

Not miR-ly small RNAs: Big potential for microRNAs in therapy

Tara M. Love, PhD,^{a,b} Howell F. Moffett, BA,^{a,b} and Carl D. Novina, MD, PhD^{a,b,c} Boston and Cambridge, Mass

RNA interference (RNAi) describes a set of natural processes in which genes are silenced by small RNAs. RNAi has been widely used as an experimental tool that has recently become the focus of drug development efforts to treat a variety of diseases and disorders. Like all molecular therapies, *in vivo* delivery is the major hurdle to realizing therapeutic RNAi. Several strategies have been developed that increase small RNA half-life in the blood, facilitate transduction across biological membranes, and mediate cell-specific delivery. Importantly, these strategies permit targeting of mRNAs as well as microRNAs (miRNAs), a class of small RNAs encoded in the genome. miRNAs are required for multiple developmental and cellular processes. Dysfunction of miRNAs can result in a host of pathologies, suggesting that miRNAs are potential targets of therapy. Recent studies of miRNA function in immune-specific pathways indicate that specific miRNAs might be exploited as therapeutic targets to treat immune disorders, including autoimmunity, allergy, and hematopoietic cancers. (J Allergy Clin Immunol 2008;121:309-19.)

Key words: RNA interference, small interfering RNA, microRNA, antagomiR

RNA interference (RNAi) is a collection of processes that use small RNAs to silence genes. As a naturally occurring pathway, one class of endogenous small RNAs, called microRNAs (miRNAs), is transcribed from cellular genes estimated to represent about 1% of all human genes.¹ More than 1/3 of the human genome is predicted to be targeted by miRNAs,² suggesting that numerous genetic networks may be affected by miRNAs. Indeed, miRNA functions are critical for several cellular processes including differentiation, apoptosis, proliferation, organogenesis, limb development, antiviral defense, and insulin secretion (see review³). It is therefore not surprising that miRNA dysfunction participates in a host of pathologies, most notably cancers. In fact, the term *oncomiR* has been coined to describe miRNAs that play a role in cancer formation, because particular miRNAs are now being considered oncogenes and tumor suppressors.⁴

Abbreviations used

AMD:	Age-related macular degeneration
CLL:	Chronic lymphocytic leukemia
CPP:	Cell-penetrating peptide
dsRNA:	Double-strand RNA
HBV:	Hepatitis B virus
miRNA:	MicroRNA
RISC:	RNA-induced silencing complex
RNAi:	RNA interference
RSV:	Respiratory syncytial virus
shRNA:	Short hairpin RNA
siRNA:	Small interfering RNA
SNALP:	Stable nucleic acid lipid particles
TP:	Target protector
UTR:	Untranslated region

As a tool, RNAi has transformed the way biomedical research is conducted and has already gained success in clinical trials. As an experimental method for discovery of gene function, RNAi uses exogenously introduced small interfering RNAs (siRNAs), which target particular mRNAs for destruction. Currently, siRNAs are the focus of RNAi-based clinical trials, although a large portion of academic, biotechnology, and big pharmaceutical efforts are now centered on the use of chemically synthesized small RNAs designed to manipulate miRNA expression. Each of these RNAi-based tools holds tremendous promise in molecular therapy. Importantly, the use of RNAi as a tool has grown up in parallel with the basic biology of RNAi. Therefore, the users of RNAi inform the scientists who investigate the biological mechanisms of RNAi in a previously unheralded manner. These and other properties of RNAi have led to the notion that the small RNA mediators of RNAi are especially good effectors and targets for therapeutic gene silencing.

Although miRNAs share many physical and biological properties with siRNAs, they produce very different functional outcomes in mammals. siRNAs are double-strand RNAs of 21 to 23 nucleotides that bind to mRNAs with perfect sequence complementarity and cause cleavage of the mRNAs (Table I; Fig 1, A). miRNAs are single-strand RNAs of 21 to 23 nucleotides and generally do not cleave mRNA targets. Instead, they bind to 3' untranslated regions (UTRs) of mRNAs with imperfect sequence complementarity and repress their translation (Table I). Notably, one siRNA cleaving its mRNA target may provide robust gene silencing (sometimes more than 90% reduction) of target mRNA expression. Conversely, a single miRNA leading to translational repression generally does not lead to robust gene silencing of its target. In fact, the subtlety and power of miRNA-based gene silencing may derive from the fact that one miRNA targets

From ^aCancer Immunology and AIDS, Dana-Farber Cancer Institute and ^bthe Department of Pathology, Harvard Medical School, Boston; and ^cthe Broad Institute of Harvard and Massachusetts Institute of Technology, Cambridge.

Disclosure of potential conflict of interest: The authors have declared that they have no conflict of interest.

Received for publication October 30, 2007; revised December 21, 2007; accepted for publication December 26, 2007.

Reprint requests: Carl D. Novina, MD, PhD, Department of Pathology, Harvard Medical School, Boston, MA 02115. E-mail: carl_novina@dfci.harvard.edu.

0091-6749/\$34.00

© 2008 American Academy of Allergy, Asthma & Immunology

doi:10.1016/j.jaci.2007.12.1167

TABLE I. Comparison of siRNA and miRNA properties

	siRNA	miRNA
Architecture	Double-strand	Single-strand
Origin	Exogenous	Endogenous
No. of intended target genes per small RNA	1	Multiple
Degree of gene silencing per small RNA	High	Low
Complementarity to mRNA target	Perfect	Imperfect
Effect on mRNA	Endonucleolytic cleavage	Repressed translation

multiple genes,⁵ many of which participate in common signaling pathways. Furthermore, several different miRNAs may target a single mRNA.⁵ Collectively, multiple miRNAs provide robust gene silencing of multiple target mRNAs. Indeed, the promiscuity of miRNAs make them attractive candidates for therapeutic gene silencing, especially because most, if not all, diseases are heterogeneous in nature and may be the product of dysregulation of more than 1 gene.

Much of our knowledge on the use of siRNAs in therapy may be directly applied to the use of small RNAs that modulate miRNA pathways. The advent of RNAi has not only altered the way biomedical research is conducted but also it has progressed to the bedside at an astounding rate from the time of its discovery less than 10 years ago! In this review, we discuss the current state of the use of siRNAs in therapy and introduce current ideas for targeting miRNAs for therapy, which are not far behind siRNAs in the race for clinical trials. We also speculate on potential therapies for immune-specific diseases by targeting specific miRNA-regulated pathways implicated in normal immune function.

siRNAs AS A NEW CLASS OF DRUGS

The use of RNAi as a therapeutic modality is making considerable progress, and siRNAs are now considered by many to be a new category of drugs. Currently there are 7 clinical trials underway that use siRNAs for treatment of a variety of diseases and disorders (Table II), and many more are in the pipeline to commence phase I clinical trials in 2008. The success of siRNAs in therapy derives in part from their powerful and efficient mechanism of gene knockdown, sequence-specific cleavage of mRNAs (Table I; Fig 1, A). Cleavage of targeted mRNAs is mediated by the RNA-induced silencing complex (RISC), an efficient, multiple turnover enzyme.⁶ That is, 1 RISC is recycled multiple times to cleave multiple copies of the same target mRNA. Consequently, very small amounts of siRNAs (nmol/L) are necessary to elicit a potent gene silencing response *in vivo*. Although a systematic and comparative analysis on multiple target mRNAs using siRNAs, antisense oligonucleotides, and ribozymes has not been performed, it is likely that siRNAs are more sensitive and potent than these other RNA-based forms of therapy. However, modifications to the newer generation of antisense molecules and ribozymes have significantly improved their performance (see reviews^{7,8}).

