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Advances in MicroRNAs: Implications for Gene Therapists

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Abstract

MicroRNAs (miRNAs) are a class of small regulatory RNAs that are thought to regulate the expression of as many as one-third of all human messenger RNAs (mRNAs). miRNAs are thought to be involved in diverse biological processes, including tumorigenesis. Analysis of miRNA levels may have diagnostic implications. Evidence shows that numerous viruses interact with the miRNA machinery, and that a number of viruses encode their own miRNAs. It seems likely that miRNAs will be implicated in many human diseases. Manipulation of miRNA levels by gene therapy provides an attractive new approach for therapeutic development. This review focuses on approaches to manipulate miRNA levels in cells and *in vivo*, and the implications for gene therapy. Furthermore, we discuss the use of endogenous miRNAs as scaffolds for the expression of RNA interference (RNAi) as well as competition between exogenous RNAi triggers and endogenous miRNAs. Because short interfering RNAs can also act as miRNAs, seed matches with the 3' untranslated regions of genes should be avoided to prevent off-target effects. Last, we discuss the use of miRNAs to avoid immune responses to viral vectors.

BACKGROUND

MICRORNAs (miRNAs) ARE SMALL, ~22-nucleotide noncoding regulatory RNAs involved in RNA-mediated gene silencing (Lagos-Quintana *et al.*, 2001). At present, more than 500 human miRNAs have been identified. miRNAs are important gene regulators during processes that include differentiation, proliferation, apoptosis, and insulin secretion as well as heart, brain, and skeletal muscle development (see Bartel [2004], Harfe [2005], Kloosterman and Plasterk [2006], and Krutzfeldt and Stoffel [2006] for comprehensive reviews). The miRNA pathway involves several processing steps to produce the mature miRNA used for gene silencing (Fig. 1). Long primary miRNA transcripts (pri-miRNAs) transcribed by polymerase II or III are cleaved by a complex of Drosha and DGCR8 (DiGeorge critical region-8) into the pre-miRNA. The pre-miRNA is exported from the nucleus to the cytoplasm by Exportin 5, where it undergoes a second cleavage step by Dicer/TRBP (HIV TAR RNA-binding protein) to produce a short, ~20-nucleotide duplex RNA with two base 3' overhangs. Usually, a single strand of this duplex RNA is incorporated into the RNA-induced silencing complex (RISC), although a subset of miRNAs incorporate both strands individually into distinct RISCs. The mature miRNA strand in RISC is called the guide strand, and the opposite strand not incorporated is referred to as the passenger strand. The minimal RISC is composed of an Argonaute (Ago) protein, Dicer, and TRBP, although it is likely that RISC interacts with numerous accessory proteins (Peters and Meister, 2007). Once the mature miRNA guide strand is loaded into the RISC, it binds to the 3' untranslated region (UTR) of its mRNA target and

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induces silencing. It is currently thought that the specificity of the miRNA for its target mRNA is primarily (although not exclusively) specified by the “seed sequence” (nucleotides 2–8 of the guide strand; Fig. 2). One implication, evidenced by the short length of the seed sequence, is that miRNAs are remarkably promiscuous. It has been suggested that some miRNAs can target hundreds of mRNAs (Farh *et al.*, 2005; Lim *et al.*, 2005; Stark *et al.*, 2005). miRNAs repress their target mRNAs by a variety of mechanisms. miRNAs with perfect complementarity to their target mRNAs cause direct mRNA cleavage, although this is thought to be rare in mammals. More often, miRNAs with partial complementarity to mRNA 3' UTRs direct translational repression, decapping, or deadenylation.

The use of RNA interference (RNAi) for gene silencing has become widespread, both for functional studies and for therapeutic purposes in a gene therapy setting. Commonly, RNAi is initiated by transfection of cells or animals with synthetic short interfering RNAs (siRNAs) or introduction of plasmids or recombinant viruses expressing short hairpin RNAs (shRNAs). It has become apparent that exogenous synthetic siRNAs can silence genes because they resemble the endogenous miRNAs generated by Dicer/TRBP, and thus can be loaded into the RISC (Fig. 1). Likewise, shRNAs resemble the pre-miRNA. Exogenous siRNA and shRNAs are designed to be perfectly complementary to their target mRNA, and therefore they direct cleavage. However, they can also act as miRNAs. The consequences for design of siRNAs/shRNAs are described below. These exogenous siRNAs and shRNAs also have the potential to overload the miRNA-processing pathway.

The last few years has witnessed an explosion of publications on miRNAs. This review is not intended as a comprehensive survey of the miRNA literature. Rather, it highlights key issues of importance to researchers who seek to integrate gene therapy approaches with our emerging understanding of miRNAs.

miRNAs and cancer: diagnosis, prognosis, and therapeutic potential

High-throughput strategies, such as miRNA microarrays (Liu *et al.*, 2004), bead-based flow cytometric miRNA expression profiling (Lu *et al.*, 2005), and quantitative polymerase chain reaction (qPCR) (Chen *et al.*, 2005; Jiang *et al.*, 2005; Murakami *et al.*, 2005; Raymond *et al.*, 2005), have been developed for miRNA profiling. These high-throughput methods have shown that miRNA levels were dramatically shifted in various cancers (Liu *et al.*, 2004; He *et al.*, 2005; Iorio *et al.*, 2005; Lu *et al.*, 2005; Murakami *et al.*, 2005) (refer to Calin and Croce [2006] and Esquela-Kerscher and Slack [2006] for a comprehensive review of miRNAs and cancer). Interestingly, most differentially expressed miRNAs were downregulated in tumors compared with normal tissues. These studies revealed that miRNA profiling could be used to distinguish between normal and cancerous tissues, developmental lineage, as well as differentiation. Identifying miRNA expression profiles during tumorigenesis will likely provide additional targets for gene therapy, while improving predictions for diagnosis and prognosis.

For most of the miRNAs found to be dysregulated in cancer, a causative relationship between differential expression and cancer has not been established; however, for a subset there is now convincing evidence that some miRNAs are directly involved in cancer formation. These “onco-miRs” can function as tumor suppressors (such as miR-15a and miR-16-1 [Calin *et al.*, 2002, 2005; Cimmino *et al.*, 2005] and the *let-7* family of miRNAs [Calin *et al.*, 2002; Takamizawa *et al.*, 2004; Johnson *et al.*, 2005, 2007]) and as oncogenes (such as the miR-17–92 cluster [He *et al.*, 2005], miR-155 [Costinean *et al.*, 2006], and miR-21 [Si *et al.*, 2007]). A few examples are provided below.

The first tumor suppressor miRNAs were identified in B cell chronic lymphocytic leukemia (CLL) (Calin *et al.*, 2002). miR-15a and miR-16-1 are located in a cluster on chromosome

13q14, which is deleted in more than half of all CLL cases. This correlates with the loss of expression of miR-15a and miR-16-1. It was later shown that miR-15a and miR-16-1 negatively regulated expression of the antiapoptotic gene *Bcl2* (Cimmino *et al.*, 2005). After discovering that miRNAs were located within a frequently deleted chromosomal region in CLL, researchers investigated the presence of miRNAs in “fragile sites” or chromosomal loci that are susceptible to chromosome breakage, amplification, and fusion with other chromosomes (Calin *et al.*, 2004). They found that 50% of miRNAs were located within human fragile sites. This provided further evidence that miRNAs are involved in cancer and that they may be useful targets for therapy. Interestingly, in one report, Donsante and coworkers found that hepatocellular carcinoma associated with adeno-associated virus integration resulted from a vector insertion near a micro-RNA cluster (Donsante *et al.*, 2007).

One of the most studied oncogenic miRNAs is the miR-17–92 cluster. The miR-17–92 cluster is at a locus that is amplified in human B cell lymphomas and therefore upregulated relative to normal tissues (He *et al.*, 2005). Furthermore, enforced expression of the miR-17–92 cluster in the context of *c-myc* overexpression accelerated tumor development in a mouse B cell lymphoma model.

Another example of an oncogenic miRNA is miR-155. miR-155 is overexpressed in various types of B cell malignancies (Metzler *et al.*, 2004; Eis *et al.*, 2005; Kluiver *et al.*, 2005). Costinean and coworkers produced a transgenic mouse that overexpresses miR-155 in B cells (Costinean *et al.*, 2006). These mice exhibited polyclonal preleukemic pre-B cell proliferation followed by B cell malignancy, which strongly suggests that miR-155 is directly implicated in the initiation and/or progression of these diseases.

Although the examples discussed above are only a small subset of miRNAs implicated in cancer development, they illustrate the point that targeting aberrantly expressed miRNAs by therapeutic approaches could have a tremendous impact on future cancer treatments. Strategies for manipulating miRNA levels are discussed below.

