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Macro and Small over Micro: Macromolecules and Small Molecules that Regulate MicroRNAs

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Introduction

MicroRNAs (miRNAs) are small endogenous RNAs of 20–22 nucleotides (nts) in length that regulate gene expression post-transcriptionally by binding to the 3′-untranslated regions (UTRs) of their mRNA targets.[1] To date, about 2600 human mature miRNA sequences are registered in miRBase, an miRNA database, and more than 60% of human coding genes are estimated to be under the control of miRNAs.[2]

MiRNAs are critical regulators of every biological process, including proliferation, differentiation, and cell survival, and so the aberrant expression or function of miRNAs often results in various diseases in humans.[3] The first human disease linked to miRNA abnormalities was chronic lymphocytic leukemia (CLL), and many more miRNAs have since been shown to be associated with different types of diseases.[4] In particular, numerous miRNAs that show variable expression in cancers have been identified. Those miRNAs with augmented or repressed expression that correlates strongly with tumor incidence and progression are referred to as oncogenic miRNAs (oncomiRs) or tumor-suppressive miRNAs, respectively.[5]

Given the correlation between the deregulation of specific miRNAs and disease onset, attempts have been made to restore the expression and function of disease-specific miRNAs to normal levels by using small-molecule-based miRNA regulators and oligonucleotide-based miRNA mimics or antagonists.[6–12] This review provides the latest update on oligonucleotide- and small-molecule-based miRNA regulators and discusses currently available in vitro and in silico assays developed to screen for small-molecule modulators of miRNA expression.

Oligonucleotide-based miRNA regulators

Antisense miRNA oligonucleotides (anti-miRs) and miRNA mimics

A new therapeutic approach for diseases caused by the deregulation of miRNAs involves restoring miRNA expression to normal levels by means of two strategies: 1) by inhibiting overexpressed miRNAs with the aid of antisense miRNA oligonucleotides (anti-miRs), and 2) by increasing miRNA levels through the exogenous introduction of miRNA mimics or miRNA expression constructs.

Single-stranded antisense oligonucleotides (ASOs) are commonly used for the sequence-specific inhibition of miRNA function. Typical ASOs are perfectly complementary to the target miRNAs and are thus able to prevent loading onto RNA-induced silencing complex (RISC), whereas some ASOs might induce miRNA cleavage by RNase H.[13] In general, ASOs are chemically modified to improve stability, biodistribution, binding affinity, and efficacy, but certain modifications can result in decreased target affinity and poor cellular uptake.[13] The chemical modifications include locked nucleic acids (LNAs), 2′-*O*-methyl-RNA, 2′-*O*-methoxyethyl-RNA, 2′-fluoro-RNA, and RNA with phosphorothioate[14,15] (this topic is reviewed in more detail in Lennox and Behlke, 2011[13] and Deleavey and Damha, 2012[15]). The first anti-miRs were 2′-*O*-methyl-RNA oligonucleotides complementary to the miRNA *let-7*, whereas antago-miRs, the first miRNA inhibitors tested in mice, were cholesterol-conjugated 2′-*O*-methyl-RNA oligonucleotides with phosphorothioate modifications.[16] LNA ASOs are the most widely explored type of miRNA inhibitor to date.

The converse approach, miRNA replacement, involves the introduction of an miRNA mimic, a chemically modified double-stranded RNA designed to mimic endogenous mature miRNAs, or the ectopic expression of miRNAs through transient or stable introduction of miRNA genes.[7] Because miRNA mimics should behave like endogenous miRNAs, the guide strands, which have mature miRNA sequences, remain relatively unmodified.[17] Instead, the passenger strands undergo rigorous modifications in order to protect the dsRNA from nucleases, to improve activity, and to block nonspecific immune responses through interferon. The use of, for example, nucleoside analogues, as found in the ASO, and the introduction of inverted bases, biotin, or alkyl groups in the terminal region are often found in miRNA mimics. In addition, liposomes and nanoparticles can be also used to deliver miRNA mimics effectively. Once inside the cells, the miRNA mimics specifically bind to their target mRNAs and mediate translational inhibition in similar manner to endogenous miRNAs.

Manipulating miRNAs by using oligonucleotide-based therapeutics has been successful in preclinical studies, and some cases have advanced to clinical trials. Miravirsen, an LNA-based

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ASO, for instance, was developed to inhibit miR-122 in the liver and to block hepatitis C virus (HCV) RNA replication (see below for more details on miR-122 function).[18–20] Miravirsen is the first miRNA-targeted drug to enter human clinical trials and is presently in phase II trials for the treatment of HCV infection.[20] Miravirsen is expected to become the first miRNA-based therapeutic with FDA approval. In addition, MRX34, a mimic of the tumor suppressor miR-34, is the first miRNA-based anticancer agent.[21] MiR-34 targets many oncogenes such as *Myc*, *Met*, *Bcl-2*, and β-catenin and controls the growth and survival of cancer cells. The expression of miR-34 is frequently reduced in numerous types of cancers, and so attempts have been made to restore miR-34 expression by using miR-34 mimics, ultimately to treat cancers. Because of the intrinsic properties of double-stranded miRNA mimics, MRX34 was encapsulated in liposome for its successful delivery. MRX34 is currently in phase I clinical trials to evaluate its safety in patients with primary liver cancer and patients with liver metastasis from other cancers.

