to our emerging grasp of their function, interest and efforts begin converging towards the assessment of their therapeutic potential. Considering their widespread presence throughout the genome, regulatory function, and cell-type specific activation, eRNAs offer novel and varied options for therapy. However, despite possible advantageous features associated to eRNA-directed therapy, several challenges still lie ahead.

The first problem consists in globally identifying and characterizing the function of eRNAs in different cell types and tissues. Easy accessibility to rapidly evolving next generation sequencing technologies will certainly help in the endeavor to identify actively transcribe eRNAs in specific contexts. So far, genome-wide mapping of regulatory elements as well as efforts to globally flag active enhancers have been initiated [28,36]. However, functional assessment of eRNAs is currently mostly confined to a single enhancer domain or few loci at the time. Attempt to globally and systematically characterize cell- and tissue-specific enhancer functions remain an essential and colossal task to tackle.

The second challenge concerns the ability to efficiently deliver therapeutic agents against eRNAs. This aspect is particularly relevant for oligonucleotide-based approaches. When introduced into the bloodstream, unmodified siRNAs are rapidly cleaved by nucleases and can also induce the innate immune system [71,72]. To reduce nuclease degradation and immunogenicity, chemical modifications such as 2′-MOE, 2′-OMe or LNA can be used. However, naked siRNAs are sufficiently small to pass through the glomerular filtration barrier and consequently get quickly eliminated out of the bloodstream by the kidney. To minimize the renal clearance and prolong their circulating time, siRNAs can be encapsulated in various lipid-based or polymer-based nanoparticles [73]. At the moment, only cationic liposome
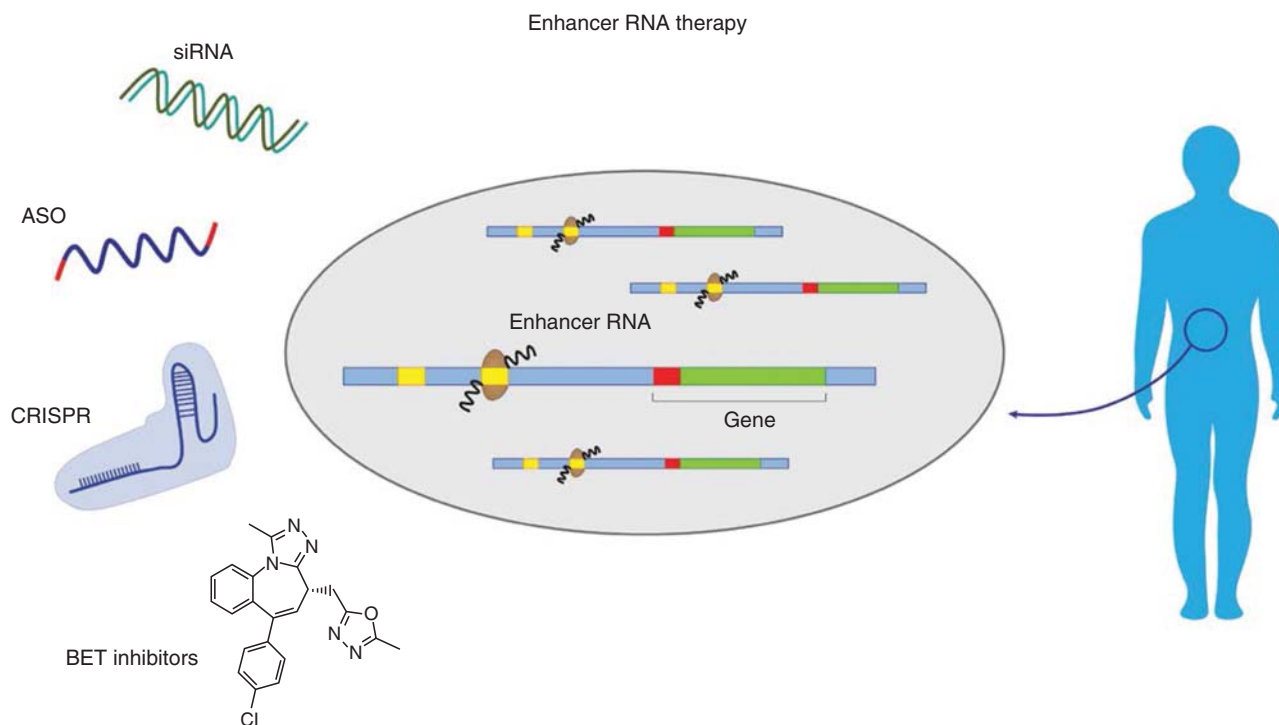
Figure 3. eRNA-directed therapy. Delivery of siRNAs and antisense oligonucleotides can specifically target eRNAs and interfere with their function. Alternatively, BET inhibitors can simultaneously influence a group of active super-enhancers. Genome editing tools such as CRISPR/Cas9 can also be used to remove specific regulatory elements.

ASO: Antisense oligonucleotide.

formulation has been used to deliver siRNA-targeting eRNAs in mice [3]. An assessment of whether this strategy is adequate or needs to be modified for human therapy purpose remains to be determined. Another important aspect of therapeutic delivery concerns the cellular uptake. How to specifically target a cell-type or a tissue is still a major challenge. Some efforts have been made to improve the specificity of the uptake by conjugating siRNAs or their encapsulating nanoparticles with antibodies or ligands [74]. However, specific delivery strategies for many cell and tissue types have not yet been developed.

The third concern associated with eRNA-directed therapy is the presence of unwanted off-target effects. When siRNAs reach their target cells, either the delivery vehicle or the siRNA itself may trigger unexpected changes. For example, siRNAs can regulate additional transcripts through complete or partial complementarity as well as affecting endogenous microRNA processing by saturating the RNAi machinery [71]. Safe therapeutic usage of siRNAs is a complex and challenging problem to solve, which will demand time and further clinical assessment.

As an alternative to RNAi-based strategy, genome engineering tools such as TALEN or CRISPR/Cas9 system are progressively emerging [72,73,75-79]. Differently from siRNAs, which intervene at the RNA level, genome editing gives the possibility to remove or insert a sequence of interest. For

instance, deletion of enhancers driving the expression of key oncogenes or mitotic genes could help in the context of cancer therapy. Although therapeutic usage of genome editing is still in its infancy, it should bring important clinical benefit in the future.

In summary, eRNA-directed therapy holds a great potential that is increasingly being considered. However, several biological and technological hurdles remain to be solved before eRNA-targeting can be proposed as a safe and efficient therapeutic alternative.

Acknowledgement

Declaration of interest

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