Viruses and miRNAs

Increasing evidence suggests that viruses interact with the miRNA machinery (reviewed in Sullivan *et al.* [2006] and Dykxhoorn [2007]). Cloning of miRNAs from virus-infected cells revealed that many latent DNA viruses encode their own miRNAs (Pfeffer *et al.*, 2004). To date, 11 viruses have been shown to encode their own miRNAs (see the miRBase Sequence Database [previously the miRNA Registry] at <http://microrna.sanger.ac.uk/sequences/index.shtml>). These include Epstein–Barr virus (EBV) (Pfeffer *et al.*, 2004), herpes simplex virus type 1 (HSV-1) (Cui *et al.*, 2006), human cytomegalovirus (Pfeffer *et al.*, 2005), human immunodeficiency virus type 1 (Omoto and Fujii, 2005), Kaposi sarcoma-associated herpesvirus (Cai *et al.*, 2005; Pfeffer *et al.*, 2005; Samols *et al.*, 2005), Marek's disease virus (Burnside *et al.*, 2006), Marek's disease virus type 2 (Yao *et al.*, 2007), mouse gammaherpesvirus 68 (Pfeffer *et al.*, 2005), rhesus lymphocryptovirus (Cai *et al.*, 2006), rhesus monkey rhadinovirus (Schafer *et al.*, 2007), and simian virus 40 (SV40) (Sullivan and Ganem, 2005). These viral miRNAs represent potential antiviral targets and could also serve as diagnostic markers for viral infection or stage of infection/latency. Below, we discuss several examples of the interplay of host and viruses via the miRNA pathway.

Viruses use miRNAs in various ways, such as to inhibit viral or host transcripts or to recruit host miRNAs for viral replication. EBV and SV40 are examples of viruses that encode miRNAs that target their own viral transcripts. EBV encodes at least 18 miRNAs (Pfeffer *et al.*, 2004; Sullivan *et al.*, 2006), one of which targets the transcript encoding the viral polymerase to negatively regulate it later during infection (Furnari *et al.*, 1993; Pfeffer *et al.*, 2005). Likewise,

SV40 encodes two viral miRNAs that target the viral T-antigen transcript (Sullivan *et al.*, 2005). This regulatory mechanism may aid the virus in immune evasion.

HSV-1 provides an example of a virus that encodes miRNAs that target host transcripts. The HSV-1 latency-associated transcript encodes an miRNA predicted to target two host transcripts involved in apoptosis (transforming growth factor- β and mothers against decapentaplegic homolog-3) (Gupta *et al.*, 2006).

Host miRNAs can also be used for viral replication as well as antiviral defense. The endogenous miRNA, miR-122, is required for replication of hepatitis C virus (Jopling *et al.*, 2005). Figure 2 shows the interaction of miR-122 with the 5' UTR of hepatitis C virus. As discussed below, two groups have successfully used antisense approaches to modulate the levels of miR-122 in mouse models (Krutzfeldt *et al.*, 2005; Esau *et al.*, 2006). Inhibition of viral replication *in vivo* using this strategy remains to be demonstrated. In contrast, primate foamy virus type 1 (PFV-1) has an mRNA that is the target of the endogenous host miRNA, miR-32 (Lecellier *et al.*, 2005). Interestingly, PFV-1 encodes a protein (Tas) that may interfere with the host miRNA pathway (Lecellier *et al.*, 2005). These examples suggest that miRNA overexpression or ablation may represent a novel antiviral therapeutic strategy.

Last, at least one commonly used gene transfer vector encodes an RNA that inhibits the miRNA pathway. The highly expressed adenoviral virus-associated type I (VAI) RNA was shown to be a competitive inhibitor of at least two steps in the miRNA pathway (Lu and Cullen, 2004; Andersson *et al.*, 2005). It is a competitive inhibitor of Exportin 5 as well as Dicer and small VAI RNAs generated by Dicer cleavage have been shown to associate with RISC. These findings suggest that saturation of the endogenous miRNA pathway should be assessed in human clinical trials with adenoviral vectors, when possible.

Other miRNA/disease associations

Although the vast majority of associations between miRNAs and human disease are related to tumorigenesis, there is increasing evidence that miRNAs could be involved in multiple diseases. For example, 28 miRNAs were found to be dysregulated in two mouse models of cardiac hypertrophy (van Rooij *et al.*, 2006). miR-9 and miR-128a were overexpressed in the brains of Alzheimer's patients (Lukiw, 2007) and 16 miRNAs were differentially expressed in the brains of schizophrenics (Perkins *et al.*, 2007). Last, a mutation in the 3' UTR of the *SLITRK1* gene that is associated with Tourette's syndrome led to enhanced repression by miR-189 (Abelson *et al.*, 2005).

THERAPEUTIC MANIPULATION OF miRNAs

As described above, miRNAs are likely to be involved in numerous genetic diseases, such as cancer, as well as some viral infections. These miRNAs represent potentially novel therapeutic targets. It would clearly be desirable to specifically modulate the levels of individual miRNAs *in vivo*. Fortunately, decades of research in the fields of antisense, ribozymes, and gene therapy provide a fertile tool chest that serves as a starting point for manipulating miRNA levels *in vivo*. Below, we discuss methods for overexpressing and ablating miRNAs and provide examples of initial efforts to apply these techniques in disease settings. Again, this is not intended to be an exhaustive review, but rather a review of important paradigms for gene therapists.

Inhibition of miRNA function through antisense and miRNA sponges

Numerous groups have now shown that antisense oligonucleotides complementary to the guide strand of miRNAs can inhibit their function in cultured cells (Esau *et al.*, 2004; Meister *et al.*, 2004; Poy *et al.*, 2004; Cheng *et al.*, 2005; Lee *et al.*, 2005; Schratt *et al.*, 2006), flies

(Boutla *et al.*, 2003; Hutvagner *et al.*, 2004; Leaman *et al.*, 2005), and mice (Hutvagner *et al.*, 2004; Esau *et al.*, 2006; Krutzfeldt *et al.*, 2007). These inhibitors, referred to as “anti-miRs” or “antago-miRs,” could have significant clinical potential (Poy *et al.*, 2004; Jopling *et al.*, 2005; Krutzfeldt *et al.*, 2005; Esau *et al.*, 2006; Weiler *et al.*, 2006). Early studies showed that 2'-*O*-methyl oligoribonucleotides were able to block miRNA function in cultured cells (Meister *et al.*, 2004) and in flies (Hutvagner *et al.*, 2004). In contrast, unmodified 2'-deoxy oligonucleotides (Meister *et al.*, 2004) and 2'-deoxy phosphorothioate oligonucleotides (Davis *et al.*, 2006) were unable to inhibit miRNA activity in cultured cells. Several reports suggest that anti-miRs interfere with miRNA-mediated silencing by binding to the mature miRNA guide strand in the RISC complex (Hutvagner *et al.*, 2004; Davis *et al.*, 2006; Krutzfeldt *et al.*, 2007). Interestingly, it appears that anti-miR binding leads to degradation of the targeted miRNA by some previously undescribed mechanism (Esau *et al.*, 2006; Krutzfeldt *et al.*, 2007).

Although 2'-*O*-methyl oligoribonucleotides appeared to be effective inhibitors of miRNA function, more potent inhibitors have been described. Davis and coworkers conducted a limited comparison of the anti-miR activities of various oligonucleotides with different backbones and 2' modifications (Davis *et al.*, 2006). They found that uniformly 2'-*O*-methoxyethyl oligonucleotides (2'-MOEs) and oligonucleotides in which every third nucleotide was substituted with a locked nucleic acid (LNA) residue (Exiqon, Woburn, MA) were the most potent. Another report also showed that LNA-substituted oligonucleotides were effective at antagonizing miRNAs (Orom *et al.*, 2006). Several vendors sell LNA-modified oligonucleotides (including Integrated DNA Technologies [Coralville, IA] and Exiqon [Vedbaek, Denmark]). 2'-*O*-Methoxyethyl oligonucleotides (Isis Pharmaceuticals, Carlsbad, CA) are not commercially available. 2'-Fluoro-oligonucleotides with a phosphorothioate backbone also showed significant activity in cultured cells (Davis *et al.*, 2006). Oligonucleotides with central stretches of 2-deoxy residues can direct RNase H-mediated degradation of RNA/oligonucleotide duplexes. However, the inability of such oligonucleotides to antagonize miRNA function suggested that RNase H may not have access to miRNA-loaded RISCs bound to anti-miRs (Davis *et al.*, 2006). Last, because transfection of cells with oligonucleotides with some chemistries can lead to a loss of cell viability at high doses (Davis *et al.*, 2006), care should be taken to control for toxicity in anti-miR studies.

