microRNAs: Master Regulators as Potential Therapeutics in Cancer

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Abstract

It has been demonstrated that all the known processes involved in cancer, including apoptosis, proliferation, survival, and metastasis, are regulated by small regulatory noncoding RNAs consisting of approximately 19–25 nucleotides; these are named microRNAs (miRNAs). Both loss and gain of miRNA function contribute to cancer development through the upregulation and silencing, respectively, of different target genes. Experimental evidence indicates that the use of miRNA mimics or anti-microRNAs may represent a powerful therapeutic strategy to interfere with key molecular pathways involved in cancer. This review provides insights about how microRNAs act as oncogenes and tumor suppressor genes and how these findings, along with our increasing understanding of miRNA regulation, can be applied to optimize recent miRNA-based technologies and make them suitable for clinical applications.

INTRODUCTION

miRNA: microRNA

mRNA: messenger RNA

UTR: untranslated region

Pol II: RNA polymerase II

Pol III: RNA polymerase III

RIIID: RNase III-like domain

dsRBD: double-stranded RNA-binding domain

Traditionally, the study of cancer has focused on protein-coding genes, considered the principal effectors and regulators of tumorigenesis. Recent discoveries, however, have highlighted the role of non-protein-coding RNA in tumor formation. The story began in 1993 when Victor Ambros and colleagues (1) discovered a gene, *lin-4*, that affected development in *Caenorhabditis elegans* and found that its product was a small noncoding RNA; this small RNA was later to be termed a microRNA (miRNA). The number of known small RNAs in different organisms such as *Caenorhabditis elegans*, *Drosophila melanogaster*, plants, and mammals—including humans—has since expanded substantially, mainly as a result of the cloning and sequencing of size-fractionated RNAs. The human genome is predicted to encode as many as 1,000 miRNAs. miRNAs are single-stranded RNAs (ssRNAs) ~19–25 nucleotides in length that are generated from endogenous hairpin transcripts (2). They play an important role in the negative regulation of gene expression by base-pairing to partially complementary sites on the target messenger RNAs (mRNAs), usually in the 3′ untranslated region (UTR). Binding of a miRNA to the target mRNA typically leads to translational repression and exonucleolytic mRNA decay, although highly complementary targets can be cleaved endonucleolytically. miRNAs have been found to regulate more than 30% of mRNAs and have roles in fundamental processes, such as development, differentiation, cell proliferation, apoptosis, and stress responses. Over the past few years, many miRNAs have been implicated in various human cancers. Both loss and gain of miRNA function contribute to cancer development through a range of different mechanisms. Because miRNAs regulate cancer cell differentiation, proliferation, survival, and metastasis, manipulating miRNA function, either by mimicking or inhibiting miRNAs implicated in cancer, could provide a powerful therapeutic strategy to interfere with cancer initiation and progression. In this review, we summarize the new discoveries about miRNAs—their functions as oncogenes, tumor suppressor genes, and drugs—and about their potential use in the treatment of cancer.

microRNA LOCALIZATION AND BIOGENESIS

A genomic analysis of miRNAs has revealed that more than 50% of mammalian miRNAs are located within the intronic regions of annotated protein-coding or non-protein-coding genes (3). These miRNAs could therefore use their host gene transcripts as carriers, although it remains possible that some are actually transcribed separately from internal promoters. Other miRNAs, located in intergenic regions, apparently have their own transcriptional regulatory elements and thus constitute independent transcription units. Animal miRNAs are identified as part of an 80-nucleotide RNA with a stem-loop structure; this RNA is known as a pre-miRNA. These animal miRNAs are included in primary miRNA precursors (pri-miRNAs) that are several hundreds or thousands of nucleotides long. The current model for maturation of the mammalian miRNAs is shown in **Figure 1**. The first step involves the transcription of the pri-miRNA mediated by RNA polymerase II (Pol II) (4), although a minor group of miRNAs can be transcribed by RNA polymerase III (Pol III) (5). The pri-miRNA is then processed to produce a second precursor (pre-miRNA) of 60–100 nucleotides in length by the nuclear protein Drosha, a large protein of ~160 kDa (6) that belongs to class II of type RNase III. The Drosha protein possesses tandem RNase III-like domains (RIIIDs) and a double-stranded RNA-binding domain (dsRBD), in addition to an extended N terminus that contains a proline-rich region and a serine/arginine-rich region of unknown function (**Figure 1**). It has been proposed that Drosha may recognize the pri-miRNA through the stem-loop structure and then cleave the stem at a fixed distance from the loop to liberate the pre-miRNA. How is the Drosha enzyme able to discriminate the pri-miRNA

