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Toward MicroRNA-Based Therapeutics for Heart Disease - the sense in antisense

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Abstract

MicroRNAs act as negative regulators of gene expression by inhibiting the translation or promoting the degradation of target mRNAs. Because individual microRNAs often regulate the expression of multiple target genes with related functions, modulating the expression of a single microRNA can, in principle, influence an entire gene network and thereby modify complex disease phenotypes. Recent studies have identified signature expression patterns of microRNAs associated with pathological cardiac hypertrophy, heart failure and myocardial infarction in humans and mouse models of heart disease. Gain- and loss-of-function studies in mice have revealed profound and unexpected functions for these microRNAs in numerous facets of cardiac biology, including the control of myocyte growth, contractility, fibrosis, and angiogenesis, providing glimpses of new regulatory mechanisms and potential therapeutic targets for heart disease. Especially intriguing is the discovery of a network of muscle-specific microRNAs embedded within myosin heavy chain genes, which control myosin expression and the response of the heart to stress and thyroid hormone signaling. Disease-inducing cardiac microRNAs can be persistently silenced *in vivo* through systemic delivery of anti-miRs, allowing for the direct therapeutic modulation of disease mechanisms. Here we summarize current knowledge of the roles of miRNAs in heart disease and consider the advantages and potential challenges of microRNA-based approaches compared to conventional drug-based therapies.

Keywords

microRNA; heart disease; remodeling; miR-based therapy

Introduction

Diseases of the cardiovascular system represent the primary cause of human morbidity and mortality, underscoring the need for innovative new therapies and diagnostics for heart disease. Recent studies have begun to unveil powerful and unexpected roles for microRNAs (miRNAs)

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in numerous forms of cardiovascular disease, providing a unique opportunity to translate this knowledge into the clinical setting in the form of miRNA-based therapeutics. Here we summarize the rapidly expanding knowledge of the involvement of miRNAs in different aspects of heart disease and consider the therapeutic feasibility and future challenges associated with the manipulation of miRNA function *in vivo*.

MiRNA biogenesis and function

MiRNAs (miRNAs) are short, noncoding RNA molecules that act as negative regulators of gene expression by inhibiting mRNA translation or promoting mRNA degradation¹. First studied in model organisms, such as *C. elegans* and *Drosophila*, where they control key steps in development², recent studies have revealed important roles for miRNAs in diseases of adult tissues, especially in cancer and cardiovascular disease, in mammals and humans.

The human genome has been estimated to encode up to 1000 miRNAs that are predicted to regulate a third of all genes³. In mammals, miRNAs originate from a primary transcript, called a pri-miR, which is transcribed by RNA polymerase II and regulated by transcription factors like conventional mRNAs. The pri-miR, which may be hundreds to thousands of nucleotides long, undergoes nuclear cleavage by a ribonuclease III called Drosha and the double-stranded DNA binding protein DGCR8/Pasha to generate a hairpin-shaped pre-miRNA. These intermediates are transported to the cytoplasm via by the nuclear export factor exportin 5. Within the cytoplasm, the ribonuclease III Dicer and its cofactors (PACT and TRBP) process the precursors into 19- to 25-nucleotide miRNA duplexes. The double-stranded RNA molecule dissociates and one strand is incorporated into the RNA Induced Silencing Complex (RISC). The miRNA-loaded RISC is capable of binding to target mRNAs and inhibits their translation by cleavage and degradation of mRNA or by blocking translation through several different mechanisms. Although most miRNAs in plants hybridize to target mRNAs with perfect complementarity, in animals the 5' proximal 'seed' region (nucleotides 2-8) appears to be the primary determinant of the pairing specificity of the miRNA to the 3' untranslated region (UTR) of a target mRNA⁴⁻⁸. In addition to Watson-Crick base pairing, the efficiency of transcriptional repression also depends on the number and configuration of mismatches between the miRNA and the target mRNA, the secondary structure of the surrounding region, and the number of target sequences on the mRNA⁹.

An especially powerful feature of miRNA-based regulation is the ability of single miRNAs to regulate multiple functionally related mRNAs, as shown for the liver-specific miR-122, which regulates multiple metabolic genes^{10, 11}. The targeting of multiple mRNAs that participate in common cellular processes contrasts with the actions of most drugs, which are directed at specific targets, and enables miRNAs to effectively regulate complex intracellular pathways, thereby potentially avoiding redundant mechanisms that might bypass a single inhibited target.