A significant barrier to the application of anti-miRs *in vivo* will concern efficient delivery of these molecules either systemically or locally. However, two groups have reported successful miRNA inhibition *in vivo*. Krutzfeldt and coworkers targeted several different miRNAs in mice, using antago-miRs (Krutzfeldt *et al.*, 2005). These are 2'-*O*-methyl oligonucleotides with two and four phosphorothioate linkages at the 5' and 3' ends, respectively. The antago-miRs were conjugated to cholesterol to reduce renal clearance and enhance uptake. Administration of three injections of high doses of antago-miR (80 mg/kg) resulted in substantial decreases in the levels of endogenous miR-16 in all tissues tested except for the brain. The authors then targeted the abundant liver miRNA, miR-122. They conducted gene expression profiling and identified hundreds of genes that were upregulated in the liver on treatment with an miR-122 antago-miR. Many of these genes were involved in cholesterol biosynthesis and, indeed, miR-122 antagomiR treatment led to a 40% reduction in plasma cholesterol levels. These findings provided the proof-of-principle that anti-miRs have the potential to be therapeutic. Because miR-122 is required for viral replication, miRNA downregulation may have antiviral activity.

In a separate article, the authors found that longer antago-miRs (25 nucleotides) were more effective *in vivo* (Krutzfeldt *et al.*, 2007). This was presumably because the phosphorothioate bases at the ends decreased the melting temperature of the miRNA-antagomiR duplex. Lengthening the antago-miR resulted in more pairing with the more stable interior 2'-deoxy

bases, while maintaining the nuclease stabilization afforded by the terminal phosphorothioate linkages. The authors also showed that some antago-miRs exhibit mismatch specificity, although this was highly position dependent (Krutzfeldt *et al.*, 2007).

In a similar study, Esau and coworkers reported that unconjugated 2'-MOE miR-122 anti-miRs reduced the levels of miR-122 in mouse liver after intraperitoneal injection of 12.5–75 mg/kg twice weekly for 4 weeks (Esau *et al.*, 2006). As with the above-described study, the authors observed elevations in the levels of predicted target mRNAs and decreases in plasma cholesterol. The authors then applied this anti-miR strategy in a disease model of diet-induced obesity in mice. Treatment with miR-122 anti-miR reduced plasma cholesterol by 35%. Treated animals also had lower levels of steatosis.

Ebert and coworkers described another approach to inhibiting the function of specific miRNAs in cells (Ebert *et al.*, 2007). They reasoned that by expressing an mRNA containing multiple binding sites for an endogenous miRNA, they could bind up the miRNA and prevent its association with its endogenous targets. They referred to these synthetic mRNAs as “miRNA sponges.” To prevent cleavage of the mRNA containing the miRNA-binding sites, they introduced central mismatches in the miRNA/mRNA duplex at positions 9–12, which were essential for activity. This should prevent release and recycling of the miRNA. They designed both polymerase (Pol) II- and Pol III-driven miRNA sponges, although they observed some nonspecific depression of luciferase expression with Pol III-driven sponges. The use of Pol II promoters should enable the creation of inducible and tissue-specific miRNA sponges. They showed that in cultured cells, their miRNA sponges were at least as effective as LNA anti-miRs. miRNA sponges were also effective at repressing multiple members of an miRNA family. Stably integrated, miRNA sponge expression cassettes yielded ~40% as much inhibition as transiently transfected plasmids. Although it remains to be demonstrated, these results suggest that the generation of transgenic animals expressing inducible miRNA sponges may be feasible. Interestingly, one report suggests that nature uses miRNA sponges. Franco-Zorrilla and coworkers showed that in *Arabidopsis*, the noncoding IPS1 RNA serves as a sponge for miR-399 (Franco-Zorrilla *et al.*, 2007). As described above, the IPS1/miR-399 hybrid contains internal mismatches that are essential for its activity.

The examples described above demonstrate the proof of principle that miRNA function can be inhibited *in vivo* and in cultured cells. However, there is clearly a need to refine and optimize these approaches. Creative methods that mimic natural biological processes may provide clues as to how to accomplish this.

Downregulating expression of miRNAs involved in cancer

Because miRNAs are often dysregulated in cancer, modulating their levels may have therapeutic benefit. miR-21 was an early example of how downregulating an miRNA inhibited cancer development. miR-21 is overexpressed in many different cancer types, such as breast cancer (Iorio *et al.*, 2005; Si *et al.*, 2007), ovarian cancer (Iorio *et al.*, 2007), hepatocellular carcinoma (Meng *et al.*, 2007), gliomas (Chan *et al.*, 2005), pancreatic cancer (Lee *et al.*, 2007), and CLL (Fulci *et al.*, 2007). miR-21 plays a critical role in cell proliferation by targeting and, therefore, inhibiting the tumor suppressor genes *TPM1* (tropomyosin 1) (Zhu *et al.*, 2007) and *PTEN* (phosphatase and tensin homolog) (Meng *et al.*, 2007). Using a xenograft carcinoma model, researchers transiently transfected MCF-7 cells with 2'-O-methyl oligonucleotides complementary to miR-21 (or negative control oligonucleotide), and then injected them into mammary pads of female nude mice (Si *et al.*, 2007). After 28 days, tumors derived from MCF-7 cells transfected with anti-miR-21 were 50% smaller in size compared with tumors of mice receiving cells transfected with negative control oligonucleotide. The authors showed that suppression of miR-21 lasted 2 weeks. Although the model system is somewhat artificial (*ex vivo* treatment of tumors), this report demonstrates that knockdown of

oncogenic miRNAs can inhibit tumor growth. Thus, knockdown of miRNAs by systemic or local delivery of anti-miRs could represent a novel anticancer therapy.

Overexpression of miRNAs

When miRNA levels are reduced in human disease, it may be desirable to use gene therapy-based approaches to restore miRNA expression in certain tissues. The considerable experience with nonviral and viral delivery of transgenes that has accumulated over the past few decades will certainly facilitate this goal. As is discussed below, the endogenous miRs miR-26a (McManus *et al.*, 2002) and miR-30 (Zeng *et al.*, 2002; Boden *et al.*, 2004) have been used as scaffolds for the expression of RNAi triggers. Clearly, these well-characterized systems could be used to produce mature miRNA guide strands in cells. For example, overexpression of an miRNA (or miRNAs) whose expression is downregulated in cancer may be beneficial as treatment. In some cases, it may be desirable to express endogenous miRNAs in their native context with their own promoters, thus maintaining proper regulation. As more complete miRNA transcription units are characterized, this may become increasingly possible.

Reexpression of miRNAs downregulated during cancer

Loss of tumor suppressor genes is a common theme during tumorigenesis. As previously discussed, most differentially expressed miRNAs in tumors were downregulated compared with normal tissues. Exogenous expression of miRNAs downregulated during tumorigenesis may have potential as a therapeutic strategy. Researchers have demonstrated that miR-34a was downregulated in 36% of human colon cancers compared with counterpart normal tissues (Tazawa *et al.*, 2007). *In vivo* reexpression of miR-34a suppressed growth of HCT 116 and RKO cells in mice. Furthermore, miR-34a functions as a potent suppressor of cell proliferation through modulation of the E2F signaling pathway. Loss of miR-34a expression could contribute to aberrant cell proliferation, leading to colon cancer development (Tazawa *et al.*, 2007). Thus, exogenous expression of miRNAs may have application as an anticancer strategy.

Synthetic miRNAs targeting genes associated with metastasis

An alternative approach for cancer therapeutics involves creating synthetic miRNAs that target genes responsible for onco-genesis or metastasis. Loss of certain miRNAs in breast tumor cells can lead to increased invasion and metastasis of breast cancer. Inhibition of chemokine (C-X-C motif) receptor 4 (CXCR4) with siRNA inhibited metastasis of breast cells *in vivo* (Liang *et al.*, 2004, 2005). Therefore, researchers designed and synthesized a pre-miRNA with an miR-155 backbone that targeted CXCR4 in order to determine whether the CXCR4/SDF-1 pathway was regulated by expression of miRNAs (Liang *et al.*, 2007). After transfecting breast tumor cells with the synthesized miRNA, they observed a significant decrease in CXCR4 in breast tumor cells and reduced migration and invasion. Furthermore, they formed fewer lung metastases *in vivo* compared with control miRNA-transfected cells.

Another example of inhibiting metastatic-related genes involves PRL-3 (protein tyrosine phosphatase of regenerating liver-3), whose high expression is associated with lymph node metastasis in gastric carcinoma. This study used an artificial miRNA with an miR-155 backbone that targeted PRL-3. PRL-3 knockdown effectively suppressed the growth of peritoneal metastases and improved the prognosis for nude mice (Li *et al.*, 2006). Therefore, synthetic miRNAs that target genes involved in metastasis serve as an alternative means for treating cancer.