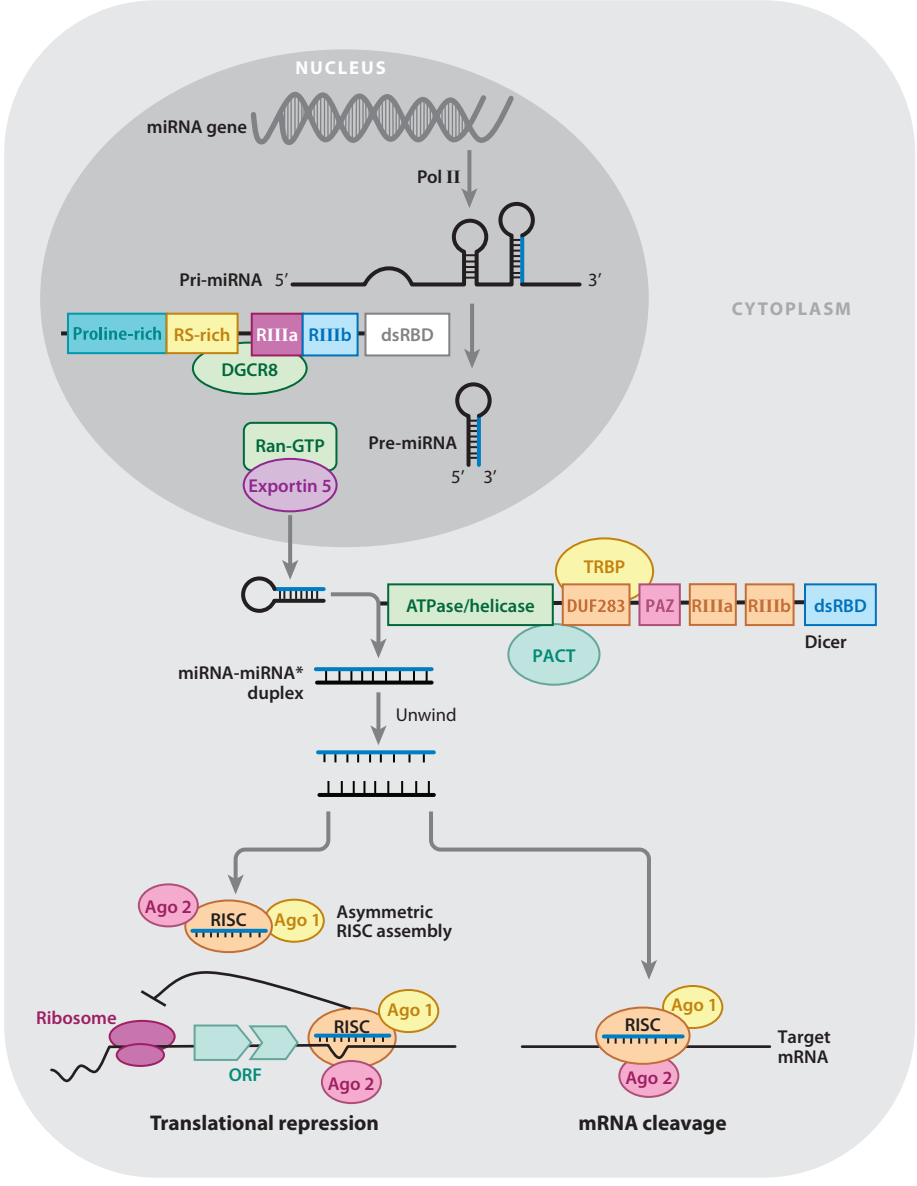
stem-loop structure from the other stem-loop cellular RNAs? Both cell culture experiments and in vitro Drosha cleavage assays have shown that proteins associated with Drosha confer specificity to this process. In fact, Drosha has been found to be part of a large, ~650-kDa protein complex known as the Microprocessor (7), where Drosha interacts with its cofactor DGCR8 (the DiGeorge syndrome critical region gene 8 protein) in the human and interacts with Pasha in *Drosophila melanogaster* (8). Drosha functions as the catalytic subunit, whereas DGCR8 recognizes the RNA substrate. After the Microprocessor nuclear activity, the produced pre-miRNA is exported to the cytoplasm by Exportin 5 (Exp5) and its Ran–guanosine triphosphate (Ran-GTP) cofactor (9). The Exp5/Ran-GTP complex has a high affinity for pre-miRNAs, protecting them from the moment they are generated in the nucleus until they are ready for the next cleavage step in the cytoplasm, where GTP is hydrolyzed to guanosine diphosphate (GDP); at that point, the Exp5/Ran-GDP complex releases its cargo. In the cytoplasm, the pre-miRNA is cleaved by another RNase III–type class III enzyme, Dicer, which is a 200-kDa multidomain protein characterized by different domains including an RNA helicase/ATPase domain, the DUF283 and PAZ signatures, two neighboring RIIIDs (RIIIa and RIIIb), and a dsRBD (**Figure 1**). The dsRBD and RIIIDs are most certainly involved in the binding and cleavage of double-stranded RNA. Because a pre-miRNA generated by Drosha already contains a 2-nucleotide, 3′ overhang, Dicer would recognize the 3′ overhang via its PAZ domain and cleave the double-stranded region approximately 20 nucleotides away, and its two RIIIDs would form a single catalytic center intramolecularly (10). The product is a miRNA duplex containing ~2-nucleotide, 3′ overhangs at both ends. Just like DGCR8 in the case of Drosha, proteins with double-stranded RNA-binding domains, such as transactivating response RNA-binding protein (TRBP) and protein activator of protein kinase R (PACT) in humans, bind to Dicer and contribute to Dicer function. TRBP and PACT are not essential for Dicer-mediated cleavage of the pre-miRNA, but they facilitate it, and TRBP stabilizes Dicer (11, 12). These proteins participate in the selection of mature miRNA strands and/or the transfer of miRNAs to their final stop, the RNA-induced silencing complex (RISC) or miRgonaute, which mediates the degradation or translation inhibition of mRNA's target gene.

DGCR8: the DiGeorge syndrome critical region gene 8 protein

RISC: RNA-induced silencing complex

At the core of RISCs are Ago family proteins (13). Of the four mammalian Ago-subfamily proteins (Ago1–4), only Ago2 possesses target cleavage (slicer) activity (14). In flies, both Ago1 and Ago2 have slicer activity; however, Ago1 is a much weaker endonuclease than Ago2 (15). The miRNA-miRNA* duplex needs to be unwound to act as a single-stranded guide in the RISC to recognize its target mRNAs. It was originally proposed that an ATP-dependent helicase (known as unwindase) separates the two small RNA strands, after which the resulting single-stranded guide is loaded into Ago proteins. However, it was later shown that *Drosophila* Ago2 (16) as well as human Ago2 (17) directly receive double-stranded small RNA from the RISC-loading complex. Ago2 then cleaves the passenger strand, thereby liberating the single-stranded guide to form mature Ago2-RISC. Thus it remains unknown when (i.e., before or after RISC loading) and, more importantly, how the two strands are separated in such slicer-independent RISC assembly pathways. Kawamata et al. (18) showed that miRNA-miRNA* duplexes are loaded into Ago1 as double-stranded RNAs in an ATP-dependent fashion. In contrast, unwinding requires neither ATP nor the slicer activity of Ago1 (18). In mammals, miRNAs guide the RISC to complementary target sites in mRNAs, where endonucleolytically active Ago proteins cleave the RNA (19) (**Figure 1**). Other miRNAs, on the other hand, predominantly bind to partially complementary target sites located in the 3′ UTRs of their specific target mRNAs. Imperfect base pairing between small RNAs and their target mRNAs leads to repression of translation and/or deadenylation (removal of the polyA tail of the target), followed by destabilization of the target (20). The mechanism by which Ago proteins mediate translational repression is still a matter of debate. Ago proteins have been shown to act on translation initiation (summarized in Reference 21), on

elongation (22), and on the degradation of nascent polypeptides (23), depending on the target being regulated. Several groups recently reported that, under specific conditions, miRNAs can do just the opposite—i.e., perform translational activation of their target mRNAs. Vasudevan and colleagues (24–26) surprisingly found that human Ago2 activates translation of target mRNAs on cell cycle arrest caused by serum starvation or contact inhibition (24, 25), whereas it normally represses translation of the same target mRNAs in proliferating cells (26). These new findings add another layer of complexity to the miRNA field. Undoubtedly, further studies are needed to understand molecular mechanisms for miRNA-mediated translational repression or activation and mechanisms for switching between the two processes.



microRNAs IMPLICATED IN CANCER

A great deal of data already establishes an important role for miRNAs among the many regulatory factors involved in the pathogenesis of cancer. The discovery of the loss of *miR-15a/16-1* at chromosome 13q14 in chronic lymphocytic leukemia (27) has prompted several groups over the past few years to study the miRNA expression profile in cancer patients. Investigators found that miRNAs are differentially expressed not only in normal and tumor tissues (28) but also in primary tumors and metastatic tissues (29). These differences are tumor specific and in some cases are associated with prognosis.