Approximately half of all known miRNA genes are clustered in the genome and transcribed as polycistronic primary transcripts with the remainder expressed as individual transcripts from intergenic or intronic locations. Regardless of genomic organization, miRNAs function in a distinct yet cooperative manner to regulate cellular processes by coordinately targeting related proteins^{12, 13}. Additionally, miRNAs often belong to families of closely related or identical sequences. Due to their homology in the seed sequence, the related miRNAs are able to target the same mRNAs, which enhances the efficiency of repression.

MiRNAs as disease determinants

It is becoming increasingly apparent that the aberrant expression of miRNAs is causally related to a variety of disease states like cancer, diabetes and heart failure. Stress-induced up-regulation of miRNAs can lead to the down-regulation of a set of targeted mRNAs, while down-regulation

of miRNAs can result in up-regulation of target mRNAs because of the loss of tonic inhibitory control of the miRNA on its target mRNA. Ultimately, it is the pattern of miRNA-induced gene expression that contributes to the resultant disease phenotype¹⁴. Stress also induces changes in miRNA function. Recently, it was shown that different forms of stress relieve mRNAs from miRNA repression by releasing mRNAs from P-bodies and promoting their entry into polysomes, thereby enhancing translation of the pre-existing mRNA¹⁵. Thus, while miRNAs appear to act as moderate regulators under baseline conditions, both changes in expression and function in response to stress increase their influence under conditions of disease states. The apparent roles of miRNAs in disease have led to an increasing interest in miRNA regulation as a therapeutic and diagnostic approach¹⁶.

MiRNAs in cardiac disease

The adult heart responds to injury or hemodynamic overload by activating a variety of intracellular signaling pathways and transcriptional mediators that promote myocyte hypertrophy, re-expression of an embryonic gene program, and remodeling of the extracellular matrix. These events lead to left ventricular dilation (remodeling) and progressive myocardial fibrosis that can culminate in cardiac arrhythmias and failure¹⁷. Several recent studies performed microarray analyses to determine whether miRNAs are dysregulated in hypertrophic and failing hearts¹⁸⁻²³. These studies have revealed signature patterns of miRNAs that are up- and down-regulated during pathological cardiac remodeling in rodents and humans. While miR-1, miR-29, miR-30, miR-133, and miR-150 have often been found to be down-regulated, miR-21, miR-23a, miR-125, miR-195, miR-199 and miR-214 are up-regulated with hypertrophy. Gain-and-loss of function studies have implicated these miRNAs in the different aspects of the remodeling process during the progression of heart disease. The continuous discovery of new miRNAs suggests that the current collection of miRNAs implicated in cardiovascular disease is likely to be incomplete.

Cardiomyocyte hypertrophy and remodeling

Cardiomyocyte hypertrophy is the dominant cellular response to virtually all forms of hemodynamic overload, endocrine disorders, myocardial injury or inherited mutations in a variety of structural and contractile proteins. Pathological hypertrophy results in loss of cardiac function and is the major predictor of heart failure and sudden death²⁴. As such, there has been intense interest in deciphering the underlying molecular mechanisms and in discovering novel therapeutic targets for suppressing adverse cardiac growth. In vitro experiments using either over-expression or knockdown of miRNAs in cultured cardiomyocytes indicate that several of these miRNAs indeed actively participate in cardiomyocyte hypertrophy^{18, 20-23, 25}.

One miRNA that is consistently induced by cardiac stress, miR-21, appears to function as a regulator of cardiac growth and fetal gene activation in primary cardiomyocytes in vitro, although its role during myocyte hypertrophy remains controversial^{18, 21}. Cheng et al. reported that oligonucleotide-mediated knockdown of this miRNA can suppress cardiomyocyte growth and fetal gene expression in response to hypertrophic agonists, while Tatsuguchi et al. showed miR-21 knockdown enhances myocyte hypertrophy with over-expression resulting in a blunted hypertrophic response. It is notable in this regard that miR-21 has been reported to modulate proliferation, both positively and negatively, and to suppress apoptosis in transformed cells²⁶. More recently Sayed *et al.* reported miR-21 to modulate the formation of cellular protrusions through the regulation of sprouty2, an intracellular inhibitor of MAP kinase signaling²⁷. Although the significance of this function of miR-21 in cardiac pathogenesis remains uncertain, these connections might function to enhance conduction during cardiac hypertrophy.