MiRNA sponges and sponge variants

In contrast to transiently delivered oligonucleotide-based antimiRs, a new method for delivering ASOs by means of a "sponge" expression vector has been developed.[22] In this method, tandem repeats (typically four to nine) of the antisense sequence for a specific miRNA are inserted into an expression cassette and transcribed into sponge RNAs. To stabilize RNA transcripts without 5′-cap and 3′-poly-A tail structure, stem-loop sequences are sometimes placed in the 3′- and 5′-terminal regions. The miRNA binding regions can be designed to match the target sequence perfectly to induce endonucleolytic cleavage, or they can contain an imperfect match in the middle position for a more stable and effective interaction with the miRNA. Sponge RNAs bind to and decrease the cellular levels of target miRNAs. Because sponge RNAs contain binding sites that are specific to a miRNA seed region, a sponge can inhibit a family of related miRNAs that share a common seed sequence. Currently, numerous sponge constructs with different vectors, promoters, and reporters are available, and their efficacies and half-lives vary from one to another. Variants of the miRNA sponge have also been reported, including the miRNA-eraser, miRNA-mower, and tough decoy (Table 1). Despite the differences in their names, they have common features in that they are RNA transcripts containing multiple miRNA binding sites and are transcribed from transgenes such as those found in the miRNA sponge.

The miRNA-eraser contains tandem repeats of a sequence that has perfect complementarity to a specific miRNA and blocks the action of the miRNA on its target.[23] The eraser construct lacks stem-loop sequences at the 5′- and 3′-ends of the tandem repeats and contains two copies of the antisense sequence, whereas the sponge has four to nine copies of the miRNA binding sites. Additionally, whereas the original sponge construct is built on a plasmid, the eraser uses a recombinant adenoviral vector for delivery and has a better inhibitory effect on miRNA levels.

The miR-mower construct contains six tandemly arrayed copies of miRNA binding sites with central mismatches.[24] Unlike the sponge construct, with multiple copies of a single miRNA binding site, the miRNA-mower construct can be designed to have binding sites for three different types of miRNAs (two copies for each miRNA). However, the major structural components of miRNA sponges, including bulges, multiple binding sites, and short linkers connecting the binding sites, are well preserved in miR-mowers.

The tough decoy (TuD) forms a stem capped by a large loop structure that contains miRNA binding sites that are perfectly complementary to target miRNAs.[25] The lentiviral vector-based TuD can inhibit miRNA expression over a significantly longer period of time than the miRNA sponge, the expression of which does not persist past one month. In addition, dual-targeting TuDs, which carry two miRNA binding sites and clustered TuD hairpins that contain as many as six miRNA-recognition sites, are available, making it possible to suppress multiple miRNAs simultaneously through the introduction of a single expression cassette.[26]

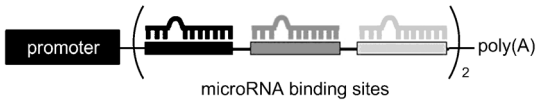
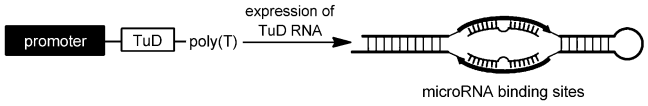
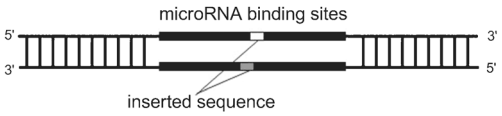
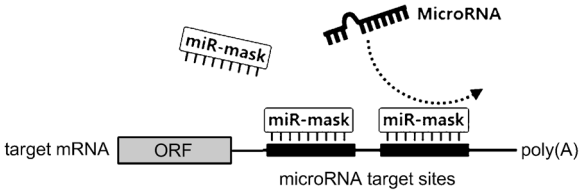
LidNA

Currently available methods for the sequence-specific inhibition of miRNAs use chemically modified oligonucleotides, because unmodified DNA is often unstable and vulnerable to nucleases and forms miRNA/DNA duplexes, which have lower affinity than miRNA/mRNA duplexes. However, given that the binding abilities of structured molecules are generally better than those of unstructured molecules, the miRNA inhibitor LidNA was designed to form unmodified but structured DNA.[27] The miR-16 inhibitor LidNA-16 has two miR-16 binding sites between two double-stranded regions and has higher affinity for its target than conventional LNA or 2′-*O*-methylated RNA. The presence of double-stranded regions near the miRNA binding sites confers an increased affinity for miRNAs, thus making LidNA the first miRNA-inhibiting oligonucleotide with no chemical modification.

Target masking by miR masks

The miR mask is a chemically modified oligoribonucleotide with perfect complementarity to the miRNA binding region in the 3′-UTR of the target mRNA.[28] Instead of blocking miRNAs, the miR-mask binds to the miRNA binding sites of the target mRNA, thereby preventing the association of an miRNA with its target. The major advantage of this method is its specificity. Because an miRNA may have multiple mRNA targets, direct scavenging of miRNAs can affect the expression of all target mRNAs and induce off-target effects. However, masking the target with miR masks only regulates the interaction between an miRNA and a single mRNA target, thus decreasing the undesirable nonspecific effects.

Table 1. Oligonucleotide-based miRNA inhibitors.[a]

Name	Structure	Feature	Ref.
sponge		– is a vector-based miRNA inhibitor – has four to nine perfect or bulged binding sites – inhibits multiple miRNAs that share the same seed – can be more stabilized with 5′- and 3′-stem-loop elements	[22]
eraser		– is a vector-based miRNA inhibitor – has two perfect binding sites – inhibits one specific miRNA	[23]
miRNA-mower		– is a vector-based miRNA inhibitor – has six bulged binding sites – inhibits one or multiple specific miRNAs	[24]
tough decoy (TuD)		– is a vector-based miRNA inhibitor – has a stem-loop structure in decoy RNA – has two bulged binding sites – inhibits one specific miRNA – provides long-term suppression of miRNAs for more than one month	[25], [26]
LidNA		– is an unmodified DNA-based miRNA inhibitor – has two bulged binding sites between double-stranded regions – inhibits one specific miRNA	[27]
miR-mask		– is a target-specific miRNA inhibitor – blocks action of a miRNA without knocking down the miRNA – modulates the interaction between miRNA and one specific target mRNA	[28]

[a] Antisense miRNA oligonucleotides and miRNA mimics are not shown. Sponge, eraser, miRNA-mower, and TuD are vector-based miRNA inhibitors, whereas LidNA and miR-mask are modified oligonucleotides.

