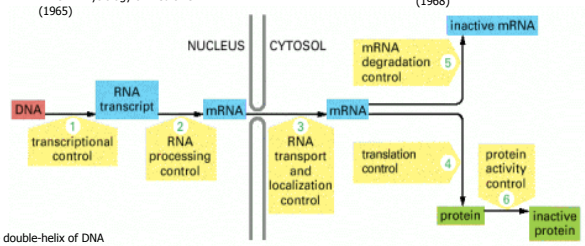
Small regulatory RNAs - gene silencing and editing

Serena Zacchigna, MD PhD
Group Leader, Cardiovascular Biology
ICGEB, Trieste
zacchign@icgeb.org

RNA in the CONTROL of GENE EXPRESSION - a Nobel story

The gene is transcribed into a specific RNA species, mRNA
F. Jacob and J. Monod; Nobel Prize in Physiology or Medicine (1965)

The genetic code
M. Nirenberg and G. Khorana; Nobel Prize in Physiology or Medicine (1968)



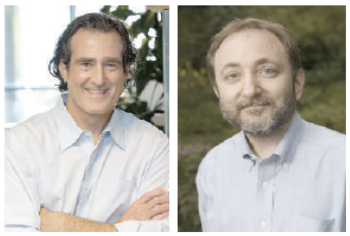
The double-helix of DNA identified as the genetic material
F.Crick, J. Watson and M. Wilkins; Nobel Prize in Physiology or Medicine (1962)

The split gene
P. Sharp and R. Roberts; Nobel Prize (1993)

RNA can act as a catalyst
S. Altman and T. Cech; Nobel Prize in Chemistry (1989)

Youthful duo snags a swift Nobel for RNA control of genes

Nobel prize 2006 Physiology and Medicine to Craig Mello and Andrew Fire for their report on RNAi.

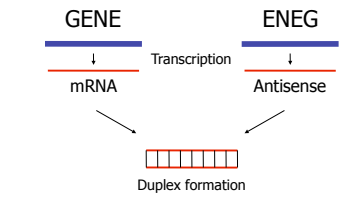


Silence is golden: Craig Mello (left) and Andrew Fire.

Short history of post-transcriptional gene silencing

- **1962, Singer, Jones, Nirenberg**
  Translation of mRNA can be blocked by complementary (**antisense**) RNA
- **1990, Jorgensen**
  Introduction of transgenes homologous to endogenous genes often results in plants with both genes suppressed (**co-suppression**)
- **1995, Guo and Kemphues**
  Injection of either antisense or sense RNAs in the germline of C.elegans is equally effective at silencing homologous target genes
- **1998, Mello and Fire**
  Combination of sense and antisense RNA (=**dsRNA**) is 10 times more effective than ssRNA

Antisense RNA

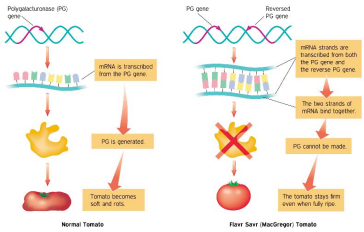


Right: Flower of a tobacco plant carrying a transgene whose transcript is antisense to one of the mRNAs needed for normal flower pigmentation. Left: Flower of another transgenic plant that failed to have its normal pigmentation altered.
(van der Krol, et. al., from Nature 333:866, 1988.)

When the aRNA binds to the complementary mRNA, it forms a double-stranded RNA (dsRNA) complex that is similar to double-stranded DNA. The dsRNA complex does not allow normal translation to occur. The exact mechanism by which translation is blocked is unknown. Several theories include:

- that the dsRNA prevents ribosomes from binding to the sense RNA and translating (Kimball, Nov 2002)
- that the dsRNA cannot be transported from within the nucleus to the cytosol, which is where translation occurs (Tritton, 1998)
- that dsRNA is susceptible to endoribonucleases that would otherwise not affect single stranded RNA, but degrade the dsRNA (Kimball, Nov 2002)

Longer lasting tomatoes by RNA antisense technology



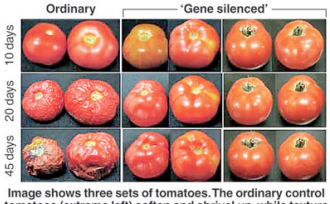


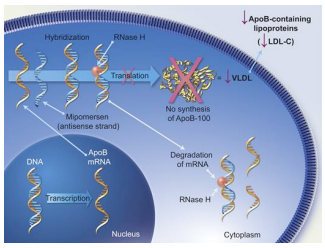
Image shows three sets of tomatoes. The ordinary control tomatoes (extreme left) soften and shrivel up, while texture of gene-silenced tomatoes remains intact for up to 45 days.
Photo credit: Asis Datta, Subhra Chakraborty, National Institute of Plant Genome Research, New Delhi

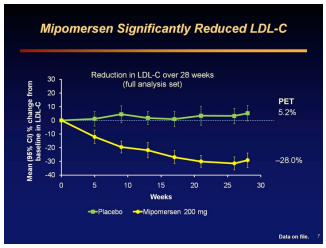
The **Flavr Savr** tomato is a genetically altered tomato developed by Calgene. It contains an antisense RNA which inhibits the expression of a gene that normally causes fruit to soften, therefore, the fruit stays firm longer. This allows producers a greater period of time for transportation and the opportunity for mechanical harvesting with little bruising.

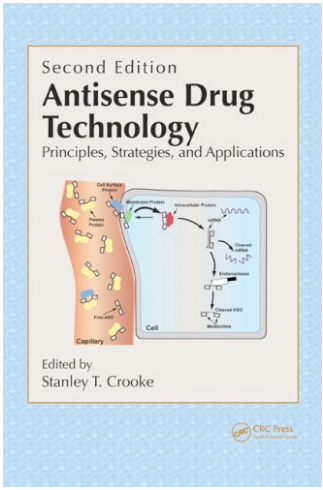
Antisense approach for lipid management

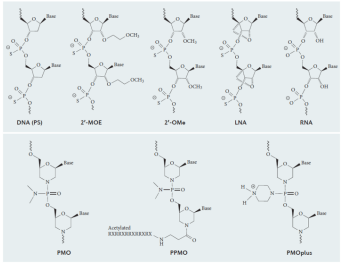
KYNAMRO® is an oligonucleotide inhibitor of apolipoprotein B-100 synthesis indicated as an adjunct to lipid-lowering medications and diet to reduce low density lipoprotein-cholesterol (LDL-C), apolipoprotein B (apo B), total cholesterol (TC), and non-high density lipoprotein-cholesterol (non-HDL-C) in patients with homozygous familial hypercholesterolemia (HoFH).

Because of the risk of hepatotoxicity, KYNAMRO is available only through a restricted program under a Risk Evaluation and Mitigation Strategy (REMS) called the KYNAMRO REMS.









RNA therapeutics: beyond RNA interference and antisense oligonucleotides

Ryszard Kole[1], Adrian R. Krainer[2] and Sidney Altman[3]

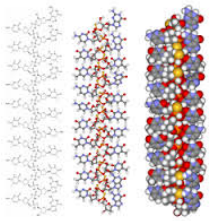
Abstract | Here, we discuss three RNA-based therapeutic technologies exploiting various oligonucleotides that bind to RNA by base pairing in a sequence-specific manner yet have different mechanisms of action and effects. RNA interference and antisense oligonucleotides downregulate gene expression by inducing enzyme-dependent degradation of targeted mRNA. Steric-blocking oligonucleotides block the access of cellular machinery to pre-mRNA and mRNA without degrading the RNA. Through this mechanism, steric-blocking oligonucleotides can redirect alternative splicing, repair defective RNA, restore protein production or downregulate gene expression. Moreover, they can be extensively chemically modified to acquire more drug-like properties. The ability of RNA-blocking oligonucleotides to restore gene function makes them best suited for the treatment of genetic disorders. Positive results from clinical trials for the treatment of Duchenne muscular dystrophy show that this technology is close to achieving its clinical potential.