Mammals tolerate very large doses of siRNAs. Mice receiving 2.0 to 2.5 mg siRNAs injected directly into their bloodstreams did not demonstrate signs of toxicity.⁹ In contrast, mice receiving high titers of adeno-associated viruses expressing multiple

different short hairpin RNAs (shRNAs) to 6 target genes died from toxicity.¹⁰ This latter strategy requires expression of shRNAs, which are double-strand RNAs that form a hairpin structure containing a stem of perfectly complementary sequences that get processed into siRNAs by endogenous endonucleases. Thus, shRNAs enter the natural RNAi pathway at an earlier point than siRNAs. The lethality in mice receiving shRNAs was attributed to saturation of the stabilizer and transporter of siRNA/miRNA precursors, Exportin 5 (limiting in cells), which, in turn, caused a global decrease in endogenous miRNAs, leading to liver toxicity and morbidity.¹⁰ Together, these observations support the notion that, if necessary, siRNAs may be administered at high doses because they enter the natural RNAi pathway at a step later than Exportin 5 function and therefore do not induce toxicity. This property of siRNAs is especially useful in cases in which higher doses manifest stronger therapeutic effects. For example, Merck's drug AGN211745 (Merck, Whitehouse Station, NJ) showed varied dose-dependent effects during phase I clinical trials to treat age-related macular degeneration (AMD; Table II). A single intravitreal injection resulted in maintenance of visual acuity at lower doses (100 μ g), but at higher doses (as much as 800 μ g), visual acuity was greatly improved without toxic side effects.¹¹

UNINTENDED EFFECTS OF siRNAs

One major concern with the use of siRNAs in therapy is the potential for off-target effects, gene silencing by siRNAs on unintended mRNA targets. In one study, introduction of an individual siRNA into cells had small (~2-fold) effects on hundreds of genes.¹² It is likely that the 2 strands of the introduced siRNA had imperfectly complementary base-pairing with certain mRNAs; that is, the siRNA strands may have mimicked miRNA targeting of mRNAs. Efforts to reduce off-target effects of siRNAs include choosing siRNA design algorithms that result in more functional siRNA targeting and avoid sequence similarities with off-target mRNAs. These algorithms aim to reduce the amount of siRNAs required for on-target silencing. Additional efforts to reduce off-target effects have focused on modifying the sense and antisense strands of siRNAs (see reviews^{13,14}). For example, 2' O-methyl modification at position 2 of the antisense strand of an siRNA duplex significantly reduces off-target effects without compromising silencing of the intended mRNA target.¹⁵ Still, some effects on unintended targets cannot be avoided; however, in gene therapy and gene discovery, small effects on unintended targets may be acceptable to achieve very large effects on intended targets.

Another concern with using siRNAs in therapy is their potential to be immunogenic. Generally, long double-strand RNAs (dsRNAs) introduced into mammalian cells activate protein kinase R, a dsRNA-dependent protein kinase and key mediator of the IFN pathway. Shorter dsRNAs, including siRNAs (<30 bp), have previously been reported to avoid induction of the IFN pathways¹⁶⁻²⁰; however, multiple reports have also demonstrated cases in which siRNAs do trigger an IFN response.²⁰⁻²² This property may depend on the cell context²⁰ and the sequences of the siRNAs.²³⁻²⁵ Therefore, any indication of IFN activation by particular siRNAs should be followed up by using siRNAs with different sequences devoid of certain immunogenic motifs (G-U-rich^{23,26}). In addition, one or two 2' O-methyl base modifications in 1 strand of an siRNA duplex can significantly decrease the immunostimulatory characteristics of that siRNA.²⁶

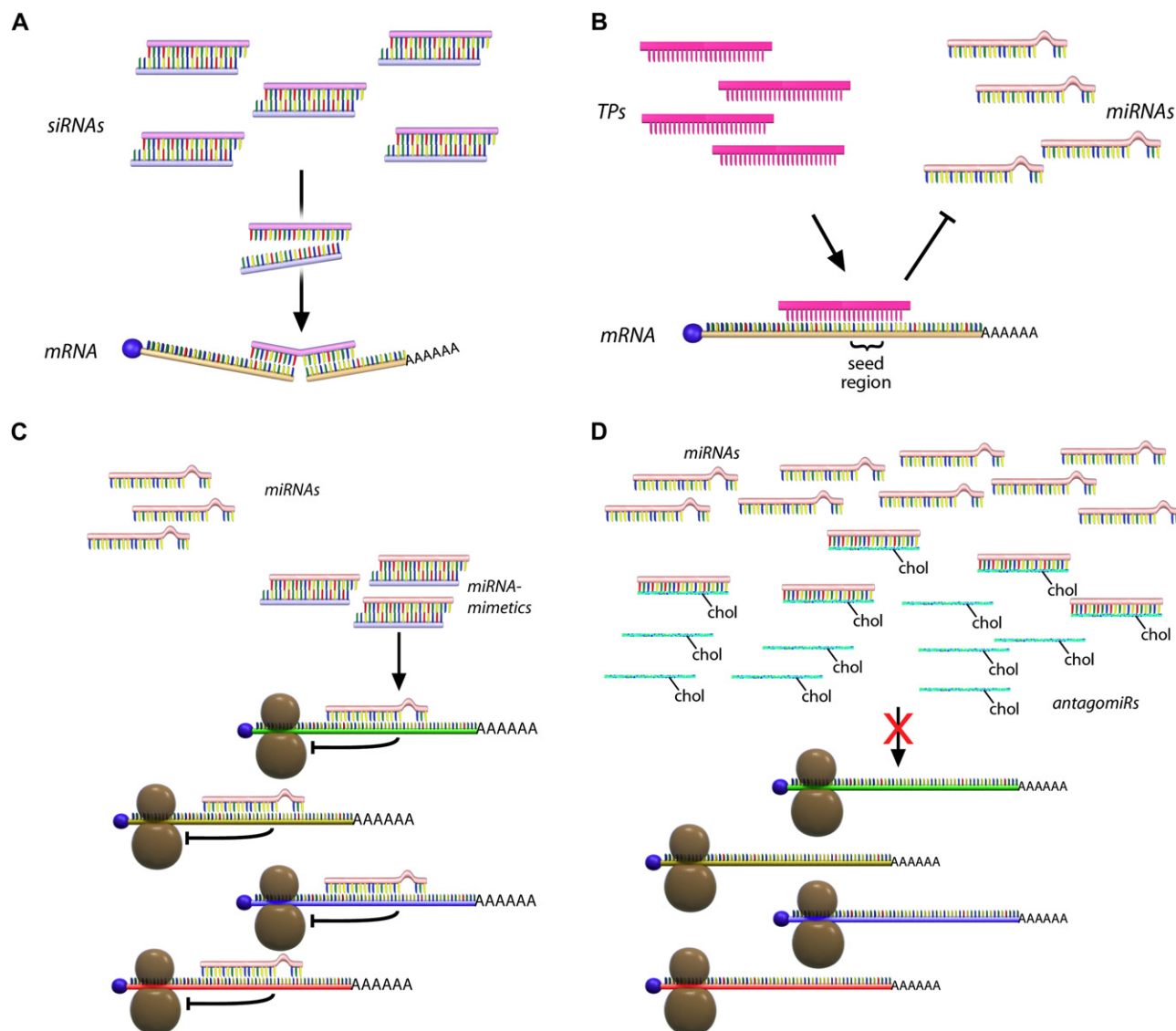


FIG 1. Schematic of RNAi-based technologies. **A**, siRNAs cleave mRNAs and reduce mRNA levels. siRNAs are 21-nucleotide to 23-nucleotide RNA duplexes with 5' recessed ends and 3' overhanging ends that bind with perfect sequence complementarity to mRNAs and destroy them by endonucleolytic cleavage. **B**, Target protectors (TPs) block miRNA binding sites and increase mRNA levels. TPs are 25-nucleotide single-strand RNAs that bind specific mRNAs at miRNA-binding sites. TPs exhibit specificity in their target recognition by exhibiting perfect sequence complementarity to the seed region of the mRNA and 16 nucleotides outside the seed region that are not conserved between different target mRNAs. **C**, miRNA-mimetics reconstitute reduced miRNAs. miRNA-mimetics are duplexed RNAs with siRNA architecture whose gene-targeting strands are composed of exact sequences of mature miRNAs. Delivery of miRNA-mimetics can result in translational inhibition of multiple mRNAs (shown in different colors). **D**, AntagomiRs inhibit increased miRNAs. AntagomiRs are single-strand antisense RNAs with perfect sequence complementarity to mature miRNAs. They permit effective inhibition of miRNA functions. Delivery of antagomiRs can result in de-repression of translation of multiple genes (shown in different colors). Addition of a cholesterol moiety (*chol*) to the 5' end facilitates their uptake *in vivo*.⁶¹

DELIVERY OF siRNAs

The delivery of siRNAs has been the biggest hurdle to overcome in RNAi-based therapies. Indeed, the success of siRNAs in therapy depends critically on efficient delivery. siRNA delivery schemes benefit from lessons learned through use of other nucleic acid-based delivery schemes. However, siRNAs have properties that might be exploited for their efficient delivery, which may reduce

the height of this hurdle. Compared with antisense oligonucleotides, siRNAs work via a more potent and efficient catalytic mechanism, suggesting that lower amounts may be required for effective gene silencing. Compared with ribozymes and plasmid-based therapies, the small size of siRNAs may better facilitate their uptake by cells. Moreover, the duplex nature of siRNAs makes them fairly resistant to exonuclease-mediated degradation,