BIC: B-cell integration cluster

CLL: chronic lymphocytic leukemia

AML: acute myelogenous leukemia

microRNAs as Oncogenes

Many miRNAs have been shown to function as oncogenes in the majority of cancers profiled to date (**Table 1**). *miR-155* was one of the first described (30). This miRNA is encoded by nucleotides 241–262 of *BIC* (B-cell integration cluster), which spans 1421 base pairs in total and is on chromosome 21. Several groups have shown that *miR-155* is highly expressed in pediatric Burkitt's lymphoma (30), Hodgkin's disease (31), primary mediastinal non-Hodgkin's lymphoma (32), chronic lymphocytic leukemia (CLL) (31), acute myelogenous leukemia (AML) (33), lung cancer (34), pancreatic cancer (35), and breast cancer (34). Our group reported that *miR-155* transgenic mice develop acute lymphoblastic leukemia/high-grade lymphoma and that most of these leukemias start at approximately nine months, irrespective of the mouse strain, preceded by a polyclonal pre-B-cell proliferation. *miR-155* downregulates Ship and c/EBPβ, initiating a chain of events that leads to the accumulation of large pre-B cells and acute lymphoblastic leukemia/high-grade lymphoma.

miR-21 was one of the first miRNAs detected in the human genome (36), and it displays a strong evolutionary conservation across a wide range of vertebrate species in mammalian, avian, and fish clades. In *Homo sapiens*, the *MIRN21* gene is located on chromosome 17, residing within the tenth intron of the gene *TMEM49* (transmembrane protein-49, also known as vacuole membrane protein-1). It has been demonstrated that a primary transcript containing *miR-21* (i.e., *pri-miR-21*) is independently transcribed from a conserved promoter that is located within the

Figure 1

microRNA biogenesis. The production of microRNAs (miRNAs) from pri-miRNA is a complex and coordinated process operated by different groups of enzymes and associated proteins in the nucleus or cytoplasm. The pri-miRNA, located in the nucleus, is converted in pre-miRNA through the cleavage activity of the Drosha enzyme. The product that results from the cropping, the pre-miRNA, presents 5′ phosphate and 3′ hydroxy termini. The produced pre-miRNA is exported to the cytoplasm by the Exportin 5 (Exp5)/Ran–guanosine triphosphate (Ran-GTP) complex. Exp5 forms a nuclear heterotrimer with Ran-GTP and pre-miRNA, which results from Drosha processing. This interaction, which is dependent on RNA structure but independent of sequence, stabilizes the nuclear pre-miRNA and promotes the export to the cytoplasm. After it arrives into the cytoplasm, the pre-miRNA is processed in ~18-22-nucleotide miRNA duplexes by the cytoplasmic RNase III Dicer. Normally, one strand of this duplex is degraded, whereas the other strand accumulates as a mature miRNA. From the miRNA-miRNA* duplex, only the miRNA enters preferentially in the protein effector complex, formed by the RNA-induced silencing complex (RISC) and miRgonaute. Perfect or nearly perfect complementarities between miRNA and its target 3′ UTR induce RISC to cleave the target mRNA, whereas imperfect base matching induces mainly translational silencing of the target. Acronyms: DGCR8, DiGeorge syndrome critical region gene 8 protein; dsRBD, double-stranded RNA-binding domain; ORF, open reading frame; PACT, protein activator of protein kinase R; Pol II, RNA polymerase II; TRBP, transactivating response RNA-binding protein.

Table 1 OncomiRs

miRNA gene	Localization	miRNA target	Dysregulation in cancer	Reference(s)
miR-155	21q21.3	Ship c/EBPβ Pu.1 Cutl1 Pcalm	Upregulated in pediatric Burkitt's lymphoma, Hodgkin's disease, non-Hodgkin's lymphoma, and CLL, as well as breast, lung, colon, and pancreatic cancer	30–35, 55, 89
miR-21	17q23.1	PTEN PDCD4	Overexpressed in breast, lung, prostate, gastric, cervical, head and neck, and colorectal cancer, as well as glioblastoma	36–42, 55, 56, 89, 93, 95, 97
miR-221&222	Xp11.3	PTEN TIMP3 p27 p57 Bim DDIT4 FOXO3A	Upregulated in hepatocarcinoma, CLL, melanoma, and glioblastoma, as well as lung, breast, thyroid, and prostate cancer	43–50, 94
miR-106b-93-25 cluster	7q22.1	P21/Cip1 Bim	Overexpressed in gastric, prostate, colon, and pancreatic cancer, as well as neuroblastoma and multiple myeloma	51
miR-17-92 cluster	13q31.3	p63 E2F1 P21 Bim	Upregulated in lung and colon cancer, as well as lymphoma, medulloblastoma, and multiple myeloma	52–54, 57, 58, 99

The most upregulated microRNAs in human cancers are reported. CLL, chronic lymphocytic leukemia.

intron of the overlapping protein-coding gene *TMEM49* (37). Several studies suggest that this miRNA is oncogenic (38–41) and that it may act as an antiapoptotic factor. In fact, antisense inhibition of *miR-21* caused significant apoptotic cell death in neuroepithelial cells through activation of caspases (42). We also can mention miR-221&222, which are among the most dysregulated miRNAs implicated in cancer. Expression of *miR-221&222* is highly upregulated in a variety of solid tumors, including thyroid cancer (43), hepatocarcinoma (44), breast estrogen receptor negative cells (45), and melanoma cells (46). Elevated *miR-221&222* expression has been causally linked to proliferation (47), apoptosis (48), and migration (49) of several cancer cell lines. We recently reported that the hepatocyte growth factor receptor (MET) oncogene, through c-Jun transcriptional activation, upregulates *miR-221&222* expression, which, in turn, by targeting *PTEN* and *TIMP3*, confers resistance to tumor necrosis factor–related apoptosis-inducing ligand (TRAIL)-induced cell death and enhances tumorigenicity of lung and liver cancer cells (49). The results suggest that therapeutic intervention involving the use of miRNAs should not only sensitize tumor cells to drug-inducing apoptosis but also inhibit their survival, proliferation, and invasion. Pineau and colleagues (50) confirmed our results by profiling miRNA expression in 104 hepatocellular carcinoma (HCC) samples, 90 adjacent cirrhotic livers, 21 normal livers, and 35 HCC cell lines. They found *miR-221&222* to be the most upregulated miRNAs in tumor samples, enhancing cell growth in vitro by targeting the cyclin-dependent kinase (CDK) inhibitor $p27^{Kip1}$ and by defining disease progression from normal liver to full-blown tumors through liver cirrhosis. The tumor growth activity was efficiently inhibited by specific antimiR-221&222 (see below).