NATURE REVIEWS | DRUG DISCOVERY VOLUME 11 | FEBRUARY 2012

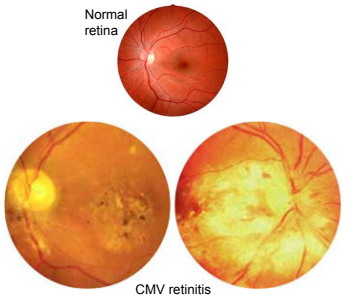
Fomivirsen

Fomivirsen sodium is a phosphorothioate oligonucleotide, twenty-one nucleotides in length, with the following sequence: 5'-GCG TTT GCT CTT CTT CTT GCG-3'

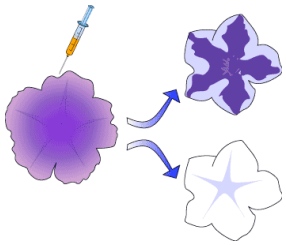
Vitravene (fomivirsen) is indicated for the local treatment of cytomegalovirus (CMV) infections, and in particular of CMV retinitis in patients with acquired immunodeficiency syndrome (AIDS), who are intolerant of or have a contraindication to other treatment(s) for CMV retinitis or who were insufficiently responsive to previous treatment(s) for CMV retinitis.



Target sequence: IE2 gene of the CMV genome



Co-suppression



Researchers were trying to deepen the purple colour of the flowers by injecting the gene responsible into the petunias, but were surprised at the result. Instead of a darker flower, the petunias were either variegated or completely white!

This phenomenon was termed **co-suppression**, since both the expression of the existing gene (the initial purple colour), and the introduced gene (to deepen the purple) were suppressed. Co-suppression has since been found in many other plant species and also in fungi. It is now known that **double stranded RNA is responsible for this effect**.

Example petunia plants in which genes for pigmentation are silenced by co-suppression. The left plant is wild-type; the right plants contain transgenes that induce suppression of both transgene and endogenous gene expression, giving rise to the unpigmented white areas of the flower

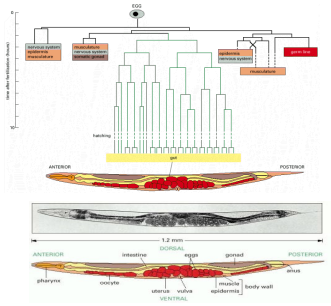
Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*

Andrew Fire*, SiQun Xu*, Mary K. Montgomery*, Steven A. Kostas*†, Samuel E. Driver† & Craig C. Mello‡

** Carnegie Institution of Washington, Department of Embryology, 115 West University Parkway, Baltimore, Maryland 21210, USA*
† Biology Graduate Program, Johns Hopkins University, 3400 North Charles Street, Baltimore, Maryland 21218, USA
‡ Program in Molecular Medicine, Department of Cell Biology, University of Massachusetts Cancer Center, Two Biotech Suite 213, 373 Plantation Street, Worcester, Massachusetts 01605, USA

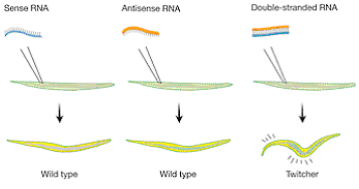
Experimental introduction of RNA into cells can be used in certain biological systems to interfere with the function of an endogenous gene[1,2]. Such effects have been proposed to result from a simple antisense mechanism that depends on hybridization between the injected RNA and endogenous messenger RNA transcripts. RNA interference has been used in the nematode *Caenorhabditis elegans* to manipulate gene expression[3,4]. Here we investigate the requirements for structure and delivery of the interfering RNA. To our surprise, we found that double-stranded RNA was substantially more effective at producing interference than was either strand individually. After injection into adult animals, purified single strands had at most a modest effect, whereas double-stranded mixtures caused potent and specific interference. The effects of this interference were evident in both the injected animals and their progeny. Only a few molecules of injected double-stranded RNA were required per affected cell, arguing against stochiometric interference with endogenous mRNA and suggesting that there could be a catalytic or amplification component in the interference process.

NATURE | VOL 391 | 19 FEBRUARY 1998



C. elegans is a precious tool in developmental biology:
- it is tiny and grow rapidly
- females are composed of 956 cells
- males are composed of 1031 cells
- the fate of every cell is characterized

Conclusions of Fire&Mello's study:

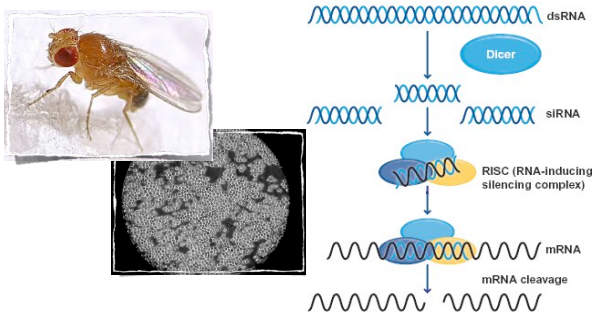


C. elegans

*Phenotypic effect after injection of single-stranded or double-stranded unc-22 RNA into the gonad of C. elegans. The **unc-22** gene encodes a myofilament protein. Decrease in unc-22 activity is known to produce severe twitching movements. Injected double-stranded RNA, but not single-stranded RNA, induced the twitching phenotype in the progeny.*

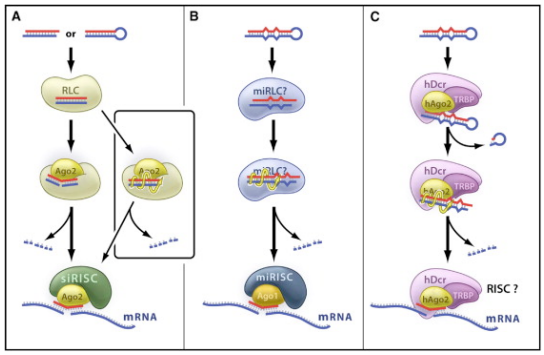
1) silencing was triggered efficiently by injected **dsRNA**, but weakly or not at all by sense or antisense single-stranded RNAs.
2) silencing was **specific** for an mRNA homologous to the dsRNA; other mRNAs were unaffected
3) the dsRNA had to correspond to the mature mRNA sequence; neither intron nor promoter sequences triggered a response. This indicated a **post-transcriptional**, presumably **cytoplasmic** mechanism
4) the targeted mRNA disappeared suggesting that it was **degraded**
5) only a few dsRNA molecules per cell were sufficient to accomplish full silencing. This indicated that the dsRNA was amplified and/or acted **catalytically** rather than stoichiometrically
6) the dsRNA effect could spread between tissues and even to the progeny, suggesting a **transmission** of the effect between cells

The RISC COMPLEX was discovered in Drosophila cultured cells



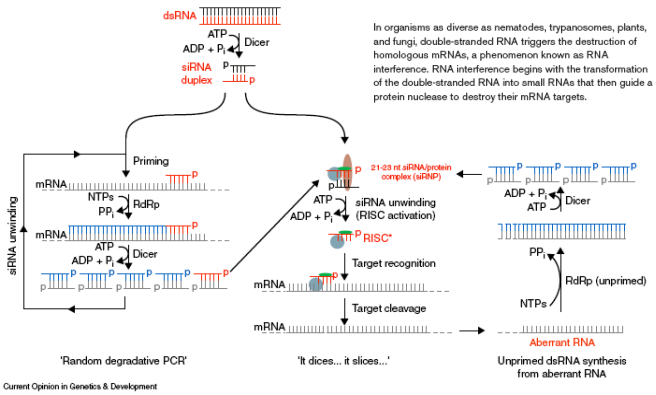
The molecular machinery responsible for RNAi involves a large complex, called RISC (RNA-induced silencing complex), which is targeted to the mRNA via the antisense RNA. The mRNA is cleaved and subsequently degraded.