MiRNA-modulating small molecules

Although oligonucleotide-based miRNA mimics or antagonists are becoming a promising therapeutic strategy, these drugs have some limitations in their ability to be manipulated to improve characteristics such as delivery, stability, and pharmacokinetics. Therefore, studies have been undertaken to identify miRNA-regulating small molecules to resolve the issues of using oligonucleotide-based drugs. Small molecules are resistant to nuclease degradation and can diffuse across cell membranes, enabling the delivery of drugs to intracellular targets. Their structures can be readily modified to change their pharmacological and pharmacokinetic properties, unlike those of oligonucleotides. They are also less expensive to manufacture. Therefore, small molecules are more suitable than oligonucleotide-based drugs for drug development.

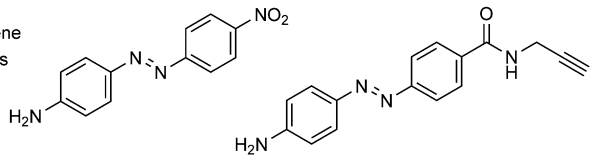
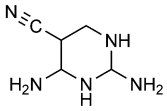
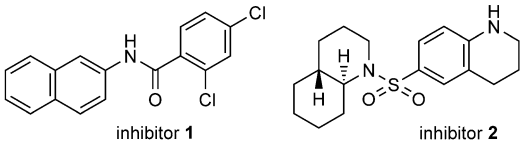
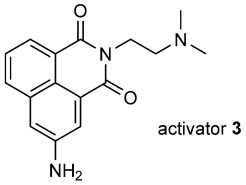
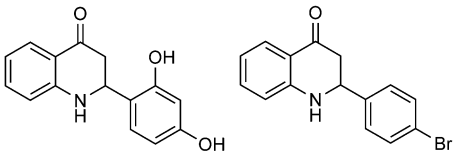
To date, dozens of chemical compounds that change the expression of various miRNAs have been identified. These small-molecule modulators can be divided into those that control the RNAi pathway generally, thereby affecting the function of the full range of siRNAs or miRNAs, and those that regulate a specific type of miRNA. In this review, we only discuss the small-molecule regulators that control the functions of specific miRNAs. The list of miRNA-modulating small molecules is presented in Table 2.

MiR-21-specific regulators

MiR-21 is one of the most widely studied miRNAs and has been implicated in the progression of many types of human cancers, including gastric cancers, breast cancers, colon cancers, and glioblastomas.[29] Because it induces an oncogenic increase in tumor cell proliferation, migration, and survival, miR-21 is designated as an oncomiR, along with miR-155, miR-233, miR-146a, and the miR-17–92a cluster, and is considered a promising anticancer therapeutic target.[30]

A diazobenzene derivative that specifically and efficiently inhibits miR-21 expression was discovered through a luciferase-based screening of more than 1000 small organic molecules (described in detail below).[8] The diazobenzene derivatives decreased the transcription of the miR-21 gene and thus the pro-

Table 2. MiRNA-modulating small molecules.

Function	Compound name and structure	Reference
miR-21 inhibitor	diazobenzene derivatives 	[8]
	streptomycin, AC1MMYR2  , estradiol	[10], [31], [34]
miR-21 activator	5-fluorouracil, estradiol	[33], [34]
miR-122 inhibitor	 inhibitor **1** inhibitor **2**	[12]
miR-122 activator	 activator **3**	[12]
miR-14/miR-4644 inhibitor	azaflavones 	[37]
miR-27a inhibitor	streptomycin, amikacin, tobramycin, neomycin	[38]

duction of primary miR-21, whereas the downstream processes of miR-21 maturation were unaffected.

Streptomycin, a well-known aminoglycoside antibiotic, has also been shown to bind directly to precursor miR-21 and to inhibit Dicer-mediated maturation of pre-miR-21.[31] Because of their particular properties of binding to RNA secondary motifs such as stem-loops and bulges, aminoglycosides are very likely to interact with precursor miRNAs (pre-miRNAs) and thus become potential candidate regulators of miRNAs.[32] Computer-aided docking, cleavage pattern, and mutation analyses have revealed that streptomycin binds to pre-miR-21 in a region close to the stem-loop junction, and that a bulge near the terminal loop is critical for this binding. However, two structural analogues, namely amikacin and dihydrostreptomycin, have no effects on miR-21 function.

Recently, AC1MMYR2 (2,4-diamino-1,3-diazinane-5-carbonitrile) was identified as a small molecular inhibitor of miR-21 that blocks the transition from pre-miR-21 to mature miR-21. The AC1MMYR2 interacts with the pre-miR-21 hairpin loop and inhibits Dicer from processing pre-miR-21 to generate mature miR-21.[10] Instead of using reporter enzyme assays, the authors performed an in silico high-throughput screen of ≈2000 compounds by molecular docking based on the 3D structure of the Dicer binding site of pre-miR-21. They assessed the validity of the initial hits in cancer cells and confirmed the strong inhibition of miR-21 expression by AC1MMYR2. The speed, efficiency, and cost-effectiveness of the computer-aided high-throughput docking method makes it an attractive alternative to traditional screening strategies.