TABLE II. Current siRNA-based clinical trials

Drug	Disease/condition	Drug target	Target tissue	Nature and method of RNAi delivery	Company	Clinical trial status
Bevasiranib	Wet AMD	VEGF	Eye	Naked siRNA, intravitreal injection	Opko (Miami, Fla)	August 2007, phase III
Sirna-027/ AGN211745	Wet AMD	VEGF receptor	Eye	Naked siRNA, intravitreal injection	Merck/Sirna (San Francisco, Calif)/Allergan (Irvine, Calif)	August 2007, phase II
ALN-RSV-01	RSV infection	RSV nucleocapsid (N)	Lung	Naked siRNA, intranasal delivery	Alnylam (Cambridge, Mass)	June 2007, phase II
RTP801i-14	Wet AMD	Hypoxia-inducible gene (RTP801)	Eye	Stabilized chemically modified siRNA, intravitreal injection	Quark (Fremont, Calif)/Silence (London, United Kingdom [UK])/Pfizer (New York, NY)	November 2007, phase I/IIa
AKIi-5	Acute renal failure	p53 gene	Kidney	Stabilized chemically modified siRNA, intravenous delivery	Quark/Silence	November 2007, phase I
CALAA-01	Solid and metastatic tumors	M2 subunit of ribonucleotide reductase	Tumor	Nanoparticle-packaged siRNA with transferrin to target tumors, intravenous delivery	Calando (Pasadena, Calif)	End of 2007, preclinical/phase I
NUCB1000	HBV infection	4 HBV genes	Liver	Cationic lipid-packaged DNA-based plasmid expressing 4 shRNAs, intravenous delivery	Nucleonics (Horsham, Pa)/Novosom (Halle, Germany)	May 2007, phase I
rHIV7-sh1-TAR-CCR5RZ	AIDS-related lymphoma	HIV tat/rev gene, TAT responsive elements, CCR5 receptor	Stem cells	DNA-based plasmid expressing anti-HIV RNA, including shRNA, lentiviral infection of stem cells with plasmid followed by stem cell transplant	Benitec (Melbourne, Australia)	June 2007, phase I

VEGF, Vascular endothelial growth factor.

although their half-life in serum is still relatively short. Several tools exist or are being developed to facilitate delivery of small RNAs. Some of these strategies are discussed here.

Most mammalian tissues do not readily take up naked siRNAs partly due to their dilution in the blood stream. However, certain tissues, such as the eye and respiratory tract, are easily accessible by clinicians and are relatively isolated compartments composed of mostly nondividing cells. Therefore, successfully delivered siRNAs will not be rapidly cleared or diluted and can generally support a sustained gene silencing response from a single dose. In addition, these sites are not exposed to the blood supply, which contains degrading enzymes detrimental to naked siRNAs. Because of all these qualities, relatively lower doses of siRNAs are necessary to elicit potent gene silencing responses in the eyes and lungs. To this end, it is not surprising that the most progress has been made in the eye-based and lung-based clinical trials using siRNAs (Table II). Naked siRNAs are successfully being delivered to the eyes by direct injection into the vitreous fluid to treat AMD, and to the lungs by inhalation to treat respiratory syncytial virus (RSV) infection.

Systemic delivery of siRNAs to tissues is certainly not as trivial as local delivery schemes for siRNAs described. The systemic delivery challenge may be conceptually broken down into 3 separate issues with different strategies under investigation to

cross the delivery hurdle: increasing the half-life of siRNAs in the blood, transducing siRNAs across biological membranes, and mediating cell-specific delivery. In our discussion, these issues are artificially separated for purposes of illustration to provide a more complete understanding of specific problems and some common solutions to these problems. It is important to note that many of the siRNA delivery strategies discussed are multifunctional and can be used to overcome multiple aspects of the delivery hurdle. In addition, the most successful siRNA delivery schemes generally make use of more than 1 modification to increase the half-life of siRNAs in the blood, to transduce siRNAs across biological membranes, and to mediate cell-specific delivery. For purposes of clarity, certain strategic modifications to siRNAs are highlighted for their applicability in 1 of the 3 sections.

Virus-mediated delivery of siRNAs has 1 major advantage over the delivery of chemically synthesized siRNAs: strong, constitutive expression of siRNAs in target tissue that will not be diluted as cells divide. However, virus-mediated delivery of any gene or small RNA presents many limitations, including immunogenicity, toxic side effects from viral infection, and in the case of retroviruses, a strong potential for insertional mutagenesis (see review²⁷). In addition, high expression of viral shRNAs may saturate the limiting RNAi machinery, causing problems with

endogenous miRNA production.¹⁰ Therefore, most efforts for delivery of small RNAs have focused on delivery of chemically synthesized siRNAs rather than on virus-delivered siRNAs. The issues concerning virus-mediated delivery of transgenes have been addressed previously²⁷ and are not further addressed here. Instead, we focus our attention on strategies to deliver chemically synthesized siRNAs, which inform strategies to deliver small RNAs that can be used to modulate miRNA pathways.

Increasing the half-life of siRNAs in the blood

Naked siRNAs in the bloodstream are easily degraded because the serum contains high amounts of nuclease activities. One way to reduce nuclease sensitivity is through chemical modification of siRNAs. Chemical modifications of siRNAs that increase their stability include phosphorothioate addition to 3' ends and 2' O-methyl, 2'-fluoro, and locked nucleic acid-type substitutions to the ribose backbone (see review¹⁴). Indeed, some of these modifications are incompatible with RNAi functioning but possess characteristics that make them extremely useful in the modulation of miRNA pathways. Some of these modifications are discussed in more detail in the section on miRNA pathway modulators.

Another means to reduce nuclease sensitivity is to enclose siRNAs in a synthetic particle, such as a liposome or polymer nanoparticle. These delivery vehicles keep siRNAs in a protected milieu until they are released into target cells. In fact, a combination of chemically modified siRNAs packaged into nonviral carriers (liposomes or polymer nanoparticles) is becoming the standard for stabilization and delivery of siRNAs *in vivo*. Liposomes are synthetic phospho-lipid bilayers containing a core of aqueous solution. They have been successfully used for *in vivo* delivery of chemical drugs,²⁸⁻³⁰ plasmid DNA,³¹ and, more recently, siRNAs.³² Typically, cationic lipids are used to create siRNA liposomes. The cationic lipids bind to the net negatively charged siRNAs and form a lipid bilayer around the siRNAs, thereby protecting them from degradation and increasing their half-life and potency.³² When administered, siRNA-liposomes fuse to target cell membranes, and siRNAs are released into the cytoplasm through endocytosis. siRNA-encapsulated liposomes are very easily absorbed by some tissues, such as the epithelium and lamina propria of the mouse vagina. In 2006, Palliser et al³³ reported topical vaginal application of siRNA-encapsulated cationic liposomes directed toward herpes simplex virus-specific genes. The result was sustained gene silencing and protection from lethal herpes simplex virus infection, suggesting that topical lipid-based siRNA creams might be an option for treating other epithelial disorders.

In some cases, cationic lipid-mediated delivery of siRNAs has been associated with toxicity^{34,35} and induction of the IFN response.^{25,36-38} These properties can be greatly improved by packaging chemically modified siRNAs into specialized cationic liposomes, as was done by Morrissey et al³⁸ in 2005 to suppress hepatitis B virus (HBV) replication in mice. Referred to as *stable nucleic acid lipid particles* (SNALP), these were refractory to immunostimulation and worked potently at dosage levels 10 times less than cationic liposomes containing unmodified siRNAs.³⁸ Another way to reduce toxicity and immunogenicity of siRNA-liposomes is to adjust lipid composition, for example, by using neutral lipids. In mouse models, the use of neutral lipids to create siRNA liposomes greatly enhanced efficiency of delivery and potency of silencing by using very low amounts of siRNAs.³⁹⁻⁴¹

Cationic polymer-based nanoparticles are ideal carriers of siRNAs because of their ability to stabilize siRNAs and efficiently deliver them in a targeted fashion. Nanoparticle formulations allow the addition of targeting ligands to surfaces of nanoparticles. Furthermore, large-scale manufacturing of such particles is feasible, and in general, nanoparticles are less toxic than their liposome counterparts, offering the advantage of being biodegradable. A widely used method of nanoparticle-based siRNA protection and delivery is polyethyleneimine complexation. Polyethyleneimines are highly positively charged, bind with high affinity to siRNAs, and have demonstrated efficient endocytotic uptake of siRNAs *in vivo* (see review⁴²).

Transducing siRNAs across biological membranes

In addition to making sure that delivered siRNAs are protected from degradation, it is important that they enter their target cells. As mentioned, most mammalian cells do not efficiently take up naked siRNAs. Instead, the siRNAs need to be packaged in some way that allows them to traverse the lipid bilayer of cell membranes. This has been achieved by using synthetic packaging particles, such as the liposomes and polymer nanoparticles described above, which can facilitate the endocytosis of packaged siRNAs. An alternative strategy for getting siRNAs into cells is to add a cholesterol moiety to the siRNAs. In 2004, Soutschek et al⁴³ showed that the addition of a cholesterol moiety to siRNAs greatly enhanced their uptake into liver cells and caused potent knockdown of their target gene, apolipoprotein B. The mechanism of uptake of cholesterol-conjugated siRNAs is thought to be facilitated by the low-density lipoprotein (LDL) receptors present on many cell types. Interestingly, this was the first demonstration of systemic delivery of siRNAs by intravenous injection into mice, which was also the first feasible and clinically relevant mode of delivery for siRNAs that had been reported.