Epigenetic gene therapy in cancer

Manipulation of miRNA levels may also be a means to alter DNA methylation. DNA methylation is a crucial mechanism associated with epigenetic regulation. Changes in the

pattern of DNA methylation, either increased (hypermethylation) or decreased (hypomethylation), have been identified in all types of cancer cells examined so far. DNA methyltransferase inhibitors, both nucleoside and nonnucleoside analogs, are currently being tested for cancer therapy (reviewed in Brueckner *et al.*, 2007). However, similar to most cancer treatments, there are restrictions associated with efficacy and cytotoxicity. Therefore developing a new means of targeting DNA hypermethylation is of great interest. The miR-29 family is downregulated in many cancers that contain aberrant DNA hypermethylation patterns, including lung cancer. It was shown that miR-29 targets DNA methyltransferase-3A (DNMT3A) and -3B (Fabbri *et al.*, 2007). On enforced expression of miR-29, normal methylation patterns in lung cells were reestablished and tumor suppressors previously silenced by promoter hypermethylation were reactivated. Furthermore, expression of miR-29 inhibited tumorigenicity both *in vitro* and *in vivo*. Epigenetic gene therapy approaches can be useful across many tumor types. miRNA-mediated therapy may be useful in combination with DNA methyltransferase inhibitors that are nontoxic, but have limited efficacy. One study showed that treatment of malignant cholangiocytes with gemcitabine, a nucleoside inhibitor, alters the miRNA expression profiles (Meng *et al.*, 2006). Furthermore, inhibiting miRNAs highly overexpressed in malignant cholangiocytes, such as miR-21, increased gemcitabine cytotoxicity. Therefore miRNAs may contribute to chemoresistance and inhibiting miRNAs overexpressed in cancer may increase the efficacy of chemotherapy.

IMPLICATIONS OF miRNAs FOR RNAi GENE THERAPY

miRNAs as scaffolds for RNAi triggers

Since the discovery of RNAi, researchers have been interested in increasing the efficacy of gene-silencing tools. One approach involves expressing RNAi triggers in the context of naturally occurring miRNAs. This can be accomplished by replacing sequences in the central stem of endogenous pre-miRNAs, such as miR-26a (McManus *et al.*, 2002) and miR-30 (Zeng *et al.*, 2002; Boden *et al.*, 2004), with sequences that will direct RNAi against a target of interest (RNAi triggers). The premise for this is that endogenous miRNA scaffolds will be recognized and efficiently processed by the miRNA/RNAi machinery. When designing miRNA-based RNAi triggers, care should be taken to maintain the native sequence around the Drosha cleavage site, as that can significantly influence silencing efficiency (Zhou *et al.*, 2005). By increasing our understanding of the processing and targeting of natural miRNAs we will be able to design a more robust silencing tool. Boden and coworkers reported that pri-miRNAs may be processed more efficiently than shRNAs, leading to 80% improvement in silencing efficiency (Boden *et al.*, 2004). Hairpins containing loop sequences derived from pri-miRNAs were more efficiently transported to the cytoplasm than were hairpins containing artificial loops (Kawasaki and Taira, 2003). Numerous groups, including our own, have now used this approach. Silencing efficiencies of >95% can be achieved with miRNA-derived RNAi triggers (A. McCaffrey, unpublished results). Importantly, pri-miRNAs can be expressed from polymerase II promoters (Boden *et al.*, 2004), which enables the use of tissue-specific promoters, as well as inducible expression. Alternatively, miRNA-based RNAi triggers can be expressed from polymerase III promoters for high-level expression. Thus, expression of RNAi triggers in the context of endogenous miRNAs may offer significant advantages.

Competition between exogenous RNAi triggers and endogenous miRNAs

All drugs have a “therapeutic window”; insufficient doses are not efficacious and excessive doses cause toxicity. In a sobering report, Grimm and coworkers showed that, when shRNAs are highly overexpressed in mice, they can cause acute toxicity (Grimm *et al.*, 2006). The authors speculate that shRNAs were saturating the essential miRNA nuclear export factor Exportin 5, and thus competing for export with endogenous miRNAs. Long-term expression of the hairpins led to a depletion of endogenous liver miRNAs such as miR-122. It should be

noted that high doses of adeno-associated virus (AAV) serotype 8 were used in these studies in order to achieve ~100% transduction of hepatocytes. These results indicate that it would be desirable to design the most potent RNAi triggers possible such that subsaturating doses are efficacious. Regulated and tissue-specific expression also seems prudent. It will be essential to monitor for saturation of the miRNA machinery in preclinical and human clinical trials with RNAi, when possible.

If Exportin 5 is the only bottleneck at which RNAi triggers compete with endogenous miRNAs for the RNAi/miRNA machinery, one would predict that siRNAs (which do not require Exportin 5) would not compete with endogenous miRNAs. However, the Rossi group showed that both exogenous shRNAs and siRNAs competed with miR-21 in cultured cells, suggesting that factors downstream of Exportin 5 can be saturated (Castanotto *et al.*, 2007). Intriguingly, an exogenous miRNA-based RNAi trigger with the same guide strand did not compete with miR-21 (Castanotto *et al.*, 2007). Although this suggests that expression of RNAi triggers in the context of endogenous miRNAs may increase safety, further studies are required to confirm this result.

John and coworkers reported that high doses of siRNAs formulated with lipid nanoparticles transiently mediated efficient silencing of two liver transcripts in mice without interfering with the levels of three liver miRNAs (John *et al.*, 2007). It is important to note that only two time points were examined in this experiment (2 days and 30 days). Silencing was observed only on day 2. In contrast, Grimm and coworkers observed toxicity only at late time points after chronic expression of the shRNA from viral vectors (greater than ~25 days) (Grimm *et al.*, 2006). John and coworkers conducted further long-term silencing studies with lipid nanoparticle-formulated siRNAs in hamsters (21 days) and observed efficient silencing of the hepatocyte-expressed sterol regulatory element-binding protein (SREBP) transcript without decreases in expression of miR-122. miR-122 is by far the most abundant liver miRNA. It would be interesting to determine whether longer term silencing (>25 days) would affect the levels of miR-122 or the levels of a less abundant liver miRNA. Competition between processing of siRNAs and endogenous miRNAs requires further investigation. Identifying the proper balance between efficacy and toxicity may be required for each individual exogenous RNAi trigger.

Seed sequence of siRNAs is critical for predicting off-target silencing

When siRNAs were first applied as gene-silencing tools, it was initially thought that they were exquisitely specific. Later, more comprehensive studies using microarray analysis indicated that some siRNAs (and presumably some shRNAs) can cause off-target cleavage of mRNAs containing closely related sequences (Jackson *et al.*, 2003; Scacheri *et al.*, 2004). More recently it has been appreciated that siRNAs can cause off-target cleavage of mRNAs with as few as seven nucleotide matches in the 3' UTR (Lin *et al.*, 2005). A bioinformatics and microarray analysis of off-target cleavage by 12 different siRNAs suggested that siRNAs can function as miRNAs during off-target silencing (Birmingham *et al.*, 2006). Complementarity between the nucleotides 2–7 or 2–8 (of either strand of the siRNA) and sequences in the 3' UTRs of mRNAs was associated with off-target cleavage. Nucleotides 2–8 correspond to the equivalent of the miRNA seed sequence in the siRNA. Multiple seed matches within the 3' UTR were even more highly correlated with off-target cleavage. Homology with the 5' UTR or coding region was not associated with off-target cleavage. Thus, when selecting siRNA sequences, special care should be taken to avoid guide or passenger strand sequences whose “seed sequence” has homology to the 3' UTR of mRNAs. It seems quite likely that this same paradigm applies to the siRNAs produced by Dicer cleavage of shRNAs. Clearly these results have implications for the selection of therapeutic siRNA or shRNA sequences. Dharmacon/Thermo Fisher Scientific (Lafayette, CO) provides a Web tool with which to search for seed matches in siRNA

sequences (see <http://www.dharmacon.com/seedlocator/default.aspx>). These results also underscore the importance of proper controls in siRNA screens. If a particular phenotype is indeed associated with the knockdown of a particular mRNA, then multiple distinct siRNAs targeting that mRNA should produce the same phenotype. A more rigorous approach is to demonstrate rescue with an “immunized” transgene that contains silent mutations in the siRNA-binding site, thus preventing RNAi silencing. Last, off-target effects are dose dependent (Birmingham *et al.*, 2007), and therefore the minimal dose required to obtain sufficient knockdown should be used.

Thus, because RNAi and miRNA use overlapping machinery, care must be taken when designing RNAi triggers. Maximal potency will reduce the dose required and reduce the possibility of competition with the endogenous miRNA machinery. Care must also be taken to avoid off-target effects due to RNAi triggers acting as miRNAs.