In contrast, 5-fluorouracil (5-FU), a chemotherapeutic agent used to treat colon and pancreatic cancers, has been shown to increase the expression of miR-21.[33] However, 5-FU changes the levels of other miRNAs, and therefore cannot be considered to be a miR-21-specific regulator. Estradiol has also been reported to modulate the expression of miR-21, but whether estradiol plays an inhibitory or stimulatory role in miR-21 expression is under debate.[34]

MiR-122-specific regulators

As the most abundant miRNA, accounting for almost 70% of all miRNAs in the liver, miR-122 is involved in the regulation of lipid and cholesterol metabolism, alcohol-associated inflammation, and HCV replication. Changes in miR-122 expression are closely associated with liver disease.[19,35] MiR-122 is specifically repressed in hepatocellular carcinoma (HCC) with poor prognosis, and the knockdown of miR-122 with antisense agents decreases HCV RNA replication in human liver cells and HCV levels in chronically infected primates.[18,36] These results indicate that miR-122 could serve as a viable target for anticancer therapy by using miR-122 activators or for antiviral therapy by using miR-122 inhibitors. To this end, a series of small-molecule-based miR-122 activators and inhibitors have recently been discovered.[12] The miR-122 inhibitors **1** and **2** decreased the expression of miR-122 and caused 50% reduction in HCV viral load in vitro. In contrast, activator **3** increased the expression of miR-122 in HCC cells and successfully decreased cell viability by increasing caspase-3 and caspase-7 activities. Along with oligonucleotide-based therapeutics such as miravirsen, the small-molecule-based miR-122 regulators have great potential as new therapeutic agents for HCV infection and liver cancer.

MiR-4644-specific regulators

Chandrasekhar et al. carried out searches among chemicals based on an azaflavone scaffold and identified a couple of

compounds that inhibit the expression of *Drosophila* miR-14 and its human equivalent miR-4644.[37] MiR-14/miR-4644 has been shown to suppress cell death, and so small-molecule inhibitors of miR-4644 could induce G1-phase arrest and cell death in human breast carcinoma cells. Moreover, when flies were fed with the inhibitory compounds, miR-14 expression was decreased, leading to the upregulation of EGFP, a reporter protein for the level of miR-14 expression. This study is intriguing because the inhibitory effects of the compounds were confirmed in two different model systems—human and fly—and in the case of the fly it provides a simple in vivo assay system.

MiR-27a-specific regulators

In addition to the inhibitory effects of streptomycin on the maturation of miR-21 as described above, many other aminoglycoside antibiotics have been identified as miRNA regulators. It has been reported that neomycin, amikacin, and tobramycin, as well as streptomycin, block miR-27a function through direct interaction with pre-miR-27a.[38] A decrease in the levels of miR-27a due to aminoglycoside treatments also resulted in the upregulation of expression of its target (prohibitin). Given that polycationic aminoglycoside antibiotics often interact with a variety of polyanionic RNA molecules in nature, it may be expected that many more aminoglycosides that can regulate pre-miRNA through direct binding will be revealed.

Screening strategies

Despite having better druggable properties, small molecules require an initial screening process to select chemical compounds with miRNA-regulating activities. Accordingly, robust, efficient, and simple assay systems for high-throughput screening need to be developed. There are currently several assay systems that employ molecular beacon probes with fluorophores and quenchers, fluorescent proteins, or enzymes that emit visible light through a biochemical reaction with a substrate. As an alternative to experimental screening methods, virtual screening by computational approaches has been used. Virtual screening allows a large number of chemical compounds to be tested more rapidly and at a lower cost, and is expected to increase in popularity as a screening tool.

Molecular-beacon-based assays for miRNA maturation

This assay is designed to make use of a molecular beacon to identify potential inhibitors that block pre-miRNA maturation. Hairpin-shaped pre-miRNAs with no 3′-overhang are labeled with a fluorophore at the 5′-end and a quencher at the 3′-end to function as molecular beacons that report Dicer activity.[39] In the natural state, the doubly labeled pre-miRNAs do not emit fluorescence, because the fluorophore is quenched internally. However, when Dicer cleaves the pre-miRNA, the fluorophore and quencher are dissociated, leading to an increase in fluorescence. In contrast, if any molecule binds to the pre-miRNA and inhibits Dicer-mediated cleavage, there is no fluorescence because there is no Dicer activity. Although this assay system is very robust and can be applied for high-throughput screening of small molecules, it has some limitations. Dicer might not properly hydrolyze the pre-miRNA beacon in the presence of the fluorophore and the quencher at the two ends of the hairpin. Additionally, this type of assay is more technically demanding than cell-based assays using fluorescent proteins or reporter enzymes. Nonetheless, this is a cell-free assay system consisting of Dicer, the pre-miRNA beacon, and the chemical compounds to be tested. This assay allows investigation of the interactions between pre-miRNA and small molecules.

In order to rule out the possible undesirable off-target effects of the fluorophore and quencher on Dicer activity, Bose et al. developed a modified version of a molecular-beacon-based assay.[38] Instead of labeling pre-miRNAs, they label hairpin-shaped DNA beacons in which the loop sequence is complementary to the miRNA of interest. When Dicer cleaves pre-miRNAs and mature miRNAs are released, the miRNAs interact with the loop of the DNA-beacon, open up the stem-loop structure, and increase the fluorescence signal. However, in the presence of Dicer inhibitors, the DNA-beacons will keep their stem-loop structures and emit no fluorescence. By this method, aminoglycosides have been shown to inhibit Dicer-mediated cleavage of pre-miR-27a as described above in the "miR-27a-specific regulator" section. The inhibitory function of aminoglycosides was further confirmed by Dicer-blocking assay, luciferase reporter assay, quantitative real-time polymerase chain reaction (qRT-PCR), western blot, and S1 nuclease footprinting, supporting the robustness and sensitivity of the DNA-beacon-based screening.

BRCA-based assay

The BRCA (branched rolling-circle amplification) assay was originally developed to quantify a specific miRNA in a simple and sensitive way through modification of the rolling-circle amplification (RCA) assay.[40,41] Both BRCA and RCA assays use padlock-shaped DNA probes and phi29 DNA polymerase for rolling-circle amplification of DNA products containing target miRNA sequences. The BRCA assay, however, involves a second primer, which is complementary to the tandem repeats of the single-stranded RCA product and initiates branched rolling-circle amplification of DNA to displace downstream growing strands. The BRCA product is double-stranded DNA (dsDNA), and so it is possible to use SYBR Green I fluorescent dye for detecting BRCA products.