Another miR that is consistently up-regulated in rodent and human hypertrophic hearts is miR-195. Intriguingly, forced expression of miR-195, in primary cardiomyocytes or in the

hearts of transgenic mice is sufficient to drive hypertrophic growth and myocyte disarray, resulting in dilated cardiomyopathy and heart failure²³, implying that up-regulation of miR-195 during cardiac hypertrophy actively contributes to the disease process. miR-195 belongs to the miR-15 family, which consists of miR-15, -16, -195 -424 and -497. Interestingly, both miR-15a and miR-16-1 are deleted or down-regulated in the majority of chronic lymphocytic leukemias and are inversely correlated to Bcl2 expression, an anti-apoptosis protein²⁸. Conversely, negative regulation of Bcl2 by miR-15 and -16 induces apoptosis in cancer cells²⁹. In the heart, Bcl2 is also involved in myocyte cell loss that contributes to a variety of cardiac pathologies, including heart failure and those related to ischemia/reperfusion injury³⁰. It will be interesting to determine whether repression of Bcl2 by members of the miR-15 family induces a loss of myocytes, thereby leading to the dilative phenotype seen in the miR-195 transgenic animals.

A recent report found both miR-1 and miR-133 to be down-regulated in human heart disease, as well as in three models of cardiac hypertrophy²⁵. Interestingly, the expression of these miRNAs was diminished during both physiological and pathological hypertrophy, suggesting they participate in a general hypertrophic program. Indeed, knockdown of miR-133 in mice by infusion of an antisense RNA oligonucleotide appeared sufficient to induce significant hypertrophic growth of the heart with induction of fetal gene expression compared to saline treated mice²⁵. Conversely, adenoviral-mediated over-expression of miR-133 in cardiomyocytes inhibited agonist-induced hypertrophy. These results suggest an active role for miR-133 in the inhibition of cardiac hypertrophy, and imply that modulation of miR-133 levels in vivo may serve as a therapy for modulating hypertrophic growth.

Cardiac fibrosis

Apart from the induction of hypertrophy of cardiomyocytes, miRNAs additionally regulate other fundamental aspects of the response of the heart to injury, such as alteration of the extracellular matrix. Cardiac myocytes are normally surrounded by a fine network of collagen fibers. In response to pathological stress or myocardial infarction, cardiac fibroblasts secrete extracellular matrix proteins disproportionately and excessively. Myocardial fibrosis, a characteristic of all forms of cardiac pathology, leads to mechanical stiffness, which contributes to contractile dysfunction³¹. A subset of miRNAs is enriched in cardiac fibroblasts compared to cardiomyocytes, including miR-21 and members of the miR-29 family³². Since it is well established that miR-21 functions as an oncogene and has a role in tumorigenesis by promoting cell proliferation³³, it is tempting to speculate that the induction of miR-21 in fibroblasts of diseased hearts might contribute, at least partially, to the increase in fibroblast proliferation. If true, inhibition of miR-21 might inhibit fibroblast proliferation and block cardiac fibrosis.

Myocardial infarction (MI) also induces severe cardiac fibrosis in the infarcted area, and hypertrophy and remodeling in the remote myocardium. Profiling of miRNA expression levels in the border zone of the infarct and remote myocardium both 3 days and 2 weeks post MI revealed a striking miR expression pattern³⁴. Among the regulated miRs, the miR-29 family is dramatically down-regulated in the border zone flanking the infarcted area. Our data indicate this down-regulation of miR-29 to be responsible for the induction of collagens and additional extracellular matrix genes, and thereby actively contributes to cardiac fibrosis in response to MI. miR-29 is also down-regulated in disease models for cardiac hypertrophy and failure²³. These data imply that therapeutic up-regulation of miR-29 in response to an ischemic event or cardiac stress might prevent the onset of cardiac fibrosis and thereby maintain cardiac function.