USING miRNAs TO AVOID IMMUNE RESPONSES TO VIRAL VECTORS

Naldini and coworkers described a clever use of miRNAs for gene therapy (Brown *et al.*, 2006). A significant hurdle to the application of gene replacement therapy is the development of transgene-specific immunity (Thomas *et al.*, 2003). For example, clinical trials for the treatment of hemophilia have been hampered by the development of immune responses against viral vectors and transgenes (High, 2005). A major cause of transgene-specific immunity is the expression of transgenes in professional antigen-presenting cells (APCs) (De Geest *et al.*, 2003). Tissue-specific promoters are not sufficient to prevent expression in APCs because they are leaky. In an elegant proof-of-principle article, Brown and coworkers (2006) exploited the fact that the expression of miR-142-3p is enriched in hematopoietic cells (Chen *et al.*, 2004; Baskerville and Bartel, 2005). They placed four sites with perfect complementarity to miR-142-3p in the 3' UTR of the lentivirus carrying the gene encoding green fluorescent protein (GFP), which under normal circumstances is a potent neoantigen (Stripecke *et al.*, 1999). When they injected mice with a control lentiviral vector lacking the miR-142-3p sites, they observed GFP expression in hepatocytes, endothelial cells, Kupffer cells (liver macrophages), and splenocytes on day 5. However, by day 14, little or no GFP expression was observed, presumably because of immune clearance. In contrast, when they injected mice with a lentivirus carrying the GFP gene and with four miR-142-3p-binding sites in the 3' UTR, they observed GFP expression in <1% of splenocytes, and then only in the marginal zone, which is not of hematopoietic lineage. Presumably because they avoided host immune responses, with this vector they observed long-term GFP expression (>120 days). Given the above-described report discussing miRNA sponges, one concern is that high-level expression of a transgene containing multiple binding sites for a particular miRNA could compete with endogenous miRNA targets for their cognate miRNA. Interestingly, when the authors performed the transfection with a second reporter containing four miR-142-3p-binding sites, they did not see an increase in GFP expression. This suggests that miR-142-3p was not limiting in this system. Although speculative, this may be because the complementarity of the miRNA to the target was perfect. Thus, one would predict that the miRNA would cleave the target and recycle rather than remain bound to it as is the case for miRNA sponges. In a separate report, Brown and coworkers applied the same strategy to achieve long-term expression of human clotting factor IX in hemophilia B mice (Brown *et al.*, 2007). It seems likely that this strategy could be applied to the long-term expression of other transgenes *in vivo*.

CONCLUDING REMARKS

There has been an explosive increase in our understanding of the role of miRNAs in normal gene regulation and in human disease. Gene therapists are in a unique position to rapidly apply approaches developed for antisense, ribozymes, and gene transfer to miRNAs. It seems likely

that miRNA expression levels can be used as novel diagnostic markers. Manipulation of miRNA levels could also have therapeutic benefit. Last, by exploiting our emerging understanding of miRNA biology, gene therapists may be able to design safer and more effective RNAi therapeutics as well as safer gene transfer vectors.

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REFERENCES

- ABELSON JF, KWAN KY, O'ROAK BJ, BAEK DY, STILL-MAN AA, MORGAN TM, MATHEWS CA, PAULS DL, RASIN MR, GUNEL M, DAVIS NR, ERCAN-SENCICEK AG, GUEZ DH, SPERTUS JA, LECKMAN JF, DURE LST, KURLAN R, SINGER HS, GILBERT DL, FARHI A, LOUVI A, LIFTON RP, SESTAN N, STATE MW. Sequence variants in *SLITRK1* are associated with Tourette's syndrome. *Science* 2005;310:317–320. [PubMed: 16224024]
- ANDERSSON MG, HAASNOOT PC, XU N, BERENJIAN S, BERKHOUT B, AKUSJARVI G. Suppression of RNA interference by adenovirus virus-associated RNA. *J. Virol* 2005;79:9556–9565. [PubMed: 16014917]
- BARTEL DP. MicroRNAs: Genomics, biogenesis, mechanism, and function. *Cell* 2004;116:281–297. [PubMed: 14744438]
- BASKERVILLE S, BARTEL DP. Microarray profiling of microRNAs reveals frequent coexpression with neighboring miRNAs and host genes. *RNA* 2005;11:241–247. [PubMed: 15701730]
- BIRMINGHAM A, ANDERSON EM, REYNOLDS A, ILSLEYTYREE D, LEAKE D, FEDOROV Y, BASKERVILLE S, MAKSIMOVA E, ROBINSON K, KARPILOW J, MARSHALL WS, KHVOROVA A. 3' UTR seed matches, but not overall identity, are associated with RNAi off-targets. *Nat. Methods* 2006;3:199–204. [PubMed: 16489337]
- BIRMINGHAM A, ANDERSON E, SULLIVAN K, REYNOLDS A, BOESE Q, LEAKE D, KARPILOW J, KHVOROVA A. A protocol for designing siRNAs with high functionality and specificity. *Nat. Protoc* 2007;2:2068–2078. [PubMed: 17853862]
- BODEN D, PUSCH O, SILBERMANN R, LEE F, TUCKER L, RAMRATNAM B. Enhanced gene silencing of HIV-1 specific siRNA using microRNA designed hairpins. *Nucleic Acids Res* 2004;32:1154–1158. [PubMed: 14966264]
- BOUTLA A, DELIDAKIS C, TABLER M. Developmental defects by antisense-mediated inactivation of micro-RNAs 2 and 13 in *Drosophila* and the identification of putative target genes. *Nucleic Acids Res* 2003;31:4973–4980. [PubMed: 12930946]
- BROWN BD, VENNERI MA, ZINGALE A, SERGI SERGI L, NALDINI L. Endogenous microRNA regulation suppresses transgene expression in hematopoietic lineages and enables stable gene transfer. *Nat. Med* 2006;12:585–591. [PubMed: 16633348]
- BROWN BD, CANTORE A, ANNONI A, SERGI SERGI L, LOMBARDO A, DELLA VALLE P, D'ANGELO A, NALDINI L. A microRNA-regulated lentiviral vector mediates stable correction of hemophilia B mice. *Blood* 2007;110:4144–4152. [PubMed: 17726165]
- BRUECKNER B, KUCK D, LYKO F. DNA methyltransferase inhibitors for cancer therapy. *Cancer J* 2007;13:17–22. [PubMed: 17464242]
- BURNSIDE J, BERNBERG E, ANDERSON A, LU C, MEYERS BC, GREEN PJ, JAIN N, ISAACS G, MORGAN RW. Marek's disease virus encodes microRNAs that map to *meq* and the latency-associated transcript. *J. Virol* 2006;80:8778–8786. [PubMed: 16912324]
- CAI X, LU S, ZHANG Z, GONZALEZ CM, DAMANIA B, CULLEN BR. Kaposi's sarcoma-associated herpesvirus expresses an array of viral microRNAs in latently infected cells. *Proc. Natl. Acad. Sci. U.S.A* 2005;102:5570–5575. [PubMed: 15800047]
- CAI X, SCHAFER A, LU S, BILELLO JP, DESROSIERS RC, EDWARDS R, RAAB-TRAUB N, CULLEN BR. Epstein-Barr virus microRNAs are evolutionarily conserved and differentially expressed. *PLoS Pathog* 2006;2:e23. [PubMed: 16557291]