A new assay system based on the BRCA assay and unmodified pre-miRNA was established to monitor Dicer-mediated miRNA maturation.[41,42] This method is designed such that no fluorescence will be emitted unless mature miRNA is generated from pre-miRNA. When Dicer is active, unmodified pre-miRNAs are cleaved into mature miRNAs; these bind to a circular DNA template and initiate DNA polymerization to yield a single-stranded DNA product. Subsequently, a secondary primer binds to the primary single-stranded product and generates a dsDNA product, which can be detected by SYBR Green I dye. This BRCA reaction is initiated only in the presence of mature

miRNA. Therefore, if a Dicer-mediated pre-miRNA cleavage is blocked, the BRCA reaction will not occur, and no fluorescence will be emitted.

Another application of the BRCA-based assay is based on pyrophosphate (PPi) molecules released during the BRCA reaction in the presence of target miRNAs.[43] During DNA polymerization, the PPi is released when nucleosides are incorporated into the growing DNA chain. The released PPi is then converted into adenosine triphosphate (ATP), which supplies energy for firefly luciferase to generate a luminescence signal. Because the number of released PPi units is correlated with the amount of target miRNAs, this bioluminescent PPi assay combined with BRCA can quantitatively analyze miRNAs by detecting the emission of luminescence. Although the sensitivity and the robustness of the assay have been demonstrated well in a proof-of-concept experiment, no miRNA regulator has yet been identified by this method in a high-throughput manner.

Luciferase reporter assay

A luciferase reporter assay system was developed to identify small-molecule inhibitors of individual miRNAs.[8] In this assay, the luciferase cDNA is appended to DNA sequences complementary to a target miRNA (such as miR-21) in the 3′-UTR of the luciferase gene. The reporter construct is then stably transfected into HeLa cells, where it serves as a sensor to monitor the presence of the miRNA of interest. In the presence of either endogenous or exogenous mature miR-21 binding, luciferase activity is diminished, whereas in the absence of mature miR-21 it remains highly active. Accordingly, if a chemical compound inhibits miR-21 expression, the corresponding decrease in miRNA expression will lead to an increase in luciferase activity. This reporter system allows high-throughput screening of small-molecule inhibitors of single miRNAs, and it can be expanded to detect inhibitors for any miRNA of interest. Moreover, this system is advantageous because the possibility of false-positive hits due to compound toxicity is greatly reduced because inhibitors increase rather than decrease the reporter signal.

Secreted alkaline phosphatase (SEAP) reporter assay

We have developed a new assay system based on secreted alkaline phosphatase (unpublished results). Similarly to the luciferase reporter assay, a short DNA fragment containing sequences complementary to the miRNA of interest is placed in the downstream region of the SEAP gene. Unlike endogenous alkaline phosphatase, SEAP is secreted into the culture medium and allows the detection of reporter activity without requiring the preparation of cell lysates.[44] In the SEAP reporter assay, compounds are assayed for their ability to inhibit miRNA expression, which leads to the increased expression of SEAP. SEAP activity is measured by detecting the yellow water-soluble reaction product of alkaline phosphatase and its substrate (*p*-nitrophenyl phosphate). The SEAP system, which leaves cells intact because only culture supernatant is needed, allows cells to be retrieved and subjected to further analysis by flow cytometry, northern blot, and western blot, thereby providing an advantage over other systems.

Enhanced green fluorescence protein (EGFP) reporter assay

The EGFP reporter system was developed to monitor the activity of the RNAi pathway rather than the expression of specific miRNAs. In the original assay system, HeLa cells were cotransfected with plasmids expressing EGFP and red fluorescence protein (RFP) together with siRNA targeting EGFP mRNA.[45] In the presence of EGFP siRNA, the EGFP/RFP ratio was lower than those in control transfections lacking siRNA, and the addition of siRNA-enhancing small molecules further lowered the EGFP/RFP ratio. However, the original EGFP reporter system used transient transfections, and the transfection efficiency was highly variable between experiments. Therefore, a modified EGFP system was developed to address this limitation and to make the screening system amenable to high-throughput application.[9]

In the advanced EGFP reporter system, cells stably express both EGFP protein and an shRNA against EGFP, and the double transfectants have reduced but variable intensities of EGFP expression depending on the efficacy of the shRNA knockdown. A clone displaying a strong reduction in EGFP expression would be selected for the identification of RNAi inhibitors, whereas a clone displaying a modest reduction would be suitable for identifying both inhibitors and enhancers. With this system, the antibacterial agent enoxacin was shown to enhance RNAi and miRNA biogenesis, thereby reducing the siRNA dosage required to achieve gene knockdown in mammalian cells.[46]

In addition to its utility in identifying RNAi regulators, the EGFP reporter system can be modified to screen for small molecules that can regulate the expression of selective miRNAs by introducing complementary sequences of the target miRNA in the downstream region of the EGFP gene, similarly to the luciferase and SEAP reporter assays.

Fluorescence-polarization-based assay

Although most methods for high-throughput screening of miRNA or siRNA regulators are cell-based and use fluorescent proteins or reporter enzymes, a new in vitro method based on the fluorescence polarization (FP) of tetramethylrhodamine-labeled (TAMRA-labeled) small RNAs was developed.[11] In the absence of Ago2, the red fluorescent TAMRA-labeled miRNA is free to rotate and has low levels of polarization. However, when TAMRA-labeled miRNA is bound to Ago2, the miRNA·Ago2 complex rotates more slowly, resulting in a higher polarization value. Accordingly, the TAMRA-labeled miRNA, recombinant Ago2, and the compound being tested are mixed in an assay plate and incubated, and the FP level is measured. Because the assay tests the loading of individual miRNAs/siRNAs onto Ago2, it can be used to identify selective modulators of miRNAs/siRNAs, as well as compounds that act as general regulators of the RNAi pathway at the RISC loading step.