Mechanisms of RNA loading and activation within RISC

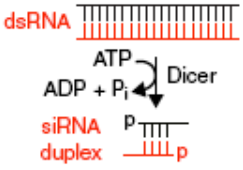


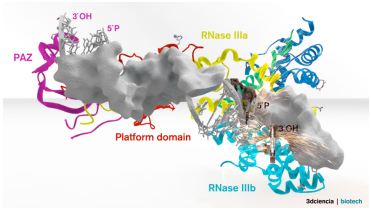
RISC contains at least one member of the argonaute protein family, which is likely to act as an endonuclease and cut the mRNA.

RNAi: nature abhors a double-strand
György Hutvágner and Phillip D Zamore*



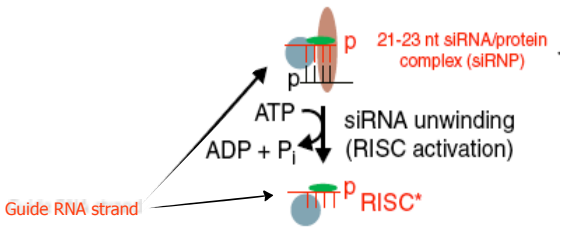
It dices…



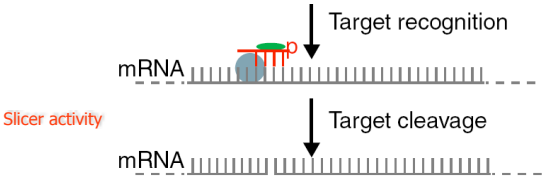


RNAi is initiated by the ATP-dependent, processive cleavage of long dsRNA into 21-25 nt ds-fragments, termed **small interfering RNAs** (**siRNAs**). This cleavage is mediated by the enzyme Dicer (a member of the RNase III family of dsRNA-specific endonucleases).

The siRNA duplexes are incorporated into a protein complex that is not yet competent to mediate RNAi.
ATP-dependent unwinding of the siRNA duplex remodels the complex to generate an active RNA-induced silencing complex (RISC - the asterisk denotes active conformation)



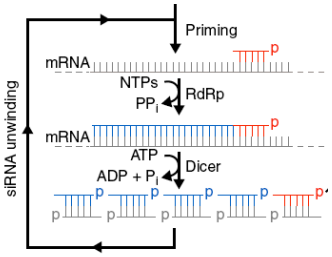
It slices…



Finally, in a step that requires little or no ATP, the RISC* can recognize and cleave a target RNA complementary to the guide strand of the siRNA.

Random degradative PCR

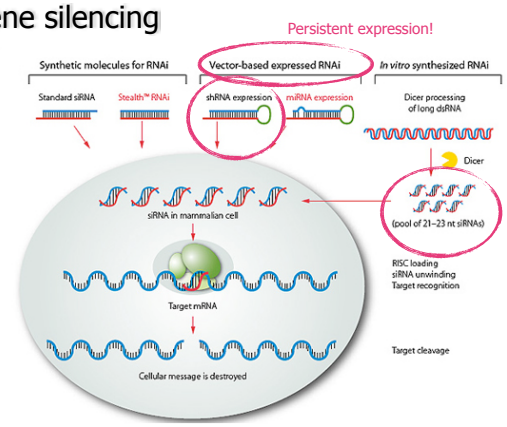
The discovery of RNA-dependent RNA polymerases (RdRPs) in plants, warms and fungi provides a possible explanation for the **remarkable efficacy** of dsRNA in gene silencing - in worms RNAi not only spread throughout the entire animal, but also can be inherited through multiple generations.



In Drosophila embryos, 35 molecules of dsRNA can silence a target mRNA thought to be present at >1000 copies per cell.

In the "random degradative PCR" model, the RdRP uses the **guide siRNA strand** as a **primer** to synthesize new RNA, using the **target RNA** as a **template** and thereby converting it into dsRNA, that can be then processed by Dicer. This in turn would release new siRNAs to prime additional rounds of synthesis and target destruction.

RNAi in mammalian cells - a precious tool for gene silencing

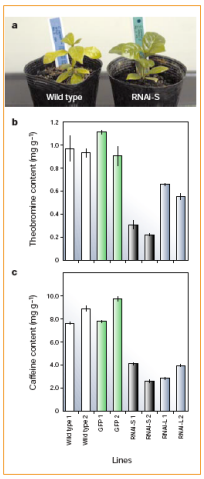


RNA interference

Producing decaffeinated coffee plants

Three N-methyltransferases are involved in caffeine biosynthesis - CaXMT1, CaMXMT1 (theobromine synthase) and CaDXMT1 (caffein synthase).

Coffee plants in which expression of CaMXMT1 is repressed by RNAi have a caffeine content reduced by up to 70%.