Another method for packaging and cellular delivery of siRNAs is the use of cationic peptides, called *cell-penetrating peptides* (CPPs). The strong positive charge on these peptides promotes binding and condensation of negatively charged biomolecules, including siRNAs. Discovered more than a decade ago, CPPs have been shown to transduce oligonucleotides and other biologically active molecules efficiently across cellular membranes in a receptor protein-independent manner (see review⁴⁴). siRNAs can be packaged with CPPs either covalently or through electrostatic interactions, and treatment of cells with siRNA/CPP complexes has been successful *in vitro*, with minimal toxicity.^{45,46} However, *in vivo* applications for siRNA/CPP complexes have yet to be successfully executed. One lesson learned from *in vitro* studies is that siRNA/CPP complexes may not always escape from endosomes once they are taken up by cells. Thus, future formulations of siRNA/CPP complexes are focused on making the CPP portion of the complexes more endosomolytic.⁴⁶

Mediating cell-specific delivery

In addition to identifying molecular targets for therapy, defining cellular targets for therapy is critical for increasing intended effects while minimizing unintended effects of siRNAs. Without targeting modules associated with siRNAs, bulk siRNAs injected into the bloodstream will eventually lead to the liver, because many drugs are metabolized by the liver. Efficient systemic delivery of siRNAs to a particular tissue other than the liver

requires a specific targeting component to be added to siRNAs. To make this work effectively, it is imperative that the target tissue expresses a cell surface protein that will recognize the targeting ligand. This bodes well for tumor cells, which tend to express cell surface receptors not found in normal cells aberrantly. For example, the transferrin receptor is highly expressed on the surfaces of a broad range of tumor cell types. Capitalizing on this characteristic of cancer cells, Calando Pharmaceuticals (Pasadena, Calif) uses nonchemically modified siRNAs coupled to transferrin receptor targeting agents, composed of specialized cyclodextrin-based nanoparticles (Table II). The siRNAs encapsulated in the nanoparticles are complimentary to the M2 subunit of the ribonucleotide reductase gene, which is a well established cancer target. Thus, the idea is to target a broad range of tumor cells via the transferrin receptor and knock down an aberrantly expressed gene important for the tumor cell's proliferative capacity.

Another method used to deliver siRNAs to specific cell addresses is coupling of siRNAs to antibody-protamine fusion proteins. Protamine is a positively charged protein that naturally packages DNA in sperm cells and thus efficiently packages siRNAs, protecting them from degradation. Because the siRNAs are not covalently bound to the protamine, cocktails of siRNAs can be used in the same delivery module. The antibody component of the fusion is responsible for targeting specific cell surface antigens and thus offers the advantage of targeting very specific subsets of cells. The siRNA payload is then delivered by receptor-mediated endocytosis. By using siRNA-protamine-antibody fusions, Song et al⁴⁷ demonstrated potent silencing of target genes in HIV-infected cells and HER2-positive cells in mice. Similarly, Wen et al⁴⁸ used siRNA-protamine-antibody fusions to inhibit HBV gene expression in mice. Next, it will be interesting to see what other cell types are targeted *in vivo* using this technology. For example, this technology might be used to deliver siRNAs specifically to acute myelogenous leukemia cells in the peripheral blood via the acute myelogenous leukemia—stem cell—specific marker, CD96.⁴⁹

Viral peptides have been exploited for their use in targeted siRNA-based therapies. Recently, it was demonstrated that siRNAs could cross the blood-brain barrier.⁵⁰ Preventing the passage of therapeutic agents to the brain, the blood-brain barrier has been a major obstacle in treating brain pathologies. To get around this obstacle, researchers used a 29-amino acid peptide from the rabies virus (which binds the acetylcholine receptor of neuronal cells) as a carrier for targeted delivery of siRNAs into the brain, thus successfully protecting mice from Japanese encephalitis virus infection.⁵⁰ This was a major breakthrough, not only from the standpoint of targeted RNAi therapeutics. It was the first successful demonstration of intravenous delivery of a therapeutic agent to the brain.

Another avenue for targeting siRNAs to specific cell types has been derived from evolutionary tools used to define novel binding motifs on surfaces of specific cells. An example of one such tool is the tryptophan cage, an independently folding 20-amino acid protein domain derived from a longer peptide isolated from the Gila monster (*Heloderma suspectum*). The tryptophan cage is a hydrophobic cluster of amino acids with tryptophan buried in a central location and has been optimized for efficient folding. Also, tryptophan cages are highly amenable to modifications at several amino acid positions. Herman et al⁵¹ exploited these properties of tryptophan cages by expressing them in a phage-display library such that different combinations of amino acids

composing the tryptophan cage form motifs that exhibit cell type-specific binding *in vivo*. Therefore, these tryptophan cage variants recognize different molecular zip codes by binding to specific cell surface proteins. Tryptophan cages may provide another avenue for siRNA conjugation and targeting to specific cells and offer the advantage of being much smaller than antibodies, making them easier to manufacture on a large scale.

miRNA PATHWAY MEDIATORS AS THE NEXT NEW CLASS OF DRUGS

Pharmaceutical companies have recently begun to move toward the use of miRNAs as a therapeutic modality. If siRNAs are efficient at targeting mRNAs by co-opting a natural endogenous pathway that is already in place for miRNA-mediated silencing of genes, one idea is to eliminate the middle man and target miRNAs directly. Several new companies are predicated on using miRNAs as diagnostic and therapeutic tools for a range of diseases. Interestingly, companies that have already developed and marketed other RNA-based therapies, such as antisense RNA drugs, are helping to accelerate the progress of therapeutic miRNA technologies. Possessing long-standing experience and expertise in RNA chemistry, these companies have jumped into the RNAi therapeutic race in full force. Lessons learned about delivery and use of antisense RNAs can be applied to siRNAs and miRNAs (see review⁵²). Then again, there has been a renaissance in antisense technologies since the discovery of RNAi. RNAi not only has benefited from antisense technologies but also may inform further development of antisense technologies as more people effectively use RNAi. Thus, collaborative efforts of old and new RNA companies pose a powerful force in small RNA therapeutics, including miRNA therapeutics.

Every cell has a unique miRNA expression signature that reflects the identity and functions of that cell. Profiling of miRNA expression patterns in numerous cell types and numerous pathways has identified differential expression of miRNAs characteristic of certain tissues under specific physiological stimuli. miRNA expression profiling can identify cell types in normal and diseased states, such as in cancer (see review⁵³). Therefore, it is possible to target miRNAs that mediate a particular cellular process without knowing all of the genes involved in that process. This presents a major advantage to using RNAi to target miRNAs rather than to target mRNAs. Globally, miRNAs are predicted to target more than 1/3 of the genome, which is roughly more than 6600 genes.² Whereas an siRNA targets 1 gene, a single miRNA is predicted to target between 100 and 200 genes.⁵

One emerging paradigm for miRNA function in mammals is that the effect of a single miRNA on translational repression of a particular mRNA containing 1 miRNA binding site is very small in degree. To obtain robust gene silencing by a miRNA, multiple miRNA binding sites must be present in a particular mRNA that act cooperatively.⁵⁴ As stated, the emerging paradigm for miRNA function in mammals is that a single miRNA may target multiple genes of a particular signaling pathway. Because some genes are involved in multiple pathways, altering expression of any 1 miRNA may lead to effects on unintended pathways. However, the effect of altering multiple gene targets of that miRNA in the intended pathway will be amplified by having small-fold intended effects become multiplicative. Because certain miRNAs and mRNAs (and subsequently the pathways they mediate) will be selectively activated, it is likely that altering a particular miRNA

will have small effects on unintended pathways and large effects on intended pathways. Thus, the power of targeting a single miRNA far exceeds that of targeting a single gene, as with siRNAs.

TOOLS FOR ALTERING CELLULAR miRNA PATHWAYS

Several tools are available that enable selective targeting of miRNA pathways, including tools that reconstitute reduced miRNAs (miRNA-mimetics; Fig 1, C) and reduce overexpressed miRNAs (antagomiRs; Fig 1, D). Recently, technologies have been described that permit selective protection from miRNAs through accessibility to cognate miRNA binding sites (target protectors; Fig 1, B).

miRNA-mimetics

For cases in which miRNAs are consistently reduced in disease processes or overexpression of miRNAs would be a therapeutic benefit, miRNA-mimetics can be used (Fig 1, C). miRNA-mimetics are synthetic small RNAs usually containing the exact sequence of the endogenous miRNA. Instead of delivering a single strand, miRNA-mimetics are delivered as perfectly complementary duplexes, like the architecture of siRNAs. This is because RISC is much more efficient in loading small RNAs originating from a duplex rather than a single strand.⁵⁵ A powerful advantage of using miRNA-mimetics is that these synthetic miRNAs can be modified to be more potent than their naturally occurring forms. For example, miRNA-mimetics have been designed to target glioma-associated antigen 1 (*Gli-1*) with a much higher binding affinity than naturally occurring miRNAs that target *Gli-1*.⁵⁶ These modified miRNA-mimetics more strongly inhibited *Gli-1*-mediated proliferation of pancreatic tumor cell lines than their natural counterparts.⁵⁶ Currently, the delivery of miRNA-mimetics has yet to be demonstrated in mouse models with the ultimate goal of replacing lost miRNA functions in human clinical models.