Finally, Pineau and colleagues (50) identified DNA damage-inducible transcript 4 (DDIT4), a modulator of the mTor pathway, as a bona fide target of miR-221. The miR-106b-25 polycistron

MET: hepatocyte growth factor receptor

TRAIL: tumor necrosis factor–related apoptosis-inducing ligand

HCC: hepatocellular carcinoma

CDK: cyclin-dependent kinase

is composed of the highly conserved miR-106b, miR-93, and miR-25 that accumulate in different types of cancer, including gastric, prostate, and pancreatic neuroendocrine tumors, as well as neuroblastoma and multiple myeloma. These miRNAs are located in intron 13 of the host gene *MCM7*, where they are cotranscribed in the context of the MCM7 primary transcript. Petrocca and collaborators (51) demonstrated that miR-106-25 polycistron functions as an oncogene by suppressing p21 and Bim. Another example of a miRNA locus with oncogenic properties is the *miR-17-92* cluster, which consists of six miRNAs: miR-17-5p, -18, -19a, -19b, -20a, and -92-1. This cluster is located within ~1 kb of an intron of the *C13orf25* locus on human chromosome 13q31, a region frequently amplified in several types of lymphoma and solid tumors (52, 53). It has been shown that mice deficient for miR-17~92 die shortly after birth with lung hypoplasia and a ventricular septal defect. This cluster is also essential for B cell development; its absence, in fact, leads to increased levels of the proapoptotic protein Bim and inhibits B cell development at the pro-B-to-pre-B transition (54). The results of these studies indicate that many miRNAs have oncogenic activity. Importantly, their knockdown through the use of antisense oligonucleotides inhibits the development of cancer-associated phenotypes, laying the groundwork for the creation of miRNA-based therapies (55, 56, 57, 58).

microRNAs as Tumor Suppressor Genes

miR-15 and *-16* were the first to establish the link between miRNAs and cancer (27) (**Table 2**). They are transcribed as a cluster (*miR-15a–miR-16-1*) that resides in the 13q14 chromosomal region. Deletions or point mutations in region 13q14 occur at high frequency in CLL, lymphoma, and several solid tumors (59). Their expression is inversely correlated to *BCL2* expression in CLL (60). The tumor suppressor function of *miR-15a/16-1* has also been addressed in vivo. In immunocompromised nude mice, ectopic expression of *miR-15a/16-1* was found to cause dramatic suppression of tumorigenicity of MEG-01 leukemic cells that exhibited a loss

Table 2 Tumor suppressor miRNAs

miRNA gene	Localization	miRNA target	Dysregulation in cancer	Reference(s)
miR-15/16	21q21.3	Bcl-2 CCND1 WNT3A	Downregulated in CLL, DLBCL, multiple myeloma, pituitary adenoma, prostate cancer, and pancreatic cancer	27, 59–63
let-7	17q23.1	Ras Myc HMGA2	Downmodulated in liposarcoma and CLL, as well as gastric, lung, prostate, breast, ovarian, and colon tumors	64–66, 76
miR-34a/b/c	Xp11.3	CCNE2 MET Bcl2 MycN Notch1/Notch2 CDK4/6	Downregulated in pancreatic cancer and Burkitt's lymphoma without Myc translocation Hypermethylated in colon tumors	67–70
miR-29	7q22.1	Mcl-1 CDC42	Downmodulated in CLL and cholangiocarcinoma, as well as colon, breast, and lung cancer	71, 72, 74
miR-122	13q31.3	ADAM17	Downregulated in HCC	73

The most downregulated microRNAs in human cancers are reported.
Abbreviations: CLL, chronic lymphocytic leukemia; DLBCL, diffuse large B cell lymphoma; HCC, hepatocellular carcinoma.

siRNA: small interfering RNA

of endogenous expression of *miR-15a/16-1*. Recently, Bonci et al. (61) reported that the *miR-15a–miR-16-1* cluster targets not only *BCL2* but also *CCND1* (encoding cyclin D1) and *WNT3A* mRNA, which promote several prostate tumorigenic features, including survival, proliferation, and invasion. Together, these data suggest that *miR-15a/16-1* genes are natural antisense interactors of *BCL2* and probably other oncogenes and that they can be used to suppress tumor growth in therapeutic application for a variety of tumors. Ongoing clinical trials are assessing the therapeutic potential of antisense oligonucleotides targeting *BCL2* gene expression in prostate cancer (62). The reintroduction of *miR-15a–miR-16* could be theoretically more effective, due to the simultaneous inhibition of *BCL2*, *CCND1*, *WNT3A*, and other possible targets, such as *MCL-1*, involved in cancer cell proliferation and resistance to apoptosis. Recently, Klein et al. (63) generated transgenic mice with a deletion of the *miR-15a–miR-16-1* cluster, causing development of indolent B-cell-autonomous, clonal lymphoproliferative disorders, recapitulating the spectrum of CLL-associated phenotypes observed in humans.

An inverse correlation exists between *let-7* and *RAS* in lung tumors; this provides a possible mechanism for let-7 in cancer (64) (**Table 2**). In 2005, Johnson and collaborators (64) reported that the loss of *let-7* family members resulted in the constitutive overexpression of *RAS*, an oncogene that contributes to the pathogenesis of several types of human tumors. Moreover, Sampson et al. (65) found that overexpression of *let-7a* decreased Myc mRNA and protein in lymphoma cells, suggesting that dysregulation of this miRNA participates in the genesis and maintenance of the lymphoma phenotype in Burkitt's lymphoma cells and other Myc-dysregulated cancers. *let-7* was also found to target the high-mobility group AT-hook 2 (HMGA2), a gene that is dysregulated in various human tumors, including liposarcoma (66).