Virtual screening

In drug discovery, "virtual screening" refers to computational methods used to search databases of small molecules in order to identify those that are likely to bind a drug target.[47] There are two types of virtual screening: structure-based and ligand-based.[48]

There are many examples of ligand-based approaches.[49] One popular approach is the pharmacophore strategy.[50] Given a set of structurally diverse ligands that bind to a common receptor, an abstract model of the receptor, termed a pharmacophore, is created. Each ligand in the set is then compared to the pharmacophore model to determine the compatibility between the ligand and receptor.[49]

In a structure-based approach, virtual screening normally consists of molecular docking, followed by scoring/ranking of potential hits in terms of their likelihood of interacting with the target site.[51] The ligand-based pharmacophore method can be combined with the structure-based docking approach.[51]

Current in silico approaches to drug discovery are primarily for protein targets and are therefore not applicable to virtual screening of RNA targets, which have docking and scoring parameters that distinguish them from proteins.[52] Integrative approaches to modeling and virtual screening of RNA inhibitors based on five docking and eleven scoring methodologies have been proposed.[52] Applying these computational virtual screening methods led to the discovery of AC1MMYR2, an inhibitor of miR-21, which reverses epithelial-to-mesenchymal transition, thus suppressing tumor growth and progression.[10]

Validation

As described above, high-throughput screening methods based on reporter assays are often used to identify candidate miRNA regulators. However, the main drawback of these methods is the detection of false positives; this occurs when small molecules directly affect reporter gene activity rather than the miRNA expression. The regulatory effects of potential candidates therefore require validation by experimental methods such as northern blot and real-time PCR analysis.

Although very well established and widely used, northern blot analysis has low sensitivity and requires large amounts of RNA, and this leads to decreased utility in detecting the expression of low-abundance miRNAs. Several studies have reported improved northern blot methods that heighten sensitivity and effectiveness. For example, crosslinking RNA to a nylon membrane by using *N*′-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide (EDC) has been shown to improve the detection of small RNAs, and LNA-modified oligonucleotides can also be used as hybridization probes to increase sensitivity tenfold or more.[53] For the detection and quantification of miRNAs with low expression levels, qRT-PCR might be more useful than northern blot analysis. Because miRNAs are very short, distinct methods for designing PCR primers and amplifying miRNAs of interest have been devised. The amplification of specific miRNAs is detected by using fluorescent compounds that can bind DNA duplexes, such as SYBR Green I dye, or by using fluorescence-labeled probes such as the Taqman probe, which relies on the sequence-specific detection of the desired product, resulting in increased specificity and sensitivity.[54] The development of multiplex PCR with use of multiple primers might be useful for amplifying many miRNAs simultaneously.[55]

Database of validated small-molecule regulators

Recently, the SM2miR database was developed to provide information on experimentally validated small molecules that control miRNA expression.[56] By August 2013, SM2miR had stored 4516 relationships between 206 small molecules and 1085 miRNAs, and these entries can be readily retrieved by searching for either the miRNA or the small molecule name. Each entry includes information about each interaction, including the detection method, miRNA expression pattern, tissues or cell lines used for detection, DrugBank Accession number, and PubChem Compound Identifier, providing a comprehensive repository of small molecules that regulate microRNAs. SM2miR is freely available on the web at http://bioinfo.hrbmu.edu.cn/SM2miR.

Outlook

Numerous studies have revealed the association between miRNAs and diseases, thus emphasizing the importance of miRNAs for disease control and diagnosis. Thanks to their ability to regulate gene expression effectively, miRNAs have attracted great attention as new drug targets, and a growing body of research has focused on the development of miRNA-targeted therapies. However, in relation to the number of known miRNA–disease associations, the numbers of identified oligonucleotide- or small-molecule-based modulators of miRNAs are minuscule. The development of small-molecule regulators remains in the early stages, whereas that of oligonucleotide-based diagnostics and therapeutics has progressed to preclinical or clinical trials. Although there are benefits of using small molecules, the screening of compound libraries and discovery of new small-molecule drug candidates is a demanding task. With the development of experiment-based or computer-aided screening systems, as well as the invention of oligonucleotides with diverse modifications, more miRNA regulators may be anticipated to emerge for the treatment of various diseases. Furthermore, it may be expected that the first therapeutic intervention targeting miRNAs will emerge soon.