AntagomiRs

For cases in which miRNAs are consistently upregulated in disease processes or reduction of miRNAs would be a therapeutic benefit, antagomiRs can be used to block those miRNAs directly and specifically. *AntagomiR* is used here as a generic term to describe any antisense oligonucleotide that directly binds to miRNAs and blocks their activities. AntagomiRs may work by stoichiometric interaction with mature miRNAs by titrating them from biologically active pools of mature miRNAs (Fig 1, D). AntagomiRs may also work by binding to miRNA precursors and inhibiting the biogenesis of mature miRNAs. Although it is not exactly clear how antagomiRs inhibit miRNA functions, their effect is to prevent incorporation of mature miRNAs into RISC. An advantage of using antagomiRs in therapy is that they potently and specifically inhibit miRNAs and therefore affect hundreds of genes. This is also a disadvantage if only a subset of those genes is the intended point of therapy.

Early reports on antagomiR use described 2' O-methyl RNA oligonucleotides that were perfectly complementary to the antisense strand of siRNAs and blocked their function *in vitro*.^{57,58} The first *in vivo* demonstration of the use of antagomiRs to block miRNA activities was in worms, in which 2' O-methyl oligonucleotides perfectly complementary to *let-7* induced a *let-7* loss-of-function phenotype.⁵⁸ Since the first reports, different types

of antagomiRs have been widely used as tools to probe the functions of miRNAs in mammalian cells. For example, miR-21 was potently inhibited in glioblastoma cells by using either 2' O-methyl or locked nucleic acid-based antagomiRs.⁵⁹ In addition, antagomiRs not only have been used to target mature miRNAs but also have been designed to block miRNA biogenesis by targeting miRNA precursors.⁶⁰

A major step toward the goal of probing miRNA functions *in vivo* was successful systemic delivery of antagomiRs in mice to knock down miR-122, a liver-specific miRNA.⁶¹ The antagomiR used in these studies was 2' O-methylated and contained a cholesterol moiety on the 5' end, which facilitated its uptake into hepatocytes, causing specific miR-122 silencing for more than 1 week after a single intravenous injection.⁶¹ In these studies, several genes involved in cholesterol biosynthesis were upregulated on miR-122 inhibition, and serum cholesterol levels went down by 44%.⁶¹ Further studies using miR-122-specific antagomiRs *in vivo* revealed specific roles for miR-122 in lipid metabolism and further supported the notion that miRNA-specific inhibition by antagomiRs holds great therapeutic promise.⁶²

Target protectors

A possible option to affect only certain miRNA target mRNAs is to use RNA-binding antisense oligonucleotides. Target protectors (TPs; Fig 1, B) are complementary to miRNA binding sites in the 3' UTR of specific genes, thus preventing miRNA access to those sites. These specialized oligonucleotides have recently been reported to interfere with miR-430-mediated repression of specific 3' UTRs in zebrafish.⁶³ The specificity of TPs for select miRNA binding sites within 3' UTRs relies on certain miRNA:mRNA binding properties. A miRNA:mRNA pair usually possesses perfect complementarity between nucleotides 2 and 7 from the 5' end of the miRNA and its binding site in the target mRNA. This is called the *seed region* of the mRNA and is highly conserved among the mRNA targets of any 1 specific miRNA. TPs take advantage of the fact that flanking sequences 5' and 3' of the seed region are much less conserved among miRNA binding sites within specific 3' UTRs. Thus, these flanking regions provide a fingerprint unique to each miRNA target. The TPs used in the zebrafish studies were 25 nucleotides long and were complementary to 9 nucleotides encompassing the seed region of miR-430 and 16 more nucleotides 5' or 3' of the seed region of the mRNA⁶² (Fig 1, B). These TPs were effective at selectively blocking miR-430-mediated inhibition of 2 different targets.⁶³ Therefore, TPs are advantageous in cases in which the mRNA targets of miRNAs are known. In the years to come, as more and more targets of miRNAs get validated, this technology will become immensely useful.

Delivery of miRNA pathway modulators

Delivery issues for miRNA-modulating small RNAs are similar to issues concerning siRNAs. For example, once the small RNA gets to its location, how can persistence of therapeutic activity be ensured? For nondividing cells, this is less of an issue. For rapidly dividing cells, like cancer cells, small RNAs will be diluted with each cell division. In these cases, dosage may be important such that high quantities of miRNA-modulating small RNAs may be needed to ensure a long enough duration of the biological effect. However, the problem with using large amounts of miRNA-modulating small RNAs might be that cellular

pathways are affected that are not germane to therapy. Constitutive expression of miRNA-modulating small RNAs using viral vectors would likely support persistent therapy in rapidly dividing cells. In these cases, it may be important to consider side effects of viral vectors. It may also be that temporary modulation of miRNAs by transient delivery of miRNA pathway-modulating small RNAs will be beneficial in some cases. For transient biological processes, like the inflammatory response in which temporary miRNA modulation is logical, delivery of miRNA-modulating small RNAs might be an effective means of changing immune responses temporarily. For these transient biological processes, the dilution of small RNAs as cells divide might make the strategy more reasonable. One might even envision a quick fix to alter miRNA levels in the treatment of disorders with acute exacerbations, such as asthma.

POTENTIAL APPLICATIONS OF miRNA PATHWAY MODULATORS IN IMMUNE DISEASES

During allergy and autoimmunity, the immune system retains a relatively normal response to pathogens. At the same time, the immune system demonstrates pathological responses to self or environmental antigens. Recent research suggests that miRNAs play important roles in regulation of innate and adaptive immune responses, as well as in the interplay between immune and nonimmune tissues. In addition, dysregulated miRNA expression has been linked to numerous hematopoietic cancers in human beings and leukemogenesis and lymphomagenesis in animal models. Together, these reports suggest that autoimmunity, allergy, and hematopoietic cancers are cases in which the subtle yet pervasive effects of miRNAs could be harnessed to affect positive clinical outcomes. We present examples of potential applications for immune-specific miRNA pathway modulators.

Autoimmunity and allergy

Much of current drug therapy for T-cell-dependent autoimmune conditions depends on nonspecific immune suppression by immunomodulating agents such as prednisone, cyclosporine, or azathioprine. Use of these agents is hampered by significant off-target side effects, hematopoietic toxicity, and immune suppression, with an attendant risk of opportunistic infection. Manipulating miRNA expression has the potential to inhibit autoimmune reactions significantly without side effects of current chemotherapeutic regimens.

miR-181a plays an important role in lymphocyte development.^{64,65} In addition, increases in miR-181a expression in T cells have been shown to lower the threshold for T-cell responses.⁶⁶ Thus, a potentially interesting target for therapy is miR-181a. Modulating miR-181a may affect regulation of T-cell responses to stimuli. The majority of autoimmune T cells have very low affinity for their cognate antigens, which allows them to escape multiple rounds of selection against autoreactive T-cell receptors during T-cell development. These autoimmune cells will thus be highly sensitive to slight changes in the threshold of T-cell activation, which is controlled by miR-181a levels. Targeted delivery of miR-181a-specific antagomiRs to peripheral T cells might preferentially inhibit low-affinity autoimmune responses while preserving high-affinity T-cell responses to pathogens.⁶⁷

Two other miRNAs that play important roles in both adaptive and innate immunity are miR-146 and miR-155. Expression of these 2 miRNAs is induced in response to activating signals,

either by T-cell and B-cell receptor ligation, or by stimulation of innate immune cells through Toll-like receptors. Recently, 2 mouse knockouts of *miR-155* were described (see review⁶⁸). Mice lacking miR-155 had severe defects in adaptive immune responses to bacterial pathogen challenge, which was associated with T_H2-biased cytokine secretion from CD4⁺ T cells and dendritic cells as well as impaired B-cell differentiation. In addition, these mice demonstrated autoimmune phenotypes in the lungs, with increased airway remodeling and leukocyte invasion.^{69,70} These observations indicate that modulation of miR-155 may allow therapeutic manipulation of the immune response.