NATURE | VOL 423 | 19 JUNE 2003 | www.nature.com/nature

siRNA/shRNA Therapeutics in Clinical Trials

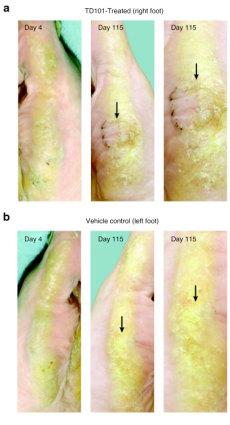
Company	Drug	Delivery route	Target	Vehicle	Disease	Phase
Santaris	SPC3649 (LNA)	SC	miR-122	Naked LNA	HCV	IIa
Opko Health	Bevasiranib	IVT	VEGF	Naked siRNA	AMD/DME	III
Allergan/Sirna	AGN-745	IVT	VEGF-R1	Naked siRNA	AMD	II
Quark/Pfizer	PF-655	IVT	RTP801	Naked siRNA	AMD/DME	II
Quark Pharma	QPI- 1007	IVT	Caspase 2	Naked siRNA	NAION	I
TransDerm/IPCC	TD101	Intralesional injection	KRT6A(N171K)	Naked siRNA	Pachyonychia Congenita	II
Sylentis	SYL040012	Ophthalmic drops	ADRB2	Naked siRNA	Intraocular Pressure	II
Sylentis	SYL1001	Ophthalmic drops	TRPV1	Naked siRNA	Dry eye syndrome	I
ZaBeCor	ExcellAirTM	Inhalation	Syk kinase	unknown	Asthma	II
Alnylam/Cubist	ALN-RSV01	Nebulization or intranasal	RSV Nucleocapsid	Naked siRNA	RSV	IIb
Marina Biotech	CEQ508	Oral	Beta catenin	tkRNAi in E. Coli	FAP/ colon cancer	I
Silenseed Ltd	siG12D LODER	EUS biopsy needle	KRASG12D	LODER polymer	PDAC	I
Tekmira	TKM-ApoB	IV	Apo B	SNALP	Hypercholesterolemia	I
Tekmira	TKM-PLK1	IV	PLK1	SNALP	Solid tumors	I
Alnylam/Tekmira	ALN-VSP02	IV	KSP and VEGF	SNALP	Solid tumors	I
Alnylam	ALN-TTR01	IV	TTR	SNALP	TTR-mediated amyloidosis (ATTR)	I
University Duisburg	Bcr-Abl siRNA	IV	Bcr-Abl	Anionic liposome	CML	I
Silence Therapeutics	Atu027	IV	PKN3	siRNA-lipoplex	Advanced solid cancer	I
Quark Pharma	IcNP	IV	P53	Naked siRNA	AKI and DGF	II
Calando Pharma	CALAA-01	IV	RRM2	Cyclodextrin nanoparticle, TF, and PEG	Solid tumors	I
Gradalis Inc.	FANG vaccine	*Ex vivo* IV	Furin and GM-CSF	Electroporation	Solid tumors	II
Duke University	IPsiRNA	*Ex vivo* intradermal injection	LMP2, LMP7, MECL1	Transfection	Metastatic melanoma	I
City of Hope/Benitec	Tat/Rev shRNA	*Ex vivo* transplant	HIV Tat and Rev	Lentivirus	HIV	o

Disease	Stage	RNAi reagent	Delivery	Company/institution
Ocular diseases				
AMD	Preclinical stage	siRNA	Direct intravitreal injection	Quark Biotech
	Clinical trial phase I	siRNA	Direct intravitreal injection	Sirna
	Clinical trial phase II	siRNA	Direct intravitreal injection	Acuity
Viral infections				
Hepatitis B and C	Preclinical stage	shRNA	Liganded nanoparticle	Nucleonics/Intradigm
RSV	Clinical trial phase I	siRNA	Aerosol	Alnylam
HIV	Clinical trial phase I (scheduled for 2007)	shRNA	Lentivirus	Benitec/City of Hope
Cancer				
Hepatic cancer	Preclinical stage	siRNA	Liganded nanoparticle	Calando
Solid tumour cancers	Preclinical stage	siRNA	Liganded nanoparticle	Intradigm
Other disease types				
ALS	Preclinical stage	siRNA	N/A	CytRx
Inflammatory diseases	Preclinical stage	siRNA	Peptide	Nastech

ALS, amyotrophic lateral sclerosis; AMD, age-related macular degeneration; RNAi, RNA interference; RSV, respiratory syncytial virus; shRNA, short hairpin RNA; siRNA, small interfering RNA.

First-in-human mutation-targeted siRNA phase Ib trial of an inherited skin disorder

TransDerm, along with the International Pachyonychia Congenita Consortium (IPCC), has designed the first mutation-specific siRNA to be used for human therapy. The **TD101** siRNA is directed at the mRNA sequence encompassing the dominant mutation (N171K) in the **keratin 6a gene** (KRT6A). This mutation causes pachyonychia congenita, a rare skin disorder characterized by **painful calluses on weight-bearing areas and hypertrophic nails** among other epidermal defects. The siRNA therapy was administered by intralesional injection in a single patient using a split body control. Since the Phase Ib therapy (NCT00716014) was well tolerated and efficacious in reducing the callus, TransDerm is developing less painful alternatives for delivering the drug, such as an ointment with lipid-based carriers (GeneCreme) and a dissolvable microneedle array (Protrusion Array Device).

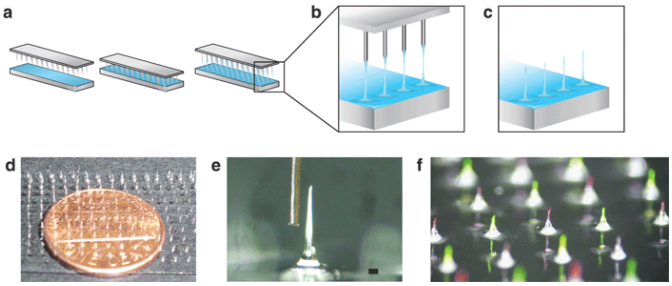


Molecular Therapy 2012

TRANSDERM, INC

Protrusion Array Device (PAD)

It consists of a loadable ordered grid of needle-like microprotrusions formed from injection-safe soluble polymers.

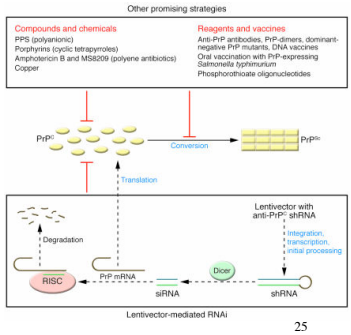


(**a**) Pin template and glass slide covered with a thin film of 20% polyvinyl alcohol (PVA) solution (left panel). The pin template is placed in contact with the PVA solution (middle panel). Microneedles are produced by withdrawing the pins as the film is drying, forming fiber-like structures (right panel). (**b**) Enlarged view of fibers. (**c**) Protrusions are subsequently trimmed to the desired length and tip shape. (**d**) PAD supported by a glass substrate, with a penny to show scale. (**e**) Micrograph of one microneedle after trimming to 1 mm length, showing beveled structure that facilitates skin penetration, with a human hair to show scale (bar = 80 μm). (**f**) PAD needles loaded alternately with fluorescein (green) and R-phycoerythrin (red).

Lentivector-mediated RNAi efficiently suppresses prion protein and prolongs survival of scrapie-infected mice

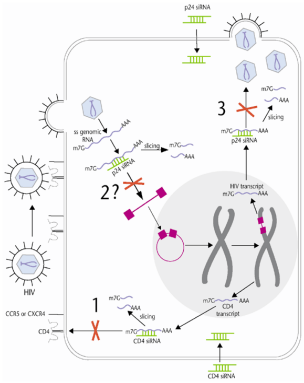
Alexander Pfeifer,[1,2] Sabina Eigenbrod,[3] Saba Al-Khadra,[1,2] Andreas Hofmann,[1,2] Gerda Mitteregger,[3] Markus Moser,[4] Uwe Bertsch,[3] and Hans Kretzschmar[3]

[1]Institute of Pharmacology and Toxicology, University of Bonn, Bonn, Germany, [2]Molecular Pharmacology, Department of Pharmacy, and [3]Center for Neuropathology and Prion Research, Ludwig-Maximilians-University of Munich, Munich, Germany. [4]Max Planck Institute of Biochemistry, Molecular Medicine, Martinsried, Germany.