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[1] D. P. Bartel, *Cell* **2004**, *116*, 281 – 297.
[2] a) R. C. Friedman, K. K. Farh, C. B. Burge, D. P. Bartel, *Genome Res.* **2009**, *19*, 92 – 105; b) S. Griffiths-Jones, R. J. Grocock, S. van Dongen, A. Bateman, A. J. Enright, *Nucleic Acids Res.* **2006**, *34*, D140 – D144.
[3] a) N. Bushati, S. M. Cohen, *Annu. Rev. Cell Dev. Biol.* **2007**, *23*, 175 – 205; b) T. C. Chang, J. T. Mendell, *Annu. Rev. Genomics Hum. Genet.* **2007**, *8*, 215 – 239.
[4] a) M. Mraz, S. Pospisilova, *Expert Rev. Hematol.* **2012**, *5*, 579 – 581; b) G. A. Calin, C. D. Dumitru, M. Shimizu, R. Bichi, S. Zupo, E. Noch, H. Aldler, S. Rattan, M. Keating, K. Rai, L. Rassenti, T. Kipps, M. Negrini, F. Bullrich, C. M. Croce, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 15524 – 15529.
[5] a) S. M. Hammond, *Cancer Chemother. Pharmacol.* **2006**, *58*, 63 – 68; b) R. Garzon, G. A. Calin, C. M. Croce, *Annu. Rev. Med.* **2009**, *60*, 167 – 179; c) W. C. Cho, *Biochim. Biophys. Acta Rev. Cancer* **2010**, *1805*, 209 – 217.
[6] a) D. M. Pereira, P. M. Rodrigues, P. M. Borralho, C. M. Rodrigues, *Drug Discovery Today* **2013**, *18*, 282 – 289; b) R. Garzon, G. Marcucci, C. M. Croce, *Nat. Rev. Drug Discovery* **2010**, *9*, 775 – 789; c) P. Gandellini, V. Profumo, M. Folini, N. Zaffaroni, *Expert Opin. Ther. Targets* **2011**, *15*, 265 – 279.
[7] Z. Wang in *Methods in Molecular Biology, Vol. 676: MicroRNA and Cancer: Methods and Protocols* (Ed.: W. Wu), **2011**, Humana, Totowa, pp. 211 – 223.
[8] K. Gumireddy, D. D. Young, X. Xiong, J. B. Hogenesch, Q. Huang, A. Deiters, *Angew. Chem. Int. Ed.* **2008**, *47*, 7482 – 7484; *Angew. Chem.* **2008**, *120*, 7592 – 7594.
[9] G. Shan, Y. Li, J. Zhang, W. Li, K. E. Szulwach, R. Duan, M. A. Faghihi, A. M. Khalil, L. Lu, Z. Paroo, A. W. Chan, Z. Shi, Q. Liu, C. Wahlestedt, C. He, P. Jin, *Nat. Biotechnol.* **2008**, *26*, 933 – 940.
[10] Z. Shi, J. Zhang, X. Qian, L. Han, K. Zhang, L. Chen, J. Liu, Y. Ren, M. Yang, A. Zhang, P. Pu, C. Kang, *Cancer Res.* **2013**, *73*, 5519 – 5531.
[11] G. S. Tan, C. H. Chiu, B. G. Garchow, D. Metzler, S. L. Diamond, M. Kiriakidou, *ACS Chem. Biol.* **2012**, *7*, 403 – 410.
[12] D. D. Young, C. M. Connelly, C. Grohmann, A. Deiters, *J. Am. Chem. Soc.* **2010**, *132*, 7976 – 7981.
[13] K. A. Lennox, M. A. Behlke, *Gene Ther.* **2011**, *18*, 1111 – 1120.
[14] a) B. Vester, J. Wengel, *Biochemistry* **2004**, *43*, 13233 – 13241; b) K. J. Friedman, J. Kole, J. A. Cohn, M. R. Knowles, L. M. Silverman, R. Kole, *J. Biol. Chem.* **1999**, *274*, 36193 – 36199; c) H. Ikeda, R. Fernandez, A. Wilk, J. J. Barchi, X. Huang, V. E. Marquez, *Nucleic Acids Res.* **1998**, *26*, 2237 – 2244.
[15] G. F. Deleavey, M. J. Damha, *Chem. Biol.* **2012**, *19*, 937 – 954.
[16] a) J. Krützfeldt, N. Rajewsky, R. Braich, K. G. Rajeev, T. Tuschl, M. Manoharan, M. Stoffel, *Nature* **2005**, *438*, 685 – 689; b) G. Hutvágner, M. J. Simard, C. C. Mello, P. D. Zamore, *PLoS Biol.* **2004**, *2*, e98.
[17] A. G. Bader, D. Brown, J. Stoudemire, P. Lammers, *Gene Ther.* **2011**, *18*, 1121 – 1126.
[18] E. S. Hildebrandt-Eriksen, V. Aarup, R. Persson, H. F. Hansen, M. E. Munk, H. Ørum, *Nucleic Acid Ther.* **2012**, *22*, 152 – 161.
[19] J. Hu, Y. Xu, J. Hao, S. Wang, C. Li, S. Meng, *Protein Cell* **2012**, *3*, 364 – 371.
[20] M. Lindow, S. Kauppinen, *J. Cell Biol.* **2012**, *199*, 407 – 412.
[21] A. G. Bader, *Front. Genet.* **2012**, *3*, 120.
[22] M. S. Ebert, J. R. Neilson, P. A. Sharp, *Nat. Methods* **2007**, *4*, 721 – 726.
[23] D. Sayed, S. Rane, J. Lypowy, M. He, I. Y. Chen, H. Vashistha, L. Yan, A. Malhotra, D. Vatner, M. Abdellatif, *Mol. Biol. Cell* **2008**, *19*, 3272 – 3282.
[24] Y. Liu, Y. Han, H. Zhang, L. Nie, Z. Jiang, P. Fa, Y. Gui, Z. Cai, *PLoS One* **2012**, *7*, e52280.
[25] T. Haraguchi, Y. Ozaki, H. Iba, *Nucleic Acids Res.* **2009**, *37*, e43.
[26] A. K. Hollensen, R. O. Bak, D. Haslund, J. G. Mikkelsen, *RNA Biol.* **2013**, *10*, 406 – 414.
[27] A. Tachibana, Y. Yamada, H. Ida, S. Saito, T. Tanabe, *FEBS Lett.* **2012**, *586*, 1529 – 1532.
[28] a) Z. Wang in *Methods in Molecular Biology, Vol. 676: MicroRNA and Cancer: Methods and Protocols* (Ed.: Wei Wu), Humana, Totowa, **2011**, pp. 43 – 49; b) Z. Wang, X. Luo, Y. Lu, B. Yang, *J. Mol. Med.* **2008**, *86*, 771 – 783.