In B cells, miR-155 is required for differentiation in response to antigen and formation of a high-avidity antibody response. Consequently, inhibition of miR-155 would be predicted to significantly repress formation of a new antibody or autoantibody response. In T cells, modulating miR-155 expression may allow for shifting the focus of an ongoing immune response. In peripheral T cells, increasing miR-155 levels with miR-155 mimetics would lead to a T_H1-like cytokine profile and enhancement of the T-cell response. Conversely, decreasing miR-155 levels with specific antagomiRs would lead to a T_H2-like cytokine profile and inhibition of the T-cell response. The role of miR-155 in innate immunity is less well defined but may also serve as a therapeutic target in autoimmune diseases involving local or systemic inflammation.

The gene encoding miR-146 has not been knocked out. However, the expression and target specificity of miR-146 suggest that this miRNA plays an important role in the regulation of cytokine responses. miR-146 is preferentially expressed in T_H1-type cells and has been shown to regulate the key cytokine signaling adapter molecules, IL-1 receptor-associated kinase (IRAK1) and TNF receptor-associated factor (TRAF) 6, in innate immune cells.^{71,72} Although additional research is needed, these results suggest that manipulation of miR-146 expression could significantly affect the T_H1/T_H2 cytokine balance.

The ability to modify cytokine secretion therapeutically should have significant implications for treatment of T_H1-biased autoimmune conditions, such as type 1 diabetes, as well as allergic diseases, including asthma, which are highly dependent on T_H2 cytokine secretion. T_H2 cytokines drive allergic responses such as asthma, whereas T_H1 cytokines can actually inhibit allergic responses. Consistent with this notion, the miR-155 knockout mice demonstrated phenotypes similar to those seen in asthma, with increased airway thickness and leukocyte invasion.^{69,70} Interestingly, *miR-155* lies within a chromosomal locus associated with asthma in human populations,⁷³ supporting a role for miR-155 in human asthma.

miRNAs also participate in regulation of immune responses by nonhematopoietic cells. miR-203 is upregulated in keratinocytes during autoimmune psoriasis and has been associated with maintenance of local inflammation in this condition.⁷⁴ One regulatory target of miR-203 is a gene known as *suppressor of cytokine signaling 3* (*Socs-3*). Transdermal delivery of siRNAs to disturbed epithelium has been reported.⁷⁵ Inhibition of miR-203 with antagomiRs or blockade of the miR-203 binding site on *Socs-3* using TPs in keratinocytes could break the cycle of inflammation that leads to psoriatic lesions.

Hematopoietic cancers

Efforts to understand biological roles of miRNAs have been bolstered by findings that miRNAs are linked to cancer. The first

report implicating miRNAs in human cancer showed that 2 miRNA genes, coding for *miR-15a* and *miR-16-1*, were located at a chromosomal region frequently deleted or downregulated in chronic lymphocytic leukemia (CLL).⁷⁶ In fact, a genome-wide analysis of 186 miRNA loci revealed that miRNA genes are often located in genomic regions that are deleted or amplified in several cancers, including follicular lymphoma and prolymphocytic leukemia.⁷⁷ In addition to loss of *miR-15a* and *miR16-1* in CLL, other hematopoietic malignancies, such as B-cell lymphomas, have been characterized by overexpression of particular miRNAs, including miR-155^{78,79} and the miR-17-92 cluster.⁸⁰ Thus, the emerging view from genomic and miRNA expression data has been that miRNAs lost in cancers might be tumor suppressors and those amplified in cancers might be oncogenes. In support of this notion, global profiling of miRNA expression levels in hundreds of different cancers revealed that miRNAs are most often reduced in cancers, with cases (albeit many fewer) in which miRNAs are increased.⁸¹ In addition, these profiling studies introduced the idea of classification of cancers on the basis of miRNA profiles, such that each profile was a fingerprint for cellular origin and differentiation state of a particular cancer.⁸¹ These studies suggested that miRNA expression profiles can be used as a diagnostic tool and to predict prognosis or response to therapy. In fact, multiple groups have reported a strong correlation between specific miRNA expression signatures and the progression and prognosis of various cancers, including CLL (see review⁵³).

Although miRNA expression profiles can serve as markers of cancer, they do not directly address the causality of miRNAs to cancer. The first direct demonstration that miRNAs could indeed cause cancer came from work showing that overexpression of RNA from the *BIC* locus, later found to encode miR-155, cooperated with *Myc* to drive erythroleukemogenesis and lymphomagenesis in chickens.⁸² Similarly, the *miR-17-92* polycistron was shown to cooperate with *Myc* to drive B-cell lymphoma formation in mice.⁸⁰ These data immediately suggest that miR-155 and miR-17-92 may be reasonable targets of antagomiR-based therapy for certain leukemias and lymphomas.

Other miRNAs may also be therapeutic targets for hematopoietic malignancies on the basis of intriguing cell culture data. For example, overexpression of miR-223 in an acute promyelocytic leukemia cell line enhanced differentiation of granulocytes.⁸³ This suggests that administering a miR-223 mimetic might be a treatment for acute promyelocytic leukemia, which is characterized by a loss of differentiation and proliferation of myeloid precursor cells, including pregranulocytes. Similarly, the administration of a miR-221/222 mimetic might be a treatment for erythroid leukemias because the overexpression of miR-221 and miR-222 reduced proliferation of a erythroleukemia cell line.⁸⁴ A recent report revealed that overexpression of let-7a in a Burkitt lymphoma cell line had a strong antiproliferative effect because of specific targeting of *Myc* by let-7a.⁸⁵ Thus, perhaps either let-7a-targeted antagomiRs or *Myc*-targeted TPs would be options for treatment of Burkitt lymphoma and possibly other MYC-dysregulated hematopoietic malignancies.

FUTURE DIRECTIONS

miRNA expression profiling efforts have provided a molecular taxonomy of cancers and, by extension, of normal cells. miRNA expression patterns may be used to classify cells and to identify

the origin of cells from a variety of pathologies. Although it is clear that miRNAs that cause disease may be targets for therapy, it is also possible that miRNAs that are not involved in the etiology of pathological processes may also be targets for therapy. That is, a characteristic miRNA expression pattern may be linked to a particular disease prognosis or susceptibility to treatment. Therefore, modulating expression of these signature miRNAs could possibly alter prognosis or susceptibility to treatment.

To illustrate this point, consider the following scenario. let-7 has been found to be globally downregulated in miRNA expression profiling analyses of hundreds of cancers⁸¹ and is known to target the *RAS* pathway.⁸⁶ *RAS* may not be directly involved in causing all of these cancers; however, there may be therapeutic benefit to targeting *RAS* signaling pathways in these cancers. In addition to *RAS*, it is possible that other genes that are regulated by let-7 may affect prognosis or susceptibility to treatment of particular cancers. Therefore, one might imagine that reconstituting let-7 with let-7 mimetics might confer a better prognosis or susceptibility to treatment in those cancers by affecting *RAS* and non-*RAS* pathways simultaneously.

In cancers in which *RAS* participates in pathogenesis directly, another therapeutic strategy might be to use siRNAs to target the *RAS* gene to confer antiproliferative properties to those cells. Using *RAS*-directed siRNAs will likely result in very robust silencing of *RAS* in cancerous cells. However, a disadvantage of using siRNAs against *RAS* might be robust silencing of *RAS*-dependent pathways in noncancerous cells. As an alternative, miRNA pathway modulators may be more effective therapeutic agents than siRNAs because of the breadth and tissue specificity of miRNA-based gene regulation. miRNAs are selectively expressed in certain tissues, and their targets are selectively expressed. Therefore, the possibilities are much lower for off-target and unintended misregulation of specific miRNA pathways compared with siRNA-based therapies. In this case, reconstituting let-7 with let-7 mimetics would selectively affect *RAS* signaling only in cells in which the *RAS* pathway is upregulated. This effect may be amplified by modulating other miRNAs affecting the *RAS* pathway. The latter example will require a more careful and accurate connectivity map that connects certain pathways by miRNA silencing. The subtlety of miRNA-mediated silencing in comparison to siRNA-mediated silencing might suggest a limitation to the use of miRNAs in therapy; however, this quality is what might make them so efficient! That is, some level of gene expression for physiological processes will be intact, but excesses will be reduced.

There may be some cases in which miRNAs work at the point of therapy in the future, but almost certainly RNAi will have multiple roles in adjunctive therapy for diseases. This could be in combination with chemical drugs, or different types of RNAi could be combined. An example of adjunctive RNAi combination therapy could be addition of an siRNA-based therapy to a miRNA pathway-modulated therapy if, for instance, stronger silencing is the goal. This might be needed in a case in which a patient is not responding to or is becoming resistant to a single type of RNAi-based therapy. In these cases, a careful risk-benefit analysis should be considered. In addition, antisense technologies might even be used in combination with siRNA and miRNA pathway modulator technologies to enhance therapeutic outcomes.