The Journal of Clinical Investigation http://www.jci.org Volume 116 Number 12 December 2006

Silencing viruses by RNAi



siRNA-directed inhibition of HIV-1 infection
Carl D. Novina *et al.*
Nature Medicine 8, 681 - 686 (2002)

Modulation of HIV-1 replication by RNA interference

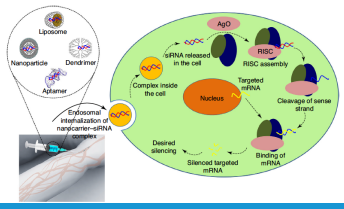
Jean-Marc Jacque, Karine Triques & Mario Stevenson

Short interfering RNA confers intracellular antiviral immunity in human cells

Leonid Gitlin*†‡, Sveta Karelsky* & Raul Andino*

NATURE | VOL 418 | 25 JULY 2002 | www.nature.com/nature

Drug Discovery Today • Volume 19, Number 12 • December 2014



siRNA nanotherapeutics: a Trojan horse approach against HIV

Vijay Mishra, Prashant Kesharwani and Narendra K. Jain

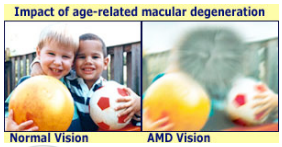
Pharmaceutics Research Laboratory, Department of Pharmaceutical Sciences, Dr H.S. Gour Central University, Sagar, MP, India

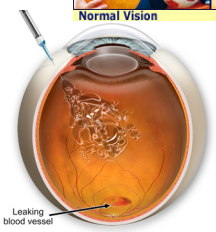
The concept of RNA interference (RNAi) is gaining popularity for the better management of various diseases, including HIV. Currently, the successful biomedical utilization of siRNA therapeutics is hampered, both ***in vivo*** and ***in vitro***, mainly by the inherent inability of naked siRNA to cross the cell membrane. RNAi can potentially improve the weakness of current highly active antiretroviral therapy (HAART) by diminishing the chances of the appearance of antiHIV-resistant strains. Here, we discuss the nanocarrier-mediated delivery of siRNA delivery as well as highlighted the scope of siRNA-mediated gene-silencing technology for improved HIV treatment.

Anti-VEGF for wet AMD

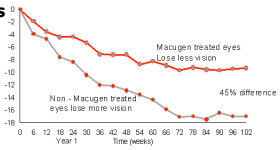
Macugen (Pegaptanib sodium - pegylated aptamer that binds VEGF165)

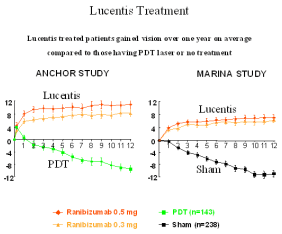
Lucentis (ranibizumab) - recombinant humanized Fab that binds all VEGF isoforms





Intravitreal injection into the back of the eye





Bevasiranib

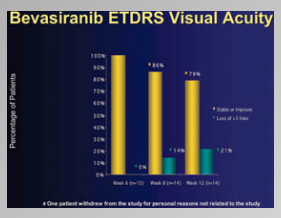
ACUITY PHARMACEUTICALS

Bevasiranib

Competitive Advantages

Bevasiranib silences the genes that produce vascular endothelial growth factor (VEGF), which has been shown to be the central stimulus in the blood vessel overgrowth and leakage that leads to vision loss in wet AMD and DME. Bevasiranib is administered directly into the eye and has demonstrated no systemic effects, an important safety consideration. In preclinical and clinical studies, its potent RNAi mechanism demonstrated the potential for efficacy, low side effects and less frequent delivery, making it potentially valuable both as monotherapy and as a complementary and synergistic agent for use with other therapies—as the **AMD Maintenance Therapy of Choice™**. Patients with wet AMD may benefit from initial treatment by a VEGF antagonist followed by long-term maintenance therapy with bevasiranib. This market positioning has attractive commercial potential.

Bevasiranib is a synthetic double-stranded RNA (dsRNA) oligonucleotide that selectively inhibits the production of all isoforms of VEGF by efficiently and effectively halting the production of VEGF on the mRNA level. VEGF is a protein that has been shown to be the central stimulus in the development of ocular neovascularization. Bevasiranib is administered locally to the eye via an intravitreal injection.



Visual acuity results from the Phase I study of bevasiranib siRNA therapy. Patients received an intravitreal injection at day 0 and week 6.

OPKO has halted Phase 3 trial with Bevasiranib in wet AMD for lack of efficacy in 2013

2010-2014 -The era of doubts and despair for siRNA-based therapeutics

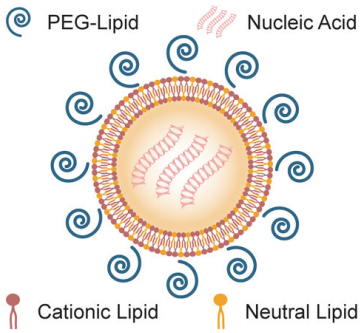
In 2010, Roche, which had invested about $500 million in RNAi, shut down its internal research program

In 2011 Pfizer and Abbott also pulled out of in-house RNAi development

In 2012 Merck shuttered the RNAi laboratory it had acquired in 2006 with its $1.1 billion purchase of Sirna Therapeutics

siRNA lipid nanoparticles (LNPs)

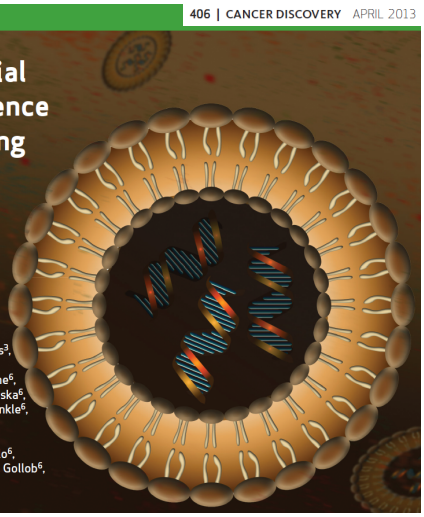
While unmodified siRNAs have been injected locally into the eye and other organs in early trials, those released directly into the bloodstream are degraded by enzymes and are unable to cross cell membranes. One strategy for smuggling siRNAs through the blood and into diseased cells is to embed them in lipid nanoparticles (LNPs).



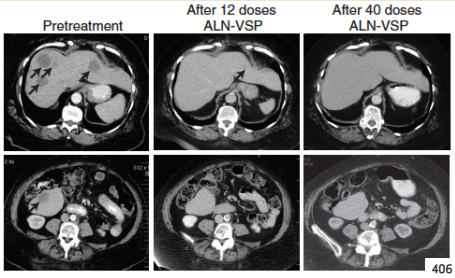
In vivo, siRNA LNPs generally end up in the liver. The liver is highly vascularized and its endothelium is peppered with pores about 100 nanometers in diameter, wide enough for 70– to 80–nanometer LNPs to slip through en route to hepatocytes. Moreover, once the LNPs are released into the bloodstream, they are rapidly coated with apolipoprotein E (ApoE), which binds to receptors on hepatocytes and eases cell entry of the nanoparticles.