The miRNA-34 family comprises three members: miRNA-34a, which is generated from a larger transcriptional unit on chromosome 1p36; and miR-34b and miR-34c, both of which are generated through the processing of a bicistronic transcript from chromosome 11q23 (termed miR-34bc) (**Table 2**). To identify miRNA components of tumor suppressor pathways, He et al. (67) compared miRNA expression profiles of wild-type and p53-deficient cells and found that miR-34a–c expression reflected p53 status. They validated different miR-34 targets as cyclin E2 (CCNE2), CDK4, and MET. Silencing these selected miR-34 targets through the use of small interfering RNAs (siRNAs) led to a substantial arrest in G1. Conversely, ectopic miR-34 delivery caused a decrease in levels of phosphorylated retinoblastoma gene product (Rb), consistent with lowered activity of both CDK4 and CCNE2 complexes. BCL2 and MYCN were identified as miR-34a targets and likely mediators of the tumor suppressor phenotypic effect in neuroblastoma (68). Another group reported that Notch1, Notch2, and CDK6 are downregulated by miR-34a in glioma cells (69). The miR-34 family members have also been identified as promising prognostic markers in non-small-cell lung cancer (NSCLC); the family is downmodulated in tumors compared with normal tissue. Restoration of miR-34 expression in the pancreatic cancer cells by either transfection of miR-34 mimics or infection with lentivirus significantly inhibited clonogenic cell growth and invasion, induced apoptosis and G1 and G2/M arrest in the cell cycle, and sensitized the cells to chemotherapy and radiation (70). Mott et al. (71) reported that miR-29, highly expressed in cholangiocytes and downregulated in malignant cells, is an endogenous regulator of Mcl-1 protein expression, and therefore apoptosis. It has been demonstrated that miR-29 family members (miR-29a, miR-29b, and miR-29c) directly suppress p85 alpha (the regulatory subunit of phosphoinositide 3-kinase) and CDC42 (a Rho family GTPase), both of which negatively regulate p53, and that they induce apoptosis in a p53-dependent manner (72) (**Table 2**). Tsai et al. (73) reported that liver-specific miR-122 is significantly downregulated in liver cancers with intrahepatic metastasis and that it negatively regulates tumorigenesis. Restoration of miR-122 in metastatic cells significantly reduced in vitro migration, invasion, and anchorage-independent growth as well

as in vivo tumorigenesis, angiogenesis, and intrahepatic metastasis in an orthotopic liver cancer model. Moreover, they found that ADAM17 (a disintegrin and metalloprotease 17) was involved in metastasis as a target of miR-122 (**Table 2**).

AMO: anti-miRNA oligonucleotide

microRNAs AND THERAPY

We are seeing the emergence of new technologies that utilize synthetic miRNAs or artificial target sites to exploit or inhibit endogenous miRNA regulation. Therapeutic strategies based on modulation of miRNA expression hold great promise owing to the ability of these small RNAs to regulate cellular behavior. The most promising therapeutic techniques tested to date are (*a*) miRNA mimics and (*b*) anti-miRNA oligonucleotides (AMOs).

microRNA MIMICS

Because the loss of a miRNA inhibitory effect contributes to oncogene activation, it could be possible to inhibit the expression of dysregulated oncogenes by using synthetic miRNA mimics. Many studies have already demonstrated how this approach works in vitro. Recently, Garzon et al. (74) found that enforced expression of miR-29a and -29b in AML cell lines and in primary AML blasts inhibited cell growth and induced apoptosis in vitro and in vivo through the downregulation of the Mcl-1 protein. The data support a tumor suppressor role for miR-29 and provide a rationale for the use of synthetic miR-29b oligonucleotides as a novel strategy to improve treatment response in AML. Furuta et al. (75) showed that *miR-124* and *miR-203* genes are hypermethylated in HCC. Restoration of miR-124 or miR-203 expression by miRNA mimics significantly reduced cell proliferation in all the HCC cell lines tested. Protein levels of CDK6, SET and MYND domain containing 3 (SMYD3), vimentin (VIM), and IQ motif containing GTPase activating protein 1 (IQGAP1) were reduced in miR-124 transfectants compared with their control counterparts. In miR-203 transfectants, the protein level of ATP-binding cassette, subfamily E, member 1 (ABCE1) and the protein level of CDK6 were decreased compared with that in the control transfectants (75). The disadvantages of this approach are that miRNA-mimic oligonucleotides have only a transient effect, they are not stable, and they may require repeated deliveries.

Another technique uses vector-based miRNA expression to produce stable expressed miRNAs. Takamizawa et al. (76) introduced *let-7* into a lung cancer cell line by using expression constructs, which were designed to synthesize the mature miRNAs of two predominant *let-7* isoforms, *let-7a* and *let-7f*, under the control of the Pol III H1-RNA gene promoter. Overexpression of *let-7f* in the A549 lung adenocarcinoma cell line resulted in a 78.6% reduction in the number of colonies, whereas the introduction of *let-7a* showed a similar but more modest growth-inhibitory effect (76). They demonstrated, for the first time, that the expression levels of the *let-7* family were significantly downregulated in lung cancers both in vitro and in vivo. Also, plasmids with Pol II promoters offer flexibility in regulating the production of miRNAs in cultured cells or in vivo. Expression simply requires the insertion of the entire predicted miRNA precursor stem-loop structure into the expression vector at an arbitrary location. Moreover, although the plasmids express only a single miRNA, the fact that Drosha cleavage independently excises each miRNA stem-loop precursor from the primary transcript to give rise to a pre-miRNA suggests that Drosha cleavage should be able to simultaneously express several artificial or authentic miRNAs by a tandem array on a precursor RNA transcript (77). McLaughlin et al. (78) designed 10 miRNA mimics directed against several sites within the *ABL* gene coding sequences using the BLOCK- iT™ Pol II miR RNAi Expression Vector from Invitrogen. In fact, a reciprocal translocation between chromosomes 9 and 22 results in chimeric Bcr-Abl gene expression, a specific chromosomal abnormality

shRNA: short hairpin RNA

that is associated with chronic myelogenous leukemia (CML). For comparison, they used a short hairpin RNA (shRNA) against the Bcr-Abl junction. The degree of suppression by the 10 miRNA constructs varied widely but had the same efficiency (90%) compared with the junction-specific shRNA. One of the miRNA constructs was remarkably efficient: It blocked Bcr-Abl protein production by ~99%, indicating greater effectiveness than the use of the Bcr-Abl tyrosine kinase inhibitor imatinib or related drugs. In addition, alternative chromosomal partners, such as *Tel*, can participate with *Abl* in the formation of chimeric oncogenes such as *Tel-Abl*. Moreover, many imatinib-resistant forms of Bcr-Abl present mutations at Thr-315. To evaluate the generality of the utility of Abl-directed miRNAs, McLaughlin et al. compared the ability of selected forms to suppress these alternative members of the Abl oncogene family and demonstrated that each could be effectively suppressed through the targeting of Abl sequences (78).