We still know very little about the basic biology of miRNA pathways. As small RNA-based tools become more widely used, we will gain more insights into basic mechanisms of miRNA

pathways. As we gain more mechanistic insights into miRNA pathways, these small RNA-based tools will improve. There is no need to reinvent the wheel. Nature has already provided us with small RNAs, which may be drugs themselves or druggable targets.

REFERENCES

- Lim LP, Glasner ME, Yekta S, Burge CB, Bartel DP. Vertebrate microRNA genes. *Science* 2003;299:1540.
- Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* 2005;120:15-20.
- Zhang B, Wang Q, Pan X. MicroRNAs and their regulatory roles in animals and plants. *J Cell Physiol* 2007;210:279-89.
- Esquela-Kerscher A, Slack FJ. Oncomirs: microRNAs with a role in cancer. *Nat Rev Cancer* 2006;6:259-69.
- Krek A, Grun D, Poy MN, Wolf R, Rosenberg L, Epstein EJ, et al. Combinatorial microRNA target predictions. *Nat Genet* 2005;37:495-500.
- Hutvagner G, Zamore PD. A microRNA in a multiple-turnover RNAi enzyme complex. *Science* 2002;297:2056-60.
- Kurreck J. Antisense technologies: improvement through novel chemical modifications. *Eur J Biochem* 2003;270:1628-44.
- Schubert S, Kurreck J. Ribozyme- and deoxyribozyme-strategies for medical applications. *Curr Drug Targets* 2004;5:667-81.
- Song E, Lee SK, Wang J, Ince N, Ouyang N, Min J, et al. RNA interference targeting Fas protects mice from fulminant hepatitis. *Nat Med* 2003;9:347-51.
- Grimm D, Streetz KL, Jopling CL, Storm TA, Pandey K, Davis CR, et al. Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. *Nature* 2006;441:537-41.
- Whelan J. First clinical data on RNAi. *Drug Discovery Today* 2005;10:1014-5.
- Jackson AL, Bartz SR, Schelter J, Kobayashi SV, Burchard J, Mao M, et al. Expression profiling reveals off-target gene regulation by RNAi. *Nat Biotechnol* 2003;21:635-7.
- Pei Y, Tuschl T. On the art of identifying effective and specific siRNAs. *Nat Methods* 2006;3:670-6.
- Peek AS, Behlke MA. Design of active small interfering RNAs. *Curr Opin Mol Ther* 2007;9:110-8.
- Jackson AL, Burchard J, Leake D, Reynolds A, Schelter J, Guo J, et al. Position-specific chemical modification of siRNAs reduces "off-target" transcript silencing. *RNA* 2006;12:1197-205.
- Manche L, Green SR, Schmedt C, Mathews MB. Interactions between double-stranded RNA regulators and the protein kinase DAI. *Mol Cell Biol* 1992;12:5238-48.
- Kariko K, Bhuyan P, Capodici J, Weissman D. Small interfering RNAs mediate sequence-independent gene suppression and induce immune activation by signaling through Toll-like receptor 3. *J Immunol* 2004;172:6545-9.
- Kim DH, Behlke MA, Rose SD, Chang MS, Choi S, Rossi JJ. Synthetic dsRNA Dicer substrates enhance RNAi potency and efficacy. *Nat Biotechnol* 2005;23:222-6.
- Siolas D, Lerner C, Burchard J, Ge W, Linsley PS, Paddison PJ, et al. Synthetic shRNAs as potent RNAi triggers. *Nat Biotechnol* 2005;23:227-31.
- Reynolds A, Anderson EM, Vermeulen A, Fedorov Y, Robinson K, Leake D, et al. Induction of the interferon response by siRNA is cell type- and duplex length-dependent. *RNA* 2006;12:988-93.
- Bridge AJ, Pebernard S, Ducraux A, Nicoulaz AL, Iggo R. Induction of an interferon response by RNAi vectors in mammalian cells. *Nat Genet* 2003;34:263-4.
- Sledz CA, Holko M, de Veer MJ, Silverman RH, Williams BR. Activation of the interferon system by short-interfering RNAs. *Nat Cell Biol* 2003;5:834-9.
- Judge AD, Sood V, Shaw JR, Fang D, McClintock K, MacLachlan I. Sequence-dependent stimulation of the mammalian innate immune response by synthetic siRNA. *Nat Biotechnol* 2005;23:457-62.
- Hornung V, Guenther-Biller M, Bourquin C, Ablasser A, Schlee M, Uematsu S, et al. Sequence-specific potent induction of IFN- α by short interfering RNA in plasmacytoid dendritic cells through TLR7. *Nat Med* 2005;11:263-70.
- Sioud M. Induction of inflammatory cytokines and interferon responses by double-stranded and single-stranded siRNAs is sequence-dependent and requires endosomal localization. *J Mol Biol* 2005;348:1079-90.
- Judge AD, Bola G, Lee AC, MacLachlan I. Design of noninflammatory synthetic siRNA mediating potent gene silencing in vivo. *Mol Ther* 2006;13:494-505.
- Tomanin R, Scarpa M. Why do we need new gene therapy viral vectors? characteristics, limitations and future perspectives of viral vector transduction. *Curr Gene Ther* 2004;4:357-72.
- Batist G, Ramakrishnan G, Rao CS, Chandrasekharan A, Gutheil J, Guthrie T, et al. Reduced cardiotoxicity and preserved antitumor efficacy of liposome-encapsulated doxorubicin and cyclophosphamide compared with conventional doxorubicin and cyclophosphamide in a randomized, multicenter trial of metastatic breast cancer. *J Clin Oncol* 2001;19:1444-54.
- Lu C, Perez-Soler R, Piperdi B, Walsh GL, Swisher SG, Smythe WR, et al. Phase II study of a liposome-entrapped cisplatin analog (L-NDDP) administered intrapleurally and pathologic response rates in patients with malignant pleural mesothelioma. *J Clin Oncol* 2005;23:3495-501.
- Gabizon AA. Pegylated liposomal doxorubicin: metamorphosis of an old drug into a new form of chemotherapy. *Cancer Invest* 2001;19:424-36.
- Patil SD, Rhodes DG, Burgess DJ. DNA-based therapeutics and DNA delivery systems: a comprehensive review. *AAPS J* 2005;7:E61-77.
- Santel A, Aleku M, Keil O, Endruschat J, Esche V, Fisch G, et al. A novel siRNA-lipoplex technology for RNA interference in the mouse vascular endothelium. *Gene Ther* 2006;13:1222-34.
- Palliser D, Chowdhury D, Wang QY, Lee SJ, Bronson RT, Knipe DM, et al. An siRNA-based microbicide protects mice from lethal herpes simplex virus 2 infection. *Nature* 2006;439:89-94.
- Dass CR. Cytotoxicity issues pertinent to lipoplex-mediated gene therapy in-vivo. *J Pharm Pharmacol* 2002;54:593-601.
- Lv H, Zhang S, Wang B, Cui S, Yan J. Toxicity of cationic lipids and cationic polymers in gene delivery. *J Control Release* 2006;114:100-9.
- Sioud M, Sorensen DR. Cationic liposome-mediated delivery of siRNAs in adult mice. *Biochem Biophys Res Commun* 2003;312:1220-5.
- Ma Z, Li J, He F, Wilson A, Pitt B, Li S. Cationic lipids enhance siRNA-mediated interferon response in mice. *Biochem Biophys Res Commun* 2005;330:755-9.
- Morrissey DV, Blanchard K, Shaw L, Jensen K, Lockridge JA, Dickinson B, et al. Activity of stabilized short interfering RNA in a mouse model of hepatitis B virus replication. *Hepatology* 2005;41:1349-56.
- Landen CN, Merritt WM, Mangala LS, Sanguino AM, Bucana C, Lu C, et al. Intraperitoneal delivery of liposomal siRNA for therapy of advanced ovarian cancer. *Cancer Biol Ther* 2006;5:1708-13.
- Thaker PH, Han LY, Kamat AA, Arevalo JM, Takahashi R, Lu C, et al. Chronic stress promotes tumor growth and angiogenesis in a mouse model of ovarian carcinoma. *Nat Med* 2006;12:939-44.
- Halder J, Kamat AA, Landen CN Jr, Han LY, Lutgendorf SK, Lin YG, et al. Focal adhesion kinase targeting using in vivo short interfering RNA delivery in neutral liposomes for ovarian carcinoma therapy. *Clin Cancer Res* 2006;12:4916-24.
- Aigner A. Gene silencing through RNA interference (RNAi) in vivo: strategies based on the direct application of siRNAs. *J Biotechnol* 2006;124:12-25.
- Soutschek J, Akinc A, Bramlage B, Charisse K, Constien R, Donoghue M, et al. Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. *Nature* 2004;432:173-8.
- Mae M, Langel U. Cell-penetrating peptides as vectors for peptide, protein and oligonucleotide delivery. *Curr Opin Pharmacol* 2006;6:509-14.
- Zhang C, Tang N, Liu X, Liang W, Xu W, Torchilin VP. siRNA-containing liposomes modified with polyarginine effectively silence the targeted gene. *J Control Release* 2006;112:229-39.
- Lundberg P, El-Andaloussi S, Sutlu T, Johansson H, Langel U. Delivery of short interfering RNA using endosomal cell-penetrating peptides. *FASEB J* 2007;21:2664-71.
- Song E, Zhu P, Lee SK, Chowdhury D, Kussman S, Dykxhoorn DM, et al. Antibody mediated in vivo delivery of small interfering RNAs via cell-surface receptors. *Nat Biotechnol* 2005;23:709-17.
- Wen WH, Liu JY, Qin WJ, Zhao J, Wang T, Jia LT, et al. Targeted inhibition of HBV gene expression by single-chain antibody mediated small interfering RNA delivery. *Hepatology* 2007;46:84-94.
- Hosen N, Park CY, Tatsumi N, Oji Y, Sugiyama H, Gramatzki M, et al. CD96 is a leukemic stem cell-specific marker in human acute myeloid leukemia. *Proc Natl Acad Sci U S A* 2007;104:11008-13.
- Kumar P, Wu H, McBride JL, Jung KE, Kim MH, Davidson BL, et al. Transvascular delivery of small interfering RNA to the central nervous system. *Nature* 2007;448:39-43.
- Herman RE, Badders D, Fuller M, Makienko EG, Houston ME Jr, Quay SC, et al. The Trp cage motif as a scaffold for the display of a randomized peptide library on bacteriophage T7. *J Biol Chem* 2007;282:9813-24.
- Corey DR. RNA learns from antisense. *Nat Chem Biol* 2007;3:8-11.
- Calin GA, Croce CM. MicroRNA signatures in human cancers. *Nat Rev Cancer* 2006;6:857-66.
- Doench JG, Petersen CP, Sharp PA. siRNAs can function as miRNAs. *Genes Dev* 2003;17:438-42.