- CALIN GA, CROCE CM. MicroRNA signatures in human cancers. *Nat. Rev. Cancer* 2006;6:857–866. [PubMed: 17060945]
- CALIN GA, DUMITRU CD, SHIMIZU M, BICHI R, ZUPO S, NOCH E, ALDLER H, RATTAN S, KEATING M, RAI K, RASSENTI L, KIPPS T, NEGRINI M, BULLRICH F, CROCE CM. 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;99:15524–15529. [PubMed: 12434020]
- CALIN GA, SEVIGNANI C, DUMITRU CD, HYSLOP T, NOCH E, YENDAMURI S, SHIMIZU M, RATTAN S, BULLRICH F, NEGRINI M, CROCE CM. 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. [PubMed: 14973191]
- CALIN GA, FERRACIN M, CIMMINO A, DI LEVA G, SHIMIZU M, WOJCIK SE, IORIO MV, VISIONE R, SEVER NI, FABBRI M, IULIANO R, PALUMBO T, PICHIORRI F, ROLDO C, GARZON R, SEVIGNANI C, RASSENTI L, ALDER H, VOLINIA S, LIU CG, KIPPS TJ, NEGRINI M, CROCE CM. A microRNA signature associated with prognosis and progression in chronic lymphocytic leukemia. *N. Engl. J. Med* 2005;353:1793–1801. [PubMed: 16251535]
- CASTANOTTO D, SAKURAI K, LINGEMAN R, LI H, SHIVELY L, AAGAARD L, SOIFER H, GATIGNOL A, RIGGS A, ROSSI JJ. Combinatorial delivery of small interfering RNAs reduces RNAi efficacy by selective incorporation into RISC. *Nucleic Acids Res* 2007;35:5154–5164. [PubMed: 17660190]
- CHAN JA, KRICHEVSKY AM, KOSIK KS. MicroRNA-21 is an antiapoptotic factor in human glioblastoma cells. *Cancer Res* 2005;65:6029–6033. [PubMed: 16024602]
- CHEN C, RIDZON DA, BROOMER AJ, ZHOU Z, LEE DH, NGUYEN JT, BARBISIN M, XU NL, MAHUVAKAR VR, ANDERSEN MR, LAO KQ, LIVAK KJ, GUEGLER KJ. Real-time quantification of microRNAs by stem-loop RT-PCR. *Nucleic Acids Res* 2005;33:e179. [PubMed: 16314309]
- CHEN CZ, LI L, LODISH HF, BARTEL DP. MicroRNAs modulate hematopoietic lineage differentiation. *Science* 2004;303:83–86. [PubMed: 14657504]
- CHENG AM, BYROM MW, SHELTON J, FORD LP. Antisense inhibition of human miRNAs and indications for an involvement of miRNA in cell growth and apoptosis. *Nucleic Acids Res* 2005;33:1290–1297. [PubMed: 15741182]
- CIMMINO A, CALIN GA, FABBRI M, IORIO MV, FERRACIN M, SHIMIZU M, WOJCIK SE, AQEILAN RI, ZUPO S, DONO M, RASSENTI L, ALDER H, VOLINIA S, LIU CG, KIPPS TJ, NEGRINI M, CROCE CM. miR-15 and miR-16 induce apoptosis by targeting BCL2. *Proc. Natl. Acad. Sci. U.S.A* 2005;102:13944–13949. [PubMed: 16166262]
- COSTINEAN S, ZANESI N, PEKARSKY Y, TILI E, VOLINIA S, HEEREMA N, CROCE CM. Pre-B cell proliferation and lymphoblastic leukemia/high-grade lymphoma in E μ -miR155 transgenic mice. *Proc. Natl. Acad. Sci. U.S.A* 2006;103:7024–7029. [PubMed: 16641092]
- CUIC, GRIFFITHS A, LI G, SILVA LM, KRAMER MF, GAASTERLAND T, WANG XJ, COEN DM. Prediction and identification of herpes simplex virus 1-encoded microRNAs. *J. Virol* 2006;80:5499–5508. [PubMed: 16699030]
- DAVIS S, LOLLO B, FREIER S, ESAU C. Improved targeting of miRNA with antisense oligonucleotides. *Nucleic Acids Res* 2006;34:2294–2304. [PubMed: 16690972]
- DE GEEST BR, VAN LINTHOUT SA, COLLEN D. Humoral immune response in mice against a circulating antigen induced by adenoviral transfer is strictly dependent on expression in antigen-presenting cells. *Blood* 2003;101:2551–2556. [PubMed: 12446451]
- DONSANTE A, MILLER DG, LI Y, VOGLER C, BRUNT EM, RUSSELL DW, SANDS MS. AAV vector integration sites in mouse hepatocellular carcinoma. *Science* 2007;317:477. [PubMed: 17656716]
- DYKXHOORN DM. MicroRNAs in viral replication and pathogenesis. *DNA Cell Biol* 2007;26:239–249. [PubMed: 17465890]
- EBERT MS, NEILSON JR, SHARP PA. MicroRNA sponges: Competitive inhibitors of small RNAs in mammalian cells. *Nat. Methods* 2007;4:721–726. [PubMed: 17694064]

- EIS PS, TAM W, SUN L, CHADBURN A, LI Z, GOMEZ MF, LUND E, DAHLBERG JE. Accumulation of miR-155 and BIC RNA in human B cell lymphomas. *Proc. Natl. Acad. Sci. U.S.A* 2005;102:3627–3632. [PubMed: 15738415]
- ESAU C, KANG X, PERALTA E, HANSON E, MARCUSSEON EG, RAVICHANDRAN LV, SUN Y, KOO S, PERERA RJ, JAIN R, DEAN NM, FREIER SM, BENNETT CF, LOLLO B, GRIFFEY R. MicroRNA-143 regulates adipocyte differentiation. *J. Biol. Chem* 2004;279:52361–52365. [PubMed: 15504739]
- ESAU C, DAVIS S, MURRAY SF, YU XX, PANDEY SK, PEAR M, WATTS L, BOOTEN SL, GRAHAM M, MCKAY R, SUBRAMANIAM A, PROPP S, LOLLO BA, FREIER S, BENNETT CF, BHANOT S, MONIA BP. miR-122 regulation of lipid metabolism revealed by *in vivo* antisense targeting. *Cell Metab* 2006;3:87–98. [PubMed: 16459310]
- ESQUELA-KERSCHER A, SLACK FJ. OncomiRs: MicroRNAs with a role in cancer. *Nat. Rev. Cancer* 2006;6:259–269. [PubMed: 16557279]
- FABBRI M, GARZON R, CIMMINO A, LIU Z, ZANESI N, CALLEGARI E, LIU S, ALDER H, COSTINEAN S, FERNANDEZ-CYMERING C, VOLINIA S, GULER G, MORRISON CD, CHAN KK, MARCUCCI G, CALIN GA, HUEBNER K, CROCE CM. MicroRNA-29 family reverts aberrant methylation in lung cancer by targeting DNA methyltransferases 3A and 3B. *Proc. Natl. Acad. Sci. U.S.A* 2007;104:15805–15810. [PubMed: 17890317]
- FARH KK, GRIMSON A, JAN C, LEWIS BP, JOHNSTON WK, LIM LP, BURGE CB, BARTEL DP. The widespread impact of mammalian microRNAs on mRNA repression and evolution. *Science* 2005;310:1817–1821. [PubMed: 16308420]
- FRANCO-ZORRILLA JM, VALLI A, TODESCO M, MATEOS I, PUGA MI, RUBIO-SOMOZA I, LEYVA A, WEIGEL D, GARCIA JA, PAZ-ARES J. Target mimicry provides a new mechanism for regulation of microRNA activity. *Nat. Genet* 2007;39:1033–1037. [PubMed: 17643101]
- FULCI V, CHIARETTIS, GOLDONI M, AZZALIN G, CARUCCIN, TAVOLARO S, CASTELLANO L, MAGRELLI A, CITARELLA F, MESSINA M, MAGGIO R, PERAGINE N, SANTANGELO S, MAURO FR, LANDGRAF P, TUSCHL T, WEIR DB, CHIEN M, RUSSO JJ, JU J, SHERIDAN R, SANDER C, ZAVOLAN M, GUARINI A, FOA R, MACINO G. Quantitative technologies establish a novel microRNA profile of chronic lymphocytic leukemia. *Blood* 2007;109:4944–4951. [PubMed: 17327404]
- FURNARI FB, ADAMS MD, PAGANO JS. Unconventional processing of the 3' termini of the Epstein-Barr virus DNA polymerase mRNA. *Proc. Natl. Acad. Sci. U.S.A* 1993;90:378–382. [PubMed: 8093641]
- GRIMM D, STREETZ KL, JOPLING CL, STORM TA, PANDEY K, DAVIS CR, MARION P, SALAZAR F, KAY MA. Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. *Nature* 2006;441:537–541. [PubMed: 16724069]
- GUPTA A, GARTNER JJ, SETHUPATHY P, HATZIGEORGIOU AG, FRASER NW. Anti-apoptotic function of a microRNA encoded by the HSV-1 latency-associated transcript. *Nature* 2006;442:82–85. [PubMed: 16738545]
- HARFE BD. MicroRNAs in vertebrate development. *Curr. Opin. Genet. Dev* 2005;15:410–415. [PubMed: 15979303]
- HE L, THOMSON JM, HEMANN MT, HERNANDO-MONGE E, MU D, GOODSON S, POWERS S, CORDON-CARDO C, LOWE SW, HANNON GJ, HAMMOND SM. A microRNA polycistron as a potential human oncogene. *Nature* 2005;435:828–833. [PubMed: 15944707]
- HIGH K. Gene transfer for hemophilia: Can therapeutic efficacy in large animals be safely translated to patients? *J. Thromb. Haemost* 2005;3:1682–1691. [PubMed: 16102034]
- HUTVAGNER G, SIMARD MJ, MELLO CC, ZAMORE PD. Sequence-specific inhibition of small RNA function. *PLoS Biol* 2004;2:E98. [PubMed: 15024405]
- IORIO MV, FERRACIN M, LIU CG, VERONESE A, SPIZZO R, SABBIONI S, MAGRI E, PEDRIALI M, FABBRI M, CAMPIGLIO M, MENARD S, PALAZZO JP, ROSENBERG A, MUSIANI P, VOLINIA S, NENCI I, CALIN GA, QUERZOLI P, NEGRINI M, CROCE CM. MicroRNA gene expression deregulation in human breast cancer. *Cancer Res* 2005;65:7065–7070. [PubMed: 16103053]