[29] a) R. Kumarswamy, I. Volkmann, T. Thum, *RNA Biol.* **2011**, *8*, 706 – 713; b) A. M. Krichevsky, G. Gabriely, *J. Cell. Mol. Med.* **2009**, *13*, 39 – 53.
[30] a) W. C. Cho, *Mol. Cancer* **2007**, *6*, 60; b) X. Pan, Z. X. Wang, R. Wang, *Cancer Biol. Ther.* **2010**, *10*, 1224 – 1232.
[31] D. Bose, G. Jayaraj, H. Suryawanshi, P. Agarwala, S. K. Pore, R. Banerjee, S. Maiti, *Angew. Chem. Int. Ed.* **2012**, *51*, 1019 – 1023; *Angew. Chem.* **2012**, *124*, 1043 – 1047.
[32] Y. Wang, R. R. Rando, *Chem. Biol.* **1995**, *2*, 281 – 290.
[33] L. Rossi, E. Bonmassar, I. Faraoni, *Pharmacol. Res.* **2007**, *56*, 248 – 253.
[34] a) N. S. Wickramasinghe, T. T. Manavalan, S. M. Dougherty, K. A. Riggs, Y. Li, C. M. Klinge, *Nucleic Acids Res.* **2009**, *37*, 2584 – 2595; b) S. D. Selcuklu, M. T. Donoghue, M. J. Kerin, C. Spillane, *Biochem. Biophys. Res. Commun.* **2012**, *423*, 234 – 239; c) P. Bhat-Nakshatri, G. Wang, N. R. Collins, M. J. Thomson, T. R. Geistlinger, J. S. Carroll, M. Brown, S. Hammond, E. F. Srour, Y. Liu, H. Nakshatri, *Nucleic Acids Res.* **2009**, *37*, 4850 – 4861.
[35] a) M. Girard, E. Jacquemin, A. Munnich, S. Lyonnet, A. Henrion-Caude, *J. Hepatol.* **2008**, *48*, 648 – 656; b) C. Jopling, *RNA Biol.* **2012**, *9*, 137 – 142.
[36] a) C. L. Jopling, *Biochem. Soc. Trans.* **2008**, *36*, 1220 – 1223; b) H. Kutay, S. Bai, J. Datta, T. Motiwala, I. Pogribny, W. Frankel, S. T. Jacob, K. Ghoshal, *J. Cell. Biochem.* **2006**, *99*, 671 – 678.
[37] S. Chandrasekhar, S. N. Pushpavalli, S. Chatla, D. Mukhopadhyay, B. Ganganna, K. Vijeender, P. Srihari, C. R. Reddy, M. Janaki Ramaiah, U. Bhadra, *Bioorg. Med. Chem. Lett.* **2012**, *22*, 645 – 648.
[38] a) D. Bose, G. G. Jayaraj, S. Kumar, S. Maiti, *ACS Chem. Biol.* **2013**, *8*, 930 – 938.
[39] a) B. P. Davies, C. Arenz, *Angew. Chem. Int. Ed.* **2006**, *45*, 5550 – 5552; *Angew. Chem.* **2006**, *118*, 5676 – 5679; b) B. P. Davies, C. Arenz, *Bioorg. Med. Chem.* **2008**, *16*, 49 – 55.
[40] S. Neubacher, C. Arenz, *ChemBioChem* **2009**, *10*, 1289 – 1291.
[41] S. Neubacher, C. M. Dojahn, C. Arenz, *ChemBioChem* **2011**, *12*, 2302 – 2305.
[42] S. Neubacher, C. Arenz in *Methods in Molecular Biology, Vol. 1095: Detection of Micro RNA Maturation Using Unmodified pre-miRNA and Branched Rolling Circle Amplification* (Ed.: C. Arenz), Humana, Totowa, **2014**, pp. 109 – 119.
[43] Y. Mashimo, M. Mie, S. Suzuki, E. Kobatake, *Anal. Bioanal. Chem.* **2011**, *401*, 221 – 227.
[44] J. Berger, J. Hauber, R. Hauber, R. Geiger, B. R. Cullen, *Gene* **1988**, *66*, 1 – 10.
[45] Y. L. Chiu, C. U. Dinesh, C. Y. Chu, A. Ali, K. M. Brown, H. Cao, T. M. Rana, *Chem. Biol.* **2005**, *12*, 643 – 648.
[46] Y. Li, C. He, P. Jin, *Chem. Biol.* **2010**, *17*, 584 – 589.
[47] U. Rester, *Curr. Opin. Drug Discovery Dev.* **2008**, *11*, 559 – 568.
[48] C. McInnes, *Curr. Opin. Chem. Biol.* **2007**, *11*, 494 – 502.
[49] H. Sun, *Curr. Med. Chem.* **2008**, *15*, 1018 – 1024.
[50] J. S. Mason, A. C. Good, E. J. Martin, *Curr. Pharm. Des.* **2001**, *7*, 567 – 597; S. Pirhadi, F. Shiri, J. B. Ghasemi, *Curr. Top. Med. Chem.* **2013**, *13*, 1036 – 1047.
[51] T. T. Talele, S. A. Khedkar, A. C. Rigby, *Curr. Top. Med. Chem.* **2010**, *10*, 127 – 141.
[52] X. Chen, C. Huang, W. Zhang, Y. Wu, C. Y. Zhang, Y. Zhang, *Chem. Commun.* **2012**; *48*, 6432 – 6434.
[53] A. Válóczi, C. Hornyik, N. Varga, J. Burgyán, S. Kauppinen, Z. Havelda, *Nucleic Acids Res.* **2004**, *32*, e175; G. S. Pall, C. Codony-Servat, J. Byrne, L. Ritchie, A. Hamilton, *Nucleic Acids Res.* **2007**, *35*, e60.
[54] a) T. D. Schmittgen, J. Jiang, Q. Liu, L. Yang, *Nucleic Acids Res.* **2004**, *32*, e43; b) G. Wan, Q. E. Lim, H. P. Too, *RNA* **2010**, *16*, 1436 – 1445; c) T. D. Schmittgen, E. J. Lee, J. Jiang, A. Sarkar, L. Yang, T. S. Elton, C. Chen, *Methods* **2008**, *44*, 31 – 38.
[55] S. Hwang, B. Kang, J. Hong, A. Kim, H. Kim, K. Kim, D. S. Cheon, *J. Med. Virol.* **2013**, *85*, 1274 – 1279.
[56] X. Liu, S. Wang, F. Meng, J. Wang, Y. Zhang, E. Dai, X. Yu, X. Li, W. Jiang, *Bioinformatics* **2013**, *29*, 409 – 411.

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