55. Martinez J, Patkaniowska A, Urlaub H, Luhrmann R, Tuschl T. Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. *Cell* 2002;110:563-74.
56. Tsuda N, Ishiyama S, Li Y, Ioannides CG, Abbruzzese JL, Chang DZ. Synthetic microRNA designed to target glioma-associated antigen 1 transcription factor inhibits division and induces late apoptosis in pancreatic tumor cells. *Clin Cancer Res* 2006;12:6557-64.
57. Meister G, Landthaler M, Dorsett Y, Tuschl T. Sequence-specific inhibition of microRNA- and siRNA-induced RNA silencing. *RNA* 2004;10:544-50.
58. Hutvagner G, Simard MJ, Mello CC, Zamore PD. Sequence-specific inhibition of small RNA function. *PLoS Biol* 2004;2:E98.
59. Chan JA, Krichevsky AM, Kosik KS. MicroRNA-21 is an antiapoptotic factor in human glioblastoma cells. *Cancer Res* 2005;65:6029-33.
60. Lee YS, Kim HK, Chung S, Kim KS, Dutta A. Depletion of human microRNA miR-125b reveals that it is critical for the proliferation of differentiated cells but not for the down-regulation of putative targets during differentiation. *J Biol Chem* 2005;280:16635-41.
61. Krutzfeldt J, Rajewsky N, Braich R, Rajeev KG, Tuschl T, Manoharan M, et al. Silencing of microRNAs in vivo with "antagomirs." *Nature* 2005;438:685-9.
62. Esau C, Davis S, Murray SF, Yu XX, Pandey SK, Pear M, et al. miR-122 regulation of lipid metabolism revealed by in vivo antisense targeting. *Cell Metab* 2006;3:87-98.
63. Choi WY, Giraldez AJ, Schier AF. Target protectors reveal dampening and balancing of nodal agonist and antagonist by miR-430. *Science* 2007;318:271-4.
64. Chen CZ, Li L, Lodish HF, Bartel DP. MicroRNAs modulate hematopoietic lineage differentiation. *Science* 2004;303:83-6.
65. Neilson JR, Zheng GX, Burge CB, Sharp PA. Dynamic regulation of miRNA expression in ordered stages of cellular development. *Genes Dev* 2007;21:578-89.
66. Li QJ, Chau J, Ebert PJ, Sylvester G, Min H, Liu G, et al. miR-181a is an intrinsic modulator of T cell sensitivity and selection. *Cell* 2007;129:147-61.
67. Zehn D, Bevan MJ. T cells with low avidity for a tissue-restricted antigen routinely evade central and peripheral tolerance and cause autoimmunity. *Immunity* 2006;25:261-70.
68. Moffett HF, Novina CD. A small microRNA makes a Bic difference. *Genome Biol* 2007;8:221:1-4.
69. Rodriguez A, Vigorito E, Clare S, Warren MV, Couttet P, Soond DR, et al. Requirement of bic/microRNA-155 for normal immune function. *Science* 2007;316:608-11.
70. Thai TH, Calado DP, Casola S, Ansel KM, Xiao C, Xue Y, et al. Regulation of the germinal center response by microRNA-155. *Science* 2007;316:604-8.
71. Taganov KD, Boldin MP, Chang KJ, Baltimore D. NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. *Proc Natl Acad Sci U S A* 2006;103:12481-6.
72. Monticelli S, Ansel KM, Xiao C, Socci ND, Krichevsky AM, Thai TH, et al. MicroRNA profiling of the murine hematopoietic system. *Genome Biol* 2005;6:R71:1-15.
73. Ober C, Cox NJ, Abney M, Di Rienzo A, Lander ES, Changyaleket B, et al. Genome-wide search for asthma susceptibility loci in a founder population. The Collaborative Study on the Genetics of Asthma. *Human Mol Genet* 1998;7:1393-8.
74. Sonkoly E, Wei T, Janson PC, Saaf A, Lundeberg L, Tengvall-Linder M, et al. MicroRNAs: novel regulators involved in the pathogenesis of Psoriasis? *PLoS ONE* 2007;2:e610.
75. Thanik VD, Greives MR, Lerman OZ, Seiser N, Dec W, Chang CC, et al. Topical matrix-based siRNA silences local gene expression in a murine wound model. *Gene Ther* 2007;14:1305-8.
76. Calin GA, Dumitru CD, Shimizu M, Bichi R, Zupo S, Noch E, et al. Frequent deletions and down-regulation of micro-RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proc Natl Acad Sci U S A* 2002;99:15524-9.
77. Calin GA, Sevignani C, Dumitru CD, Hyslop T, Noch E, Yendamuri S, et al. Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. *Proc Natl Acad Sci U S A* 2004;101:2999-3004.
78. Metzler M, Wilda M, Busch K, Viehmann S, Borkhardt A. High expression of precursor microRNA-155/BIC RNA in children with Burkitt lymphoma. *Genes Chromosomes Cancer* 2004;39:167-9.
79. Eis PS, Tam W, Sun L, Chadburn A, Li Z, Gomez MF, et al. Accumulation of miR-155 and BIC RNA in human B cell lymphomas. *Proc Natl Acad Sci U S A* 2005;102:3627-32.
80. He L, Thomson JM, Hemann MT, Hernando-Monge E, Mu D, Goodson S, et al. A microRNA polycistron as a potential human oncogene. *Nature* 2005;435:828-33.
81. Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, et al. MicroRNA expression profiles classify human cancers. *Nature* 2005;435:834-8.
82. Tam W, Hughes SH, Hayward WS, Besmer P. Avian bic, a gene isolated from a common retroviral site in avian leukosis virus-induced lymphomas that encodes a noncoding RNA, cooperates with c-myc in lymphomagenesis and erythroleukemogenesis. *J Virol* 2002;76:4275-86.
83. Fazi F, Rosa A, Fatica A, Gelmetti V, De Marchis ML, Nervi C, et al. A microRNA circuitry comprised of microRNA-223 and transcription factors NFI-A and C/EBPalpha regulates human granulopoiesis. *Cell* 2005;123:819-31.
84. Felli N, Fontana L, Pelosi E, Botta R, Bonci D, Facchiano F, et al. MicroRNAs 221 and 222 inhibit normal erythropoiesis and erythroleukemic cell growth via kit receptor down-modulation. *Proc Natl Acad Sci U S A* 2005;102:18081-6.
85. Sampson VB, Rong NH, Han J, Yang Q, Aris V, Soteropoulos P, et al. MicroRNA Let-7a down-regulates MYC and reverts MYC-induced growth in Burkitt lymphoma cells. *Cancer Res* 2007;67:9762-70.
86. Johnson SM, Grosshans H, Shingara J, Byrom M, Jarvis R, Cheng A, et al. RAS is regulated by the let-7 microRNA family. *Cell* 2005;120:635-47.