- IORIO MV, VISIONE R, DI LEVA G, DONATI V, PETROCCA F, CASALINI P, TACCIOLI C, VOLINIA S, LIU CG, ALDER H, CALIN GA, MENARD S, CROCE CM. MicroRNA signatures in human ovarian cancer. *Cancer Res* 2007;67:8699–8707. [PubMed: 17875710]
- JACKSON AL, BARTZ SR, SCHELTER J, KOBAYASHI SV, BURCHARD J, MAO M, LI B, CAVET G, LINSLEY PS. Expression profiling reveals off-target gene regulation by RNAi. *Nat. Biotechnol* 2003;21:635–637. [PubMed: 12754523]
- JIANG J, LEE EJ, GUSEV Y, SCHMITTGEN TD. Real-time expression profiling of microRNA precursors in human cancer cell lines. *Nucleic Acids Res* 2005;33:5394–5403. [PubMed: 16192569]
- JOHN M, CONSTIEN R, AKINC A, GOLDBERG M, MOON YA, SPRANGER M, HADWIGER P, SOUTSCHEK J, VORNLOCHER HP, MANOHARAN M, STOFFEL M, LANGER R, ANDERSON DG, HORTON JD, KOTELIANSKY V, BUMCROT D. Effective RNAi-mediated gene silencing without interruption of the endogenous microRNA pathway. *Nature* 2007;449:745–747. [PubMed: 17898712]
- JOHNSON CD, ESQUELA-KERSCHER A, STEFANI G, BYROM M, KELNAR K, OVCHARENKO D, WILSON M, WANG X, SHELTON J, SHINGARA J, CHIN L, BROWN D, SLACK FJ. The let-7 microRNA represses cell proliferation pathways in human cells. *Cancer Res* 2007;67:7713–7722. [PubMed: 17699775]
- JOHNSON SM, GROSSHANS H, SHINGARA J, BYROM M, JARVIS R, CHENG A, LABOURIER E, REINERT KL, BROWN D, SLACK FJ. RAS is regulated by the let-7 microRNA family. *Cell* 2005;120:635–647. [PubMed: 15766527]
- JOPLING CL, YI M, LANCASTER AM, LEMON SM, SARNOW P. Modulation of hepatitis C virus RNA abundance by a liver-specific microRNA. *Science* 2005;309:1577–1581. www.sciencemag.org [PubMed: 16141076]
- KAWASAKI H, TAIRA K. Short hairpin type of dsRNAs that are controlled by tRNA^{Val} promoter significantly induce RNAi-mediated gene silencing in the cytoplasm of human cells. *Nucleic Acids Res* 2003;31:700–707. [PubMed: 12527779]
- KLOOSTERMAN WP, PLASTERK RH. The diverse functions of microRNAs in animal development and disease. *Dev. Cell* 2006;11:441–450. [PubMed: 17011485]
- KLUIVER J, POPPEMA S, DE JONG D, BLOKZIJL T, HARMS G, JACOBS S, KROESEN BJ, VAN DEN BERG A. BIC and miR-155 are highly expressed in Hodgkin, primary mediastinal and diffuse large B cell lymphomas. *J. Pathol* 2005;207:243–249. [PubMed: 16041695]
- KRUTZFELDT J, STOFFEL M. MicroRNAs: A new class of regulatory genes affecting metabolism. *Cell Metab* 2006;4:9–12. [PubMed: 16814728]
- KRUTZFELDT J, RAJEWSKY N, BRAICH R, RAJEEV KG, TUSCHL T, MANOHARAN M, STOFFEL M. Silencing of microRNAs *in vivo* with “antagomiRs.”. *Nature* 2005;438:685–689. [PubMed: 16258535]
- KRUTZFELDT J, KUWAJIMA S, BRAICH R, RAJEEV KG, PENA J, TUSCHL T, MANOHARAN M, STOFFEL M. Specificity, duplex degradation and subcellular localization of antagomiRs. *Nucleic Acids Res* 2007;35:2885–2892. [PubMed: 17439965]
- LAGOS-QUINTANA M, RAUHUT R, LENDECKEL W, TUSCHL T. Identification of novel genes coding for small expressed RNAs. *Science* 2001;294:853–858. [PubMed: 11679670]
- LEAMAN D, CHEN PY, FAK J, YALCIN A, PEARCE M, UNNERSTALL U, MARKS DS, SANDER C, TUSCHL T, GAUL U. Antisense-mediated depletion reveals essential and specific functions of microRNAs in *Drosophila* development. *Cell* 2005;121:1097–1108. [PubMed: 15989958]
- LECELLIER CH, DUNOYER P, ARAR K, LEHMANN-CHE J, EYQUEM S, HIMBER C, SAIB A, VOINNET O. A cellular microRNA mediates antiviral defense in human cells. *Science* 2005;308:557–560. [PubMed: 15845854]
- LEE EJ, GUSEV Y, JIANG J, NUOVO GJ, LERNER MR, FRANKEL WL, MORGAN DL, POSTIER RG, BRACKETT DJ, SCHMITTGEN TD. Expression profiling identifies microRNA signature in pancreatic cancer. *Int. J. Cancer* 2007;120:1046–1054. [PubMed: 17149698]
- LEE YS, KIM HK, CHUNG S, KIM KS, DUTTA A. Depletion of human micro-RNA 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;280:16635–16641. [PubMed: 15722555]

- LI Z, ZHAN W, WANG Z, ZHU B, HE Y, PENG J, CAI S, MA J. Inhibition of PRL-3 gene expression in gastric cancer cell line SGC7901 via microRNA suppressed reduces peritoneal metastasis. *Biochem. Biophys. Res. Commun* 2006;348:229–237. [PubMed: 16875667]
- LIANG Z, WU T, LOU H, YU X, TAICHMAN RS, LAU SK, NIE S, UMBREIT J, SHIM H. Inhibition of breast cancer metastasis by selective synthetic polypeptide against CXCR4. *Cancer Res* 2004;64:4302–4308. [PubMed: 15205345]
- LIANG Z, YOON Y, VOTAW J, GOODMAN MM, WILLIAMS L, SHIM H. Silencing of CXCR4 blocks breast cancer metastasis. *Cancer Res* 2005;65:967–971. [PubMed: 15705897]
- LIANG Z, WU H, REDDY S, ZHU A, WANG S, BLEVINS D, YOON Y, ZHANG Y, SHIM H. Blockade of invasion and metastasis of breast cancer cells via targeting CXCR4 with an artificial microRNA. *Biochem. Biophys. Res. Commun* 2007;363:542–546. [PubMed: 17889832]
- LIM LP, LAU NC, GARRETT-ENGELE P, GRIMSON A, SCHELTER JM, CASTLE J, BARTEL DP, LINSLEY PS, JOHNSON JM. Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. *Nature* 2005;433:769–773. [PubMed: 15685193]
- LIN X, RUAN X, ANDERSON MG, MCDOWELL JA, KROEGER PE, FESIK SW, SHEN Y. siRNA-mediated off-target gene silencing triggered by a 7 nt complementation. *Nucleic Acids Res* 2005;33:4527–4535. [PubMed: 16091630]
- LIU CG, CALIN GA, MELOON B, GAMLIEL N, SEVIGNANI C, FERRACIN M, DUMITRU CD, SHIMIZU M, ZUPO S, DONO M, ALDER H, BULLRICH F, NEGRINI M, CROCE CM. An oligonucleotide microchip for genome-wide microRNA profiling in human and mouse tissues. *Proc. Natl. Acad. Sci. U.S.A* 2004;101:9740–9744. [PubMed: 15210942]
- LU J, GETZ G, MISKA EA, ALVAREZ-SAAVEDRA E, LAMB J, PECK D, SWEET-CORDERO A, EBERT BL, MAK RH, FERRANDO AA, DOWNING JR, JACKS T, HORVITZ HR, GOLUB TR. MicroRNA expression profiles classify human cancers. *Nature* 2005;435:834–838. [PubMed: 15944708]
- LU S, CULLEN BR. Adenovirus VA1 noncoding RNA can inhibit small interfering RNA and microRNA biogenesis. *J. Virol* 2004;78:12868–12876. [PubMed: 15542639]
- LUKIW WJ. Micro-RNA speciation in fetal, adult and Alzheimer's disease hippocampus. *Neuroreport* 2007;18:297–300. [PubMed: 17314675]
- MCMANUS MT, PETERSEN CP, HAINES BB, CHEN J, SHARP PA. Gene silencing using microRNA designed hairpins. *RNA* 2002;8:842–850. [PubMed: 12088155]
- MEISTER G, LANDTHALER M, DORSETT Y, TUSCHL T. Sequence-specific inhibition of microRNA- and siRNA-induced RNA silencing. *RNA* 2004;10:544–550. [PubMed: 14970398]
- MENG F, HENSON R, LANG M, WEHBE H, MAHESHWARI S, MENDELL JT, JIANG J, SCHMITTGEN TD, PATEL T. Involvement of human micro-RNA in growth and response to chemotherapy in human cholangiocarcinoma cell lines. *Gastroenterology* 2006;130:2113–2129. [PubMed: 16762633]
- MENG F, HENSON R, WEHBE-JANEK H, GHOSHAL K, JACOB ST, PATEL T. MicroRNA-21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer. *Gastroenterology* 2007;133:647–658. [PubMed: 17681183]
- 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;39:167–169. [PubMed: 14695998]
- MURAKAMI Y, YASUDA T, SAIGO K, URASHIMA T, TOYODA H, OKANOUE T, SHIMOTOHNO K. Comprehensive analysis of microRNA expression patterns in hepatocellular carcinoma and non-tumorous tissues. *Oncogene* 2005;25:2537–2545. [PubMed: 16331254]
- OMOTO S, FUJII YR. Regulation of human immunodeficiency virus 1 transcription by *nef* microRNA. *J. Gen. Virol* 2005;86:751–755. [PubMed: 15722536]
- OROM UA, KAUPPINEN S, LUND AH. LNA-modified oligonucleotides mediate specific inhibition of microRNA function. *Gene* 2006;372:137–141. [PubMed: 16503100]
- PERKINS DO, JEFFRIES CD, JARSKOG LF, THOMSON JM, WOODS K, NEWMAN MA, PARKER JS, JIN J, HAMMOND SM. MicroRNA expression in the prefrontal cortex of individuals with schizophrenia and schizoaffective disorder. *Genome Biol* 2007;8:R27. [PubMed: 17326821]