First-in-Humans Trial of an RNA Interference Therapeutic Targeting VEGF and KSP in Cancer Patients with Liver Involvement

Josep Tabernero[1], Geoffrey I. Shapiro[4], Patricia M. LoRusso[7], Andres Cervantes[2], Gary K. Schwartz[8], Glen J. Weiss[9], Luis Paz-Ares[3], Daniel C. Cho[5], Jeffrey R. Infante[10], Maria Alsina[1], Mrinal M. Gounder[8], Rick Falzone[6], Jamie Harrop[6], Amy C. Seila White[6], Iva Toudjarska[6], David Bumcrot[6], Rachel E. Meyers[6], Gregory Hinkle[6], Nenad Svrzikapa[6], Renta M. Hutabarat[6], Valerie A. Clausen[6], Jeffrey Cehelsky[6], Saraswathy V. Nochur[6], Christina Gamba-Vitalo[6], Akshay K. Vaishnaw[6], Dinah W.Y. Sah[6], Jared A. Gollob[6], and Howard A. Burris III[10]



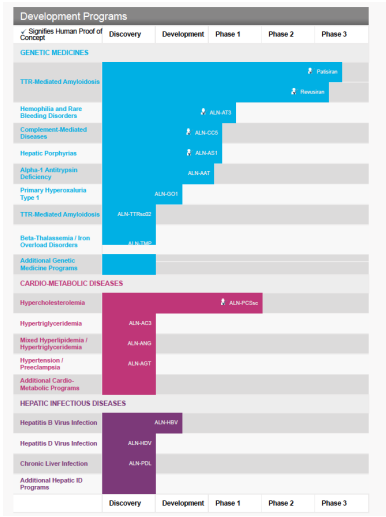
ABSTRACT RNA interference (RNAi) is a potent and specific mechanism for regulating gene expression. Harnessing RNAi to silence genes involved in disease holds promise for the development of a new class of therapeutics. Delivery is key to realizing the potential of RNAi, and lipid nanoparticles (LNP) have proved effective in delivery of siRNAs to the liver and to tumors in animals. To examine the activity and safety of LNP-formulated siRNAs in humans, we initiated a trial of ALN-VSP, an LNP formulation of siRNAs targeting *VEGF* and kinesin spindle protein (KSP), in patients with cancer. Here, we show detection of drug in tumor biopsies, siRNA-mediated mRNA cleavage in the liver, pharmacodynamics suggestive of target downregulation, and antitumor activity, including complete regression of liver metastases in endometrial cancer. In addition, we show that biweekly intravenous administration of ALN-VSP was safe and well tolerated. These data provide proof-of-concept for RNAi therapeutics in humans and form the basis for further development in cancer.



Alnylam PHARMACEUTICALS

Alnylam now concentrates on liver-based diseases, with more than 15 RNAi therapies in clinical development for 3 strategic areas:

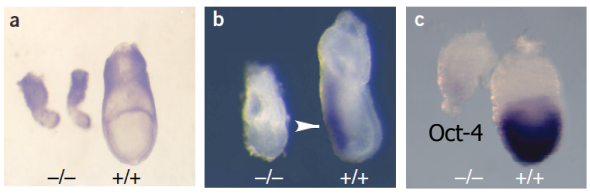
1. Genetic Medicine: treatment of rare diseases
2. Cardio-Metabolic: liver-expressed disease targets for unmet needs in dyslipidemia, hypertension, non-alcoholic steatohepatitis (NASH), type 2 diabetes
3. Hepatic Infectious Diseases: HBV, HDV



The endogenous role of RNAi

Dicer is essential for mouse development

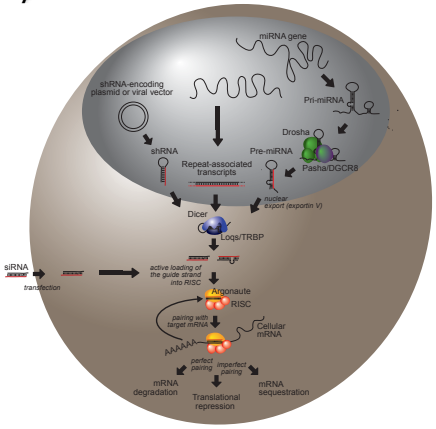
Emily Bernstein[1,2], Sang Yong Kim[1], Michelle A Carmell[1,2], Elizabeth P Murchison[1], Heather Alcorn[3], Mamie Z Li[4], Alea A Mills[1], Stephen J Elledge[4], Kathryn V Anderson[3] & Gregory J Hannon[1]



E7.5 embryos - lack of stem cell development

NATURE GENETICS VOLUME 35 | NUMBER 3 | NOVEMBER 2003

RNAi in mammalian cells works by siRNAs and by miRNAs

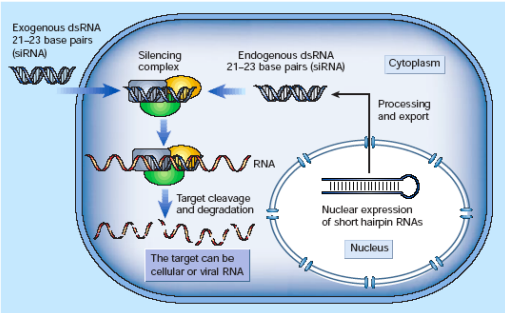


miRNAs are produced by the successive action of two RNaseIII ribonucleases. After transcription, **primary miRNAs** are cleaved in the nucleus by **Drosha**. Pre-miRNAs bind **exportin V** and is exported to the cytoplasm, where **Dicer** is thought to bind the base of the pre-miRNA stem defined in the nucleus by Drosha. Dicer cleavage liberates a duplex comprising the miRNA and miR*.

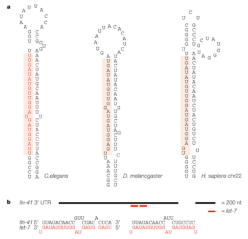
The miRNA must then be unwound and selectively incorporated into RISC to search for targets by its **seed sequence**.

miRNAs, siRNAs and shRNAs differ in their biogenesis, not in their function

Short dsRNAs can be introduced into cells from the outside, or are produced within the cell nucleus from longer precursors forming hairpin structures, which are cleaved to generate shorter RNAs (21-23 bp), that are then exported to the cytoplasm

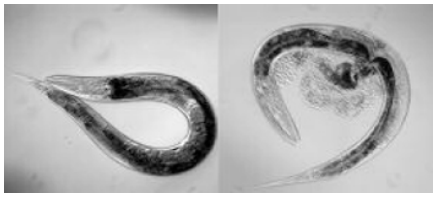


Developmental control by miRNAs



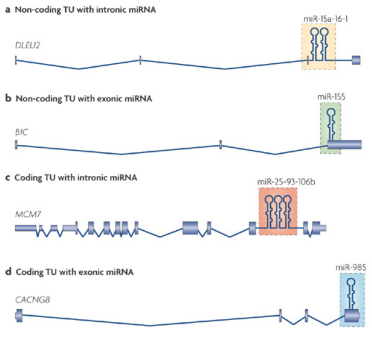
Predicted *let-7* precursor RNAs of *Caenorhabditis elegans*, *Drosophila melanogaster* and *Homo sapiens*. The region that corresponds to the mature *let-7* RNA is shaded pink

Worms with a mutated form of the microRNA let-7 (right) have severe growth problems, rupturing as they develop.