Liang et al. (79) reported another study on the miR-155-based BLOCK- iT Pol II miR RNAi Expression Vector from Invitrogen. They used this construct to silence CXCR4 in MDA-231 cells. CXCR4 interacts with SDF-1, inducing the phosphorylation of Akt. Ctrl-miRNA-transfected MDA-MB-231 cells expressed high levels of CXCR4 protein. Conversely, phosphorylation of Akt in MDA-MB-231 cells transfected with CXCR4 miRNA cannot be induced even at 30 min after SDF-1 addition because the expression of CXCR4 was silenced completely with the CXCR4 miRNA. Taken together, the data suggest that artificial miRNA may potentially be a therapeutic agent for breast cancer metastasis (79). Chung and colleagues (80) described a new Pol II vector system for RNAi, the SIBR (synthetic inhibitory BIC-derived RNA) vectors, based on *BIC*, the primary transcript for the miR-155 miRNA. The stem-loop precursor for the mouse miR-155 miRNA is located within the third exon of the mouse *BIC* gene that is an evolutionarily conserved noncoding RNA. The ability to express functional *BIC* (and presumably miR-155) from a heterologous Pol II promoter in a retroviral vector suggested that *BIC*/miR-155 might be a good sequence framework for construction of Pol II–based RNAi vectors. The replacement of the miR-155 precursor stem loop with other synthetic miRNA (i.e., siRNA) allows these vectors to provide effective inhibition of different targets in mammalian cells. miR-155-based SIBR vectors provide similar levels of inhibition to Pol III shRNA vectors, but, unlike the Pol III shRNA vectors, SIBR vectors can be used to express multiple miRNA expression cassettes from a single transcript (80). Although significant focus in the area of miRNAs and therapy has been directed toward antisense-mediated inhibition of tumorigenic and antiapoptotic miRNAs (81), several studies indicate that miRNA replacement represents an equally viable, if not more efficacious, strategy. Vector-based miRNA expression, which produces stable expressed miRNAs, can inhibit endogenous oncogene expression; this capability demonstrates the efficiency of miRNAs as therapeutic tools.

Numerous preclinical studies have demonstrated the efficacy of recombinant adeno-associated virus (rAAV) gene delivery vectors, and recent clinical trials have shown promising results. However, the efficiency of these vectors is hindered by the need to convert the single-stranded DNA (ssDNA) genome into double-stranded DNA (dsDNA) before expression. This critical step can be effectively bypassed through the use of self-complementary AAV (scAAV) vectors. AAV is a linear ssDNA molecule with dsDNA hairpin structures at each end; these function as replication origins for the synthesis of a complementary strand. Kota and collaborators (82) tested the hypothesis whether miRNAs and scAAV vectors could be used as general anticancer therapeutics. They found that miR-26a is abundantly expressed in normal liver and downregulated in human and murine liver tumors. Furthermore, it directly downregulates cyclins D2 and E2, inducing G1 arrest in human cancer cells in vitro. To assess the therapeutic efficacy of this miRNA, they constructed a scAAV vector system, which included enhanced green fluorescent protein (eGFP) driven by the ubiquitously expressed elongation factor 1 alpha (EF1α). scAAV.eGFP and scAAV.miR-26a.eGFP were packaged with the AAV8 serotype for in vivo delivery and were

administered with a single tail-vein injection in mice with established liver tumors. Mice transduced with scAAV8.miR-26a.eGFP presented high expression levels of miR-26a in the liver. Importantly, scAAV8.miR-26a.eGFP administration did not cause the inhibition of the miRNA pathway as previously reported with the delivery of shRNA (82). Moreover, no inflammation, fibrosis, or histologic evidence of toxicity was observed, which demonstrates that scAAV8 provides a nontoxic tool to deliver miRNAs to the liver. This is an important point because the use of AAV2 for in vivo therapy is limited by early immunologic memory of AAV infections (83). Most importantly, 6 out of 8 mice treated with control virus developed tumors, and 8 of 10 scAAV8.miR-26a.eGFP-treated animals exhibited only small tumors or a complete absence of tumors. Intriguingly, the remaining 2 of the 10 scAAV8.miR-26a.eGFP-treated mice presented significantly lower transduction efficiency than the successfully treated animals. This important study highlights the therapeutic promise of this approach. Many other miRNAs with therapeutic potential remain to be functionally characterized, so there clearly remains significant work to be done. The experiments have been performed in liver that is well suited for such alternative strategies—i.e., it is easily targeted by viral genes, nonviral genes, and small-molecule delivery systems (84)—but the liver is not the only organ where the AAV vectors could be used. Direct injection into muscle results in high local concentrations of vector at the injection site. There have been numerous reports of long-term expression in the adult central nervous system from AAV vectors. Injection of vector into the ventricular space can achieve a much broader distribution of central nervous system transduction because of the diffusion in the cerebral spinal fluid. The retina is an attractive application for AAV gene therapy for several reasons, including small volume and low dose requirements (85). AAV vectors have also been shown to stably integrate in long-term-regenerating cell populations within bone marrow (86). Thus AAV vectors are currently used in many gene transfer applications; however, the respiratory epithelium remains a challenging target. Recently, Fein et al. (87) synthesized two cationic sterol-based lipids, dexamethasone-spermine and disubstituted spermine, for pulmonary gene targeting. When the AAV vectors were formulated with these cationic lipids, the transduction efficiency in cultured A549 cells increased by sevenfold and sixfold, respectively. Intranasal administration of 10^{11} genome copies of AAV2/9 and AAV2/6.2 coformulated with lipid formulations resulted in an average fourfold increase in transgene expression for both vectors (87).

ANTI-microRNAs

2′-*O*-Methyl Anti-microRNA Oligonucleotides

The 2′-*O*-methyl (2′-OMe) group is one of the oldest, simplest, and most often used modifications to oligonucleotides. The methyl group contributes a limited amount of nuclease resistance and improves binding affinity to RNA compared with unmodified sequences. Lee et al. (88) used 2′-*O*-methyl antisense oligonucleotide to knock down miR-125b in PC-3 (prostate cancer) and HeLa (cervical cancer) cells. They observed a reduction in cell proliferation in both cell lines. A library of 2′-*O*-methyl anti-microRNA oligonucleotide (OMe-AMO) inhibitors in functional screening assays was used to identify miRNAs that affect cell proliferation and apoptosis. They validated the efficacy of the miRNA inhibitors using a luciferase reporter bearing miRNA target sequences cloned into its 3′ UTR (89). Despite the successful knockdown of a miRNA in vitro and in vivo, 2-*O*-methyl antisense oligonucleotide has several limitations. First, a direct measurement of the depletion of miRNAs is difficult, because miRNAs bind to the miRNA and sequester it from its target rather than induce its degradation. Therefore, the only possible method to confirm the decrease in number of miRNAs is to measure the level of expression of a reporter gene containing the target sequence of the miRNA. In addition, adding back miRNA in the presence of the

2′-*O*-methyl antisense oligonucleotide cannot rescue the knockdown phenotype. Taking into account that each miRNA could have more than hundred target mRNAs in the cell, this multitarget regulation might be responsible for adverse or nontarget effects in a future miRNA-mediated therapy (see below).