Arrhythmia

In diseased hearts, regional changes in electrophysiology can result in non-uniform impulse propagation, which can lead to arrhythmias. Although arrhythmias usually develop as a result

of cardiac disease or inherited gene mutations in ion channels, several miRNAs, including miR-1 and -133, have recently been implicated in electrophysiological abnormalities. While both *in vitro* and *in vivo* data suggest a possible role for miR-1 in myocyte hypertrophy^{20, 25, 35}, miR-1 appears to play a major role in cardiac development and conductance. Targeted deletion of miR-1-2 in mice resulted in 50% lethality largely due to ventricular-septal defects³⁶. However, approximately half of the surviving mutant animals experienced electrophysiological defects and suffered sudden death. This effect was attributed to the up-regulation of the mRNA encoding the *Irx5* transcription factor, a direct target of miR-1. Prior studies showed that *Irx5* negatively regulates the Kv4.2 potassium channel and is thereby critical for maintaining the ventricular repolarization gradient³⁷. The involvement of miR-1 in cardiac conductance was confirmed in a study by Yang et al. who reported miR-1 expression to be up-regulated in humans with coronary artery disease³⁸. *In vivo* gene transfer was employed to either enhance or inhibit miR-1 expression in the infarcted myocardium. While injection of miR-1 into the infarcted myocardium exacerbated arrhythmogenesis, miR-1 specific knockdown suppressed arrhythmias. These data imply miR-1 to be involved in electrical remodeling and arrhythmias, effects that were attributed to the transcriptional repression of *KCNJ2* and *GJA1*³⁸. In addition to miR-1, miR-133 is also known to influence cardiac conductance. Xiao et al. found the up-regulation of miR-133 in a diabetic rabbit model to be responsible for the down-regulation of the ether-a-go-go related gene *ERG*, which is likely responsible for the arrhythmias in diabetic hearts due to QT prolongation³⁹. On the other hand, work from the same group showed that the pacemaker channel gene *HCN2* can be regulated *in vitro* by miR-133 manipulation and that down-regulation of miR-133 during cardiac hypertrophy induces an increase in expression of the *HCN2* gene, while over-expression of miR-133 inhibits *HCN2* induction during myocyte hypertrophy³⁹⁻⁴¹.

Cardiac contractility

Another hallmark of pathological hypertrophy and heart failure is the re-activation of a set of fetal cardiac genes, including those encoding atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP) and fetal isoforms of contractile proteins, such as skeletal α -actin and β -myosin heavy chain (MHC). These genes are typically repressed post-natally and replaced by the expression of a set of adult cardiac genes⁴². The consequences of fetal gene expression on cardiac function and remodeling (e.g. fibrosis) are not completely understood, but the up-regulation of β MHC, a slow ATPase, and down-regulation of α MHC, a fast contracting ATPase, in response to stress has been implicated in the diminution of cardiac function⁴². Relatively minor changes in the ratio of α - to β MHC have been shown to have profound effects on cardiac contractility in humans and rodents⁴³⁻⁴⁶. Thus, much attention has focused on understanding the mechanisms that regulate α - and β MHC switching and on potential approaches for therapeutically manipulating these mechanisms.

We discovered that miR-208, a miRNA encoded within intron 27 of the α MHC gene, plays a key role in the expression of β MHC in response to cardiac stress⁴⁷. Although the expression level of miR-208 remains stable during cardiac stress, this miRNA appears to fulfill a dominant function in regulating cardiac hypertrophy and remodeling. In response to pressure overload by thoracic aortic constriction or signaling by calcineurin, a calcium, calmodulin-dependent phosphatase that drives pathological remodeling of the heart, miR-208 null mice showed virtually no hypertrophy of cardiomyocytes or fibrosis and were unable to up-regulate β MHC expression. In contrast, other stress responsive genes, such as those encoding ANF and BNP, were strongly induced in miR-208 mutant animals, demonstrating that miR-208 is dedicated specifically to the control of β MHC expression, which can be uncoupled from other facets of the cardiac stress response. These data indicate that miR-208 regulates cardiac remodeling, at least in part, by regulating the stress-induced increase in β MHC expression. Because miR-208 expression does not change in response to stress, its requirement for stress-dependent cardiac

remodeling suggests that it cooperates with stress