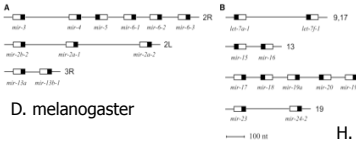


Identification of novel genes coding for small expressed RNAs (micro RNAs - miRNA)

Lagos-Quintana M, Rauhut R, Lendeckel W, Tuschl T.
2001, Science 294, 853



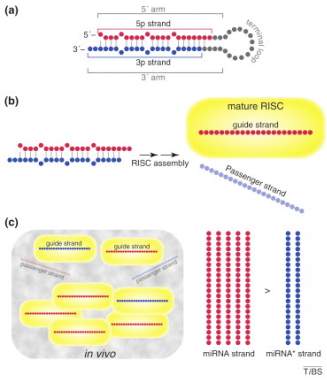
Gene clusters for miRNAs:



D. melanogaster

H. sapiens

Nomenclature for small RNA strands

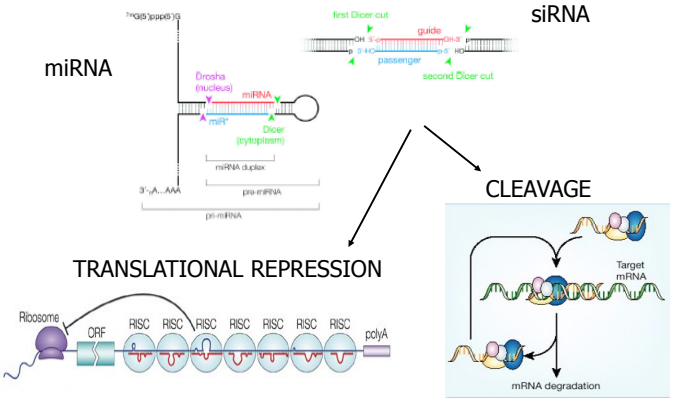


5p and 3p strands: 5p and 3p designate the strands derived from the 5′ arm and 3′ arm of a pre-miRNA, respectively.

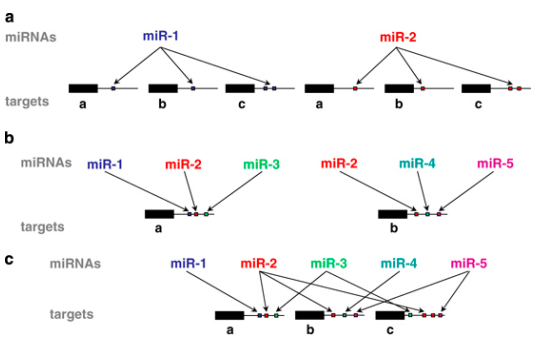
Guide and passenger strands: the guide strand is retained in the mature RISC whereas the passenger strand is discarded upon unwinding. Which strand is selected as the guide is independent of the original orientation within the pre-miRNA (i.e. 5p or 3p) or long dsRNA precursors, but does depend on the thermodynamic asymmetry, the 5′ nucleotide identity and the structure of the small RNA duplex.

miRNA and miRNA* strands: the miRNA strand is the more abundant (and thereby more frequently cloned) strand overall in vivo whereas the miRNA* strand is the less abundant strand. Note that a passenger strand is quickly degraded as soon as it is discarded from pre-RISC whereas a guide strand is protected from nucleases in the mature RISC. Consequently, the strand that is more likely to serve as the guide strand tends to accumulate and therefore become the 'miRNA strand'.

Like siRNAs, miRNAs can cleave their mRNA targets when the two are extensively complementary, but repress mRNA translation when they are not

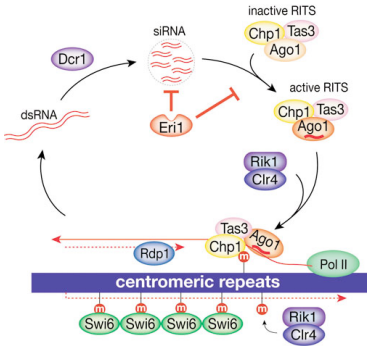


miRNAs target multiple genes and genes are targeted by multiple miRNAs



(**a**) miRNAs have multiple targets. (**b**) Many genes have seed matches for multiple miRNAs in their 3′UTRs. (**c**) A complex network of mutual interactions between miRNAs and mRNAs.

RITS Connects RNAi and Heterchromatin Formation Machinery



Transcriptionally silent transgenes within the centromeric heterochromatin of *S. pombe* are activated in mutants lacking *Argonaute*, Dicer or RdRp.

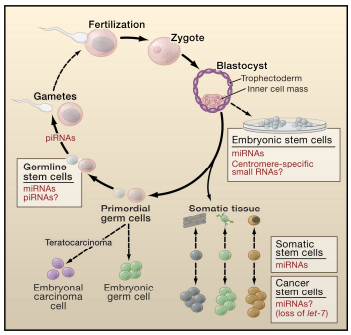
RdRp is physically associated with centromeric heterochromatin. The transcriptional silencing of centromeric heterochromatin is mediated by the RNAi machinery and transcripts encoded by centromeric DNA.

Quite how RNAi initiates chromatin silencing has not been clarified yet. One possibility is that the localised production of small dsRNA molecules enables unspecified chromodomain proteins to recruit histone methyltransferases to the pre-heterochromatic region.

Small RNAs: Keeping Stem Cells in Line

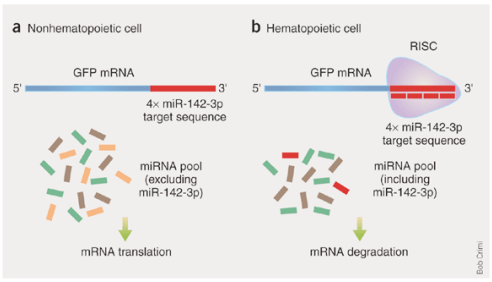
Bradford M. Stadler[1] and Hannele Ruohola-Baker[1,*]
[1]Department of Biochemistry and Institute for Stem Cell and Regenerative Medicine, University of Washington, 1705 NE Pacific Street, Health Science Building, Room J-587, Seattle, WA 98195, USA
*Correspondence: hannele@u.washington.edu
DOI 10.1016/j.cell.2008.02.005

Stem cells and RNA silencing have emerged as areas of intense interest for both basic and clinical research. Recently these fields have converged with reports implicating small regulatory RNAs in the maintenance and pluripotency of stem cells.



Cell 132, February 22, 2008 ©2008 Elsevier Inc.

RISC control for gene therapy



miR-142-3p is specifically expressed by hematopoietic cells

(**a**) In nonhematopoietic cells, expression of tagged green fluorescent protein (GFP) proceeds because endogenous miR-142-3p is absent. (**b**) In antigen-presenting cells, miR-142-3p is incorporated into a RNA-induced silencing complex (RISC), which recognizes the tag and silences gene expression through mRNA cleavage or translational repression.

Endogenous microRNA regulation suppresses transgene expression in hematopoietic lineages and enables stable gene transfer

Brian D Brown[1], Mary Anna Venneri[1], Anna Zingale[1], Lucia Sergi Sergi[1] & Luigi Naldini[1,2]

NATURE MEDICINE VOLUME 12 | NUMBER 5 | MAY 2006

Eat Less, Live Longer? miRNAs Link Calorie Restriction To Longevity

Caloric restriction (CR) is the most effective environmental method to increase lifespan (and to prevent late-onset diseases!)

Dietary restriction extends lifespan in S. cerevisiae, C. elegans, D. melanogaster, rodents and primates.

CR = 60-70% of what an animal would eat at libitum

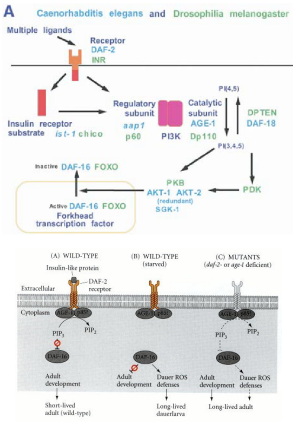
In rodents CR results in as much as a 50% increase in rodent longevity

Physiological effects of CR: acute phase followed by an adaptive period of several weeks to reach a stable, altered physiological state characterized by lower body temperature, lower blood glucose and insulin levels and reduced fat and weight.