- PETERS L, MEISTER G. Argonaute proteins: Mediators of RNA silencing. *Mol. Cell* 2007;26:611–623. [PubMed: 17560368]
- PFEFFER S, ZAVOLAN M, GRASSER FA, CHIEN M, RUSSO JJ, JU J, JOHN B, ENRIGHT AJ, MARKS D, SANDER C, TUSCHL T. Identification of virus-encoded micro-RNAs. *Science* 2004;304:734–736. [PubMed: 15118162]
- PFEFFER S, SEWER A, LAGOS-QUINTANA M, SHERIDAN R, SANDER C, GRASSER FA, VAN DYK LF, HO CK, SHUMAN S, CHIEN M, RUSSO JJ, JU J, RANDALL G, LINDENBACH BD, RICE CM, SIMON V, HO DD, ZAVOLAN M, TUSCHL T. Identification of microRNAs of the herpesvirus family. *Nat. Methods* 2005;2:269–276. [PubMed: 15782219]
- POY MN, ELIASSEN L, KRUTZFELDT J, KUWAJIMA S, MA X, MACDONALD PE, PFEFFER S, TUSCHL T, RAJEWSKY N, RORSMAN P, STOFFEL M. A pancreatic islet-specific microRNA regulates insulin secretion. *Nature* 2004;432:226–230. [PubMed: 15538371]
- RAYMOND CK, ROBERTS BS, GARRETT-ENGELE P, LIM LP, JOHNSON JM. Simple, quantitative primer-extension PCR assay for direct monitoring of microRNAs and short-interfering RNAs. *RNA* 2005;11:1737–1744. [PubMed: 16244135]
- SAMOLS MA, HU J, SKALSKY RL, RENNE R. Cloning and identification of a microRNA cluster within the latency-associated region of Kaposi's sarcoma-associated herpesvirus. *J. Virol* 2005;79:9301–9305. [PubMed: 15994824]
- SCACHERI PC, ROZENBLATT-ROSEN O, CAPLEN NJ, WOLFSBERG TG, UYAM L, LEE JC, HUGHES CM, SHANMUGAM KS, BHATTACHARJEE A, MEYERSON M, COLLINS FS. Short interfering RNAs can induce unexpected and divergent changes in the levels of untargeted proteins in mammalian cells. *Proc. Natl. Acad. Sci. U.S.A* 2004;101:1892–1897. [PubMed: 14769924]
- SCHAFER A, CAI X, BILELLO JP, DESROSIER RC, CULLEN BR. Cloning and analysis of microRNAs encoded by the primate gamma-herpesvirus rhesus monkey rhadinovirus. *Virology* 2007;364:21–27. [PubMed: 17451774]
- SCHRATT GM, TUEBING F, NIGH EA, KANE CG, SABATINI ME, KIEBLER M, GREENBERG ME. A brain-specific microRNA regulates dendritic spine development. *Nature* 2006;439:283–289. [PubMed: 16421561]
- SIML, ZHU S, WU H, LU Z, WU F, MO YY. miR-21-mediated tumor growth. *Oncogene* 2007;26:2799–2803. [PubMed: 17072344]
- STARK A, BRENNECKE J, BUSHATI N, RUSSELL RB, COHEN SM. Animal microRNAs confer robustness to gene expression and have a significant impact on 3' UTR evolution. *Cell* 2005;123:1133–1146. [PubMed: 16337999]
- STRIECKE R, CARMEN VILLACRES M, SKELTON D, SATAKE N, HALENE S, KOHN D. Immune response to green fluorescent protein: Implications for gene therapy. *Gene Ther* 1999;6:1305–1312. [PubMed: 10455440]
- SULLIVAN CS, GANEM D. MicroRNAs and viral infection. *Mol. Cell* 2005;20:3–7. [PubMed: 16209940]
- SULLIVAN CS, GRUNDHOFF AT, TEVETHIA S, PIPAS JM, GANEM D. SV40-encoded microRNAs regulate viral gene expression and reduce susceptibility to cytotoxic T cells. *Nature* 2005;435:682–686. [PubMed: 15931223]
- SULLIVAN CS, GRUNDHOFF A, TEVETHIA S, TREISMAN R, PIPAS JM, GANEM D. Expression and function of microRNAs in viruses great and small. *Cold Spring Harb. Symp. Quant. Biol* 2006;71:351–356. [PubMed: 17381316]
- TAKAMIZAWA J, KONISHI H, YANAGISAWA K, TOMIDA S, OSADA H, ENDOH H, HARANO T, YATABE Y, NAGINO M, NIMURA Y, MITSUDOMI T, TAKAHASHI T. Reduced expression of the *let-7* microRNAs in human lung cancers in association with shortened postoperative survival. *Cancer Res* 2004;64:3753–3756. [PubMed: 15172979]
- TAZAWA H, TSUCHIYA N, IZUMIYA M, NAKAGAMA H. Tumor-suppressive miR-34a induces senescence-like growth arrest through modulation of the E2F pathway in human colon cancer cells. *Proc. Natl. Acad. Sci. U.S.A* 2007;104:15472–15477. [PubMed: 17875987]
- THOMAS CE, EHRHARDT A, KAY MA. Progress and problems with the use of viral vectors for gene therapy. *Nat. Rev. Genet* 2003;4:346–358. [PubMed: 12728277]

- VAN ROOIJ E, SUTHERLAND LB, LIU N, WILLIAMS AH, MCANALLY J, GERARD RD, RICHARDSON JA, OLSON EN. A signature pattern of stress-responsive micro-RNAs that can evoke cardiac hypertrophy and heart failure. *Proc. Natl. Acad. Sci. U.S.A* 2006;103:18255–18260. [PubMed: 17108080]
- WEILER J, HUNZIKER J, HALL J. Anti-miRNA oligonucleotides (AMOs): Ammunition to target miRNAs implicated in human disease? *Gene Ther* 2006;13:496–502. [PubMed: 16195701]
- YAO Y, ZHAO Y, XU H, SMITH LP, LAWRIE CH, SEWER A, ZAVOLAN M, NAIR V. Marek's disease virus type 2 (MDV-2)-encoded microRNAs show no sequence conservation with those encoded by MDV-1. *J. Virol* 2007;81:7164–7170. [PubMed: 17459919]
- ZENG Y, WAGNER EJ, CULLEN BR. Both natural and designed micro RNAs can inhibit the expression of cognate mRNAs when expressed in human cells. *Mol. Cell* 2002;9:1327–1333. [PubMed: 12086629]
- ZHOU H, XIA XG, XU Z. An RNA polymerase II construct synthesizes short-hairpin RNA with a quantitative indicator and mediates highly efficient RNAi. *Nucleic Acids Res* 2005;33:e62. [PubMed: 15805121]
- ZHU S, SI ML, WU H, MO YY. MicroRNA-21 targets the tumor suppressor gene tropomyosin 1 (*TPM1*). *J. Biol. Chem* 2007;282:14328–14336. [PubMed: 17363372]

FIG. 1.

The miRNA/RNAi pathway. miRNAs are transcribed from Pol II or Pol III promoters as long transcripts. These pri-miRNAs are processed by Drosha/DGCR8 to form pre-miRNAs. Pre-miRNAs are exported to the cytoplasm by Exportin 5, where they are further processed into a short duplex by Dicer/TRBP. One strand of the duplex (the guide strand) is incorporated into RISC and the other strand is destroyed. RISC mediates gene silencing by inhibition of translation, decapping, deadenylation, or direct target cleavage. shRNAs or siRNAs enter the miRNA/RNAi pathway and mediate target cleavage. Ago, Argonaute protein; DGCR8, DiGeorge critical region-8; miRNA, microRNA; Pol, RNA polymerase; pri-miRNA, primary miRNA transcript; RISC, RNA-induced silencing complex; shRNA, short hairpin RNA; siRNA, short interfering RNAs; TRBP, HIV-1 TAR RNA-binding protein.

FIG. 2.

The miRNA seed sequence determines specificity. The seed sequence (nucleotides 2–8 of the miRNA, boxed and in boldface) is the primary determinant of miRNA specificity. The predicted base pairing of the hepatitis C virus 5' UTR with miR-122 is shown. [Adapted from Jopling *et al.*, *Science* 309:1577 (9/2/2005). Reprinted with permission from AAAS.]