2′-*O*-Methoxyethyl Anti-microRNA Oligonucleotides

2′-*O*-methoxyethyl (2′-MOE)-modified oligonucleotides have higher affinity and specificity to RNA than their OMe analogs. Esau et al. (90) inhibited a panel of 86 miRNAs in preadipocytes using 2′-*O*-methoxyethyl anti-microRNA oligonucleotides (MOE-AMOs) and evaluated the effect on adipocyte differentiation. The comparison of the miRNA expression profile in differentiated versus nondifferentiated adipocytes showed that a miRNA, miR-143, was found to be involved in the differentiation process through the regulation of the ERK5 protein. Treatment of adipocytes with a MOE-AMO complementary to miR-143 effectively inhibited this process, as compared with the cells transfected with the miRNA negative control (90).

The Locked Nucleic Acid Antisense Oligonucleotides

The success of siRNA as a potent and specific inhibitor of gene expression in vitro has fueled the interest for in vivo applications of siRNA as a genetic therapeutic. The therapeutic efficacy of siRNAs depends on their ability to migrate through the body and reach diseased organs in therapeutically relevant levels. Serum nuclease degradation, inadequate organ distribution, and a high level of off-target effects have limited the development of siRNA therapeutics. The half-life of unmodified siRNAs in vivo is in the range of minutes, but chemical modifications can significantly improve this range without a major loss of silencing activity (91). A locked nucleic acid (LNA) is an oligonucleotide that contains conformationally locked nucleotide monomers with a methylene bridge connecting the 2′-oxygen and 4′-carbon atoms of the ribose ring. Incorporation of LNA monomers into oligonucleotides and siRNA constructs has been shown to increase the nuclease resistance significantly, stabilize the duplex structure, and improve mismatch discrimination (92). LNA is substantially compatible with the siRNA machinery, and it reduces sequence-related off-target effects either by lowering incorporation of the siRNA sense strand and/or by reducing the ability of inappropriately loaded sense strands to cleave the target RNA. The application of 2′-*O*-methyl- and/or DNA/LNA-mixed oligonucleotides to specifically inhibit miR-21 in glioblastoma and breast cancer cells suppressed cell growth and enhanced caspase activation in vitro (39, 93).

Recently, Pineau et al. (50) identified DNA damage-inducible transcript 4 (DDIT4), a modulator of the mTor pathway, as a bona fide target of miR-221. They introduced into liver cancer cells, by lipofection, LNA-modified oligonucleotides specifically designed for miR-221 (antimiR-221) and miR-222 (antimiR-222) knockdown. Treatment by antagomiRs, but not scrambled oligonucleotide, reduced cell growth in liver cancer cell lines that overexpressed miR-221 and miR-222 by 35% and 22%, respectively. When introduced in combination, antimiR-221 and -222 did not increase growth inhibition, which suggests that a saturation threshold is reached in the four cell lines by a single antagomiR. Thus the use of synthetic inhibitors of miR-221 may prove to be a promising approach to liver cancer treatment (50). Galardi et al. (94) showed that miR-221 and miR-222 knockdown through antisense LNA oligonucleotides increases $p27^{Kip1}$ in human prostate cancer (PC3) cells and strongly reduces their clonogenicity in vitro. Corsten et al. (95) found that silencing miR-21 using locked-nucleic-acid-modified oligonucleotide (LNA-antimiR) molecules in glioma cells followed by TRAIL treatments increased caspase activity in vitro and reduced tumor growth in vivo. Despite recent progress in silencing of miRNAs in rodents, the

development of effective and safe approaches for sequence-specific antagonism of miRNAs in vivo remains a significant scientific and therapeutic challenge. Recently, Elmén and collaborators (96) showed for the first time that the simple systemic delivery of an unconjugated, PBS-formulated LNA-antimiR effectively antagonizes the liver-expressed miR-122 in nonhuman primates. Administration by intravenous injections of LNA-antimiR into African green monkeys resulted in the formation of stable heteroduplexes between the LNA-antimiR and miR-122, accompanied by depletion of mature miR-122 and dose-dependent lowering of plasma cholesterol. These findings demonstrate the utility of systemically administered LNA-antimiRs in exploring miRNA function in primates and show the impressive potential of this strategy to overcome a major hurdle for clinical miRNA therapy (96).

microRNA Decoy or Sponge

A number of groups have shown that vectors expressing miRNA target sites can be used to saturate an endogenous miRNA, preventing the downregulation of its natural target. This technology, which has been defined with different names [decoy (97), sponge (98), eraser (99), and antagomiR (100)], utilizes different gene delivery systems, including plasmids and vectors based on adenoviruses, retroviruses, and lentiviruses. Overexpression of a miRNA target can be achieved through the use of high vector copy, strong promoters, or stable transcript. The sponge strategy fills the gaps of the antagomiR approach in different ways. First, genetic knockout of miRNA is a powerful tool to identify its function, but knockouts are limited to studies in mice and take time. Second, up to 40% of miRNA genes are located in protein-coding genes, and this might create an artifact. Third, this strategy makes it possible to silence the effect of an entire miRNA family through a single member because family members have the same seed sequence. Finally, unlike oligonucleotide-based miRNA knockdown, the decoy vectors, mainly when based on lentiviral vectors, can stably antagonize a miRNA without requiring multiple administrations (101). Recently, Valastyan et al. (102) deployed a stable miRNA sponge strategy to inhibit miR-31 in vivo in noninvasive MCF7-Ras cells using retroviral miRNA sponges that carried miR-31 recognition motifs in their 3′ UTRs. The miR-31 sponge reduced miR-31 function by a factor of 2.5 but did not affect the activity of other known antimetastatic miRNAs. This approach allowed the otherwise nonaggressive breast cancer cell to metastasize. However, the decoy vector technology has its limitations. The overexpression of the target genes could be toxic for the cells and is not always achieved in some cells and tissue types. Moreover, it is difficult to determine the degree of miRNA inhibition mediated by a sponge vector, so genetic knockouts are still required to guarantee a complete loss of miRNA activity.