The CR animals are more resistant to external stressors, including heat and oxidative stress; organs are typically smaller (except for the brain)

CR animals are resistant to disease, including **cancer** and **infections**

Mutants in the IIS pathway with extended lifespan (~50-80%) in C. elegans and D. melanogaster



C. elegans

age-1: catalitic subunit of PI3 kinase

daf-2: Insulin/IGF1 receptor

daf-16: fork-head (FOXO) transcription factor

Mutations in the GH axis, which in turn impair the IIS activity

Drosophila

IIS receptor

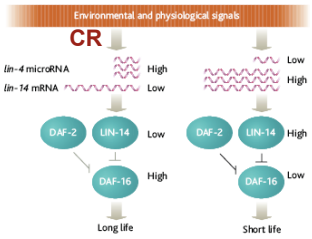
Insulin receptor substrate (chico)

Ablation of neurosecretory cells producing insulin-like ligands

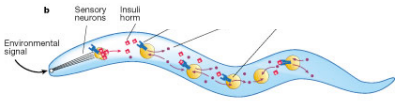
Overexpression of the forkhead transcription factor (dFOXO) in the fat body

all increase lifespan up to 85%

Eat Less, Live Longer? miRNAs Link Calorie Restriction To Longevity



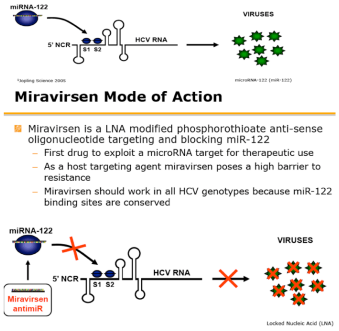
Regulation of adult life span by the *lin-4* microRNA. (Left) When *lin-4* microRNA activity is high, expression of *lin-14* mRNA and protein are low. Hence, the DAF-16 transcription factor is active and promotes long life. **(Right)** When *lin-4* activity is low, *lin-14* activity is high, and DAF-16 is inhibited, resulting in short life. *lin-4* and *lin-14* gene products may work downstream of, or in parallel to, DAF-2 (the insulin-like receptor) to modulate DAF-16. Proteins are depicted as oval shapes.



Treatment of HCV Infection by Targeting MicroRNA

Harry L.A. Janssen, M.D., Ph.D., Hendrik W. Reesink, M.D., Ph.D., Eric J. Lawitz, M.D., Stefan Zeuzem, M.D., Maribel Rodriguez-Torres, M.D., Keyur Patel, M.D., Adriaan J. van der Meer, M.D., Amy K. Patick, Ph.D., Alice Chen, B.A., Yi Zhou, Ph.D., Robert Persson, Ph.D., Barney D. King, M.D., Sakari Kauppinen, Ph.D., Arthur A. Levin, Ph.D., and Michael R. Hodges, M.D.

N Engl J Med 2013; 368:1685-1694May 2, 2013DOI: 10.1056/NEJMoa1209026



HCV replication depends on miR-122 expression.

The use of miravirsen in patients with chronic HCV genotype 1 infection showed prolonged dose-dependent reductions in HCV RNA levels without evidence of viral resistance. (Funded by Santaris Pharma; ClinicalTrials.gov number, NCT01200420.)

miRNA THERAPEUTICS

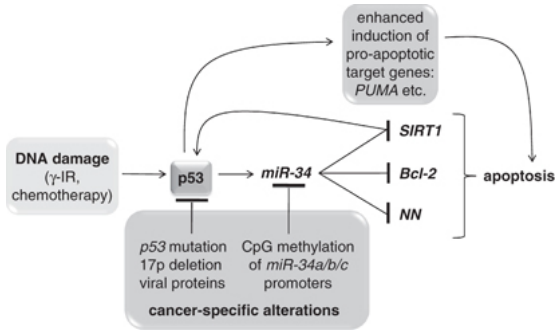
pioneering microRNA Replacement Therapy

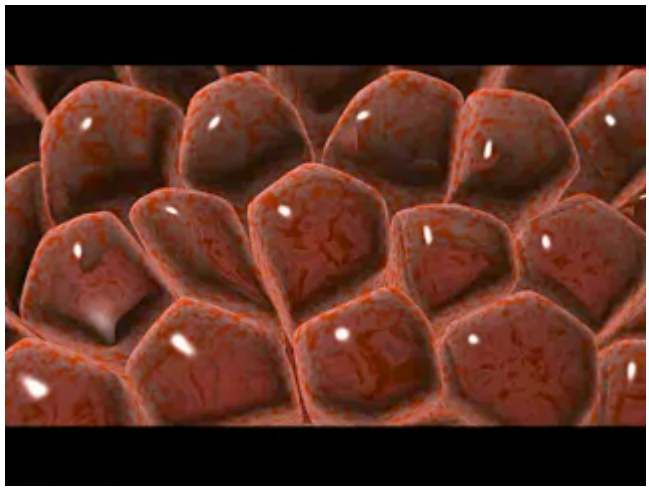
MRX34 is a first-in-class cancer therapy and the first microRNA mimic to enter clinical trials.

Mirna has secured an exclusive license from Marina Biotech, Inc. to the patent estate covering the SMARTICLES® liposomal delivery technology for several of our lead microRNA product candidates, including miR-34, let-7 and two other undisclosed targets. The SMARTICLES formulation offers key efficacy and safety benefits, including the ability to deliver high numbers of microRNA mimic molecules to cancers cells in the liver, spleen and other highly vascularized tissues, as well as bone marrow and malignant lymphocytes.

Program	Key Oncogenic Targets	Indication	In Vivo Formulation: Discovery	Preclinical	Phase 1	Phase 2
MRX34 miR-34 mimic	BCL2, E2F3, HDAC1, MET, MEK1, CDK4/6, PDGFR-α, WNT1/3, NOTCH-1	Primary liver cancer & solid cancers with liver metastases			●	
		Hematological malignancies			●	
miR-Rxlet-7 let-7 mimic	RAS, MYC, HMGA2, TGFBR1, MYCN, Cyclin D2, IL6, ITGB3			●		
miR-Rx06	UNDISCLOSED			●		
miR-Rx07	UNDISCLOSED			●		
miR-Rx16 miR-16 mimic	BCL2, VEGF-A, Cyclin-D1, HMGA1, FGFR1, CDK6, BMI1			●		

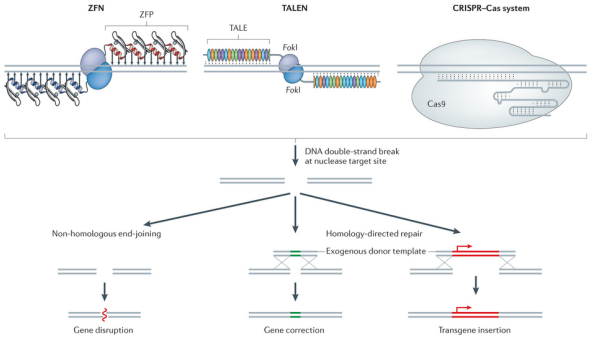
The miR-34 gene family is a mediator of tumor suppression by p53





Genome editing technology

- zinc finger nucleases (ZFNs)
- transcription activator-like effector nucleases (TALENs)
- clustered regularly interspaced short palindromic repeat (CRISPR)/Cas system



Nature Reviews Genetics **15**, 541–555 (2014)