CONCLUSIONS

Targeted molecular therapeutics based on miRNAs hold great promise for the development of less toxic and more effective personalized treatment strategies for cancer. This approach requires a deeper understanding of the molecular changes that drive tumor formation and also requires development of therapeutic agents that specifically inhibit the genes and pathways activated by these changes. Many, if not all, of the miRNAs highlighted in this review may very well play important roles in the development of novel cancer treatment strategies. miRNAs constitutively dysregulated in cancer represent optimal miRNA-therapy targets. The field of small RNAs is rapidly advancing toward in vivo delivery for therapeutic purposes. Advanced molecular therapies aimed at downmodulating (96) or upmodulating the level of a given miRNA in model organisms have been successfully established (82) (**Figure 2**). In theory, if the activation of an oncogene

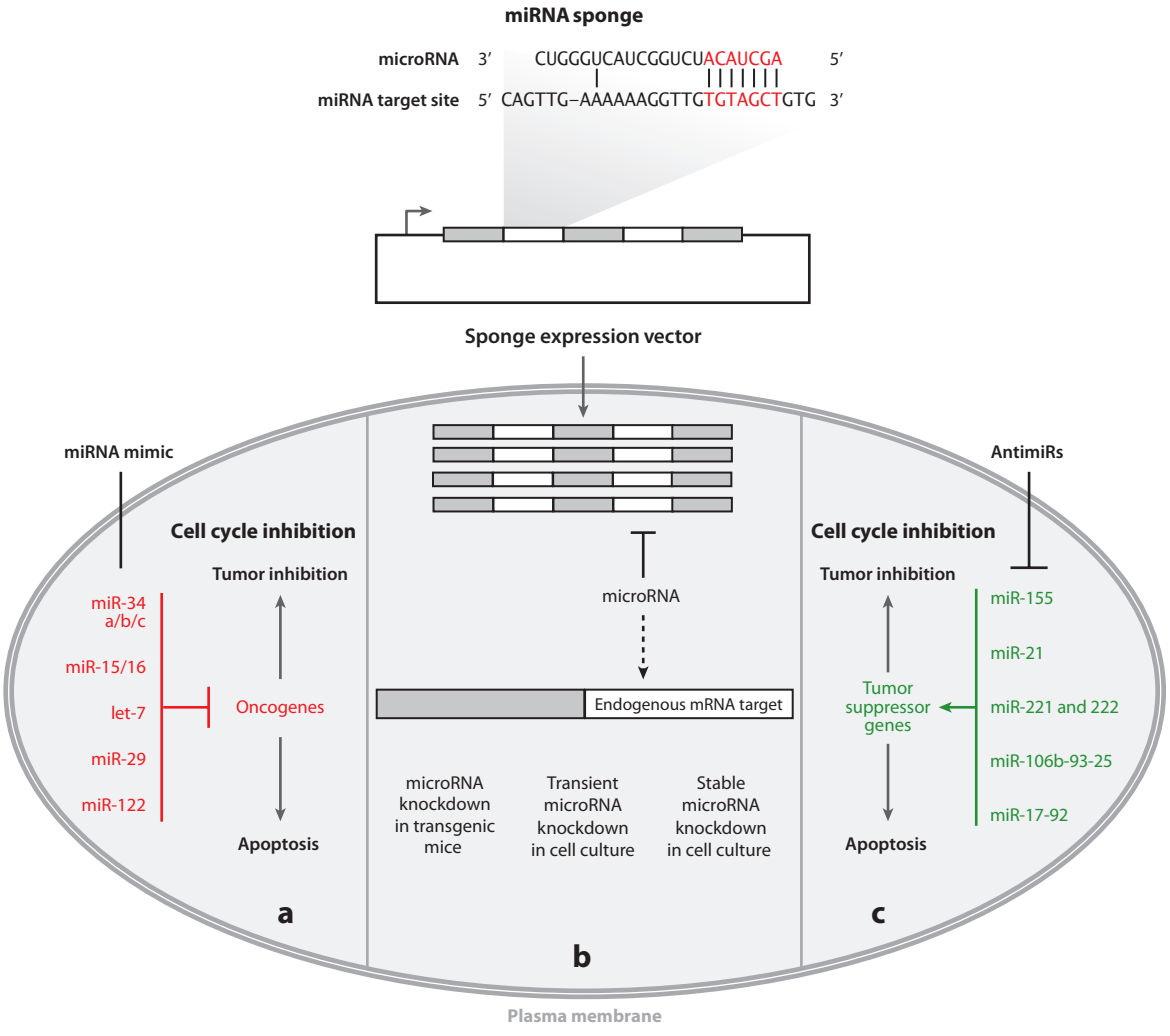


Figure 2

microRNAs (miRNAs) as therapeutics. (*a*) The miRNA mimic technology is an innovative approach for gene silencing. This approach uses synthetic miRNAs that, once introduced into cells, mimic endogenous miRNAs, bind specifically to their target genes, and produce posttranscriptional repression—more specifically, translational inhibition—of the genes. (*b*) Sponges are ectopically expressed mRNAs that contain multiple miRNA target sites (*white*). These target sites compete miRNAs away from their natural mRNA targets. miRNA sponges are suitable for use in a variety of experimental systems, including cultured cells and transgenic animals. (*c*) Knockdown of oncomiRs through anti-microRNA oligonucleotides (2′-*O*-methyl anti-microRNA oligonucleotides, 2′-*O*-methoxyethyl anti-microRNA oligonucleotides, and locked nucleic acid antisense oligonucleotides) leads to the upregulation of tumor suppressor proteins, inducing apoptosis and blocking tumor formation in vitro and in vivo.

promotes tumor growth and spread, then the ability to specifically reduce oncogene expression may slow cancer growth. The discovery of small RNAs and their functions has revitalized the prospect of controlling expression of specific genes in vivo, with the ultimate hope of building a new class of gene-specific medical therapies. miRNAs have been shown to target the expression of important cancer-related genes without associated toxicities or histopathological changes in

animals. Precise delivery to the cancer cell may be needed to avoid unwanted miRNA effects that could result from targeting important genes in other healthy tissues.

In addition, miRNA has several targets, and different genes can be regulated by several miRNAs, so this multitarget modulation might be responsible for adverse or nontarget effects. In this regard, Xiao et al. (101) have already suggested two promising approaches—a gene-specific miRNA mimic approach and a miRNA-masking antisense approach—to explore the possibility of using miRNA's mechanism of action in a gene-specific manner. Specifically, they showed that gene-specific miRNA mimics of 22 nucleotides, designed on the miR-1 and miR-133 target sites in the 3′ UTRs of HCN2 and HCN4 cardiac channels, were efficient in abrogating expression and function of these two proteins without affecting the proteins' transcript levels; thus they eliminated the possible miR-1 and miR-133 multitarget effects. Meanwhile, the miRNA-masking antisense oligonucleotides, designed on the miR-1 and miR-133 target sites in the 3′ UTRs of HCN2 and HCN4, hybridized with the target mRNA to mask the miRNA binding sites and block the action of miR-1 and miR-133. This mechanism enhanced HCN2/HCN4 expression and function (101). Promising miRNA formulations should be further evaluated by detailed pharmacokinetics and pharmacodynamics studies in animal models. In conclusion, the latest findings discussed in this review indicate that miRNAs could become powerful therapeutic tools in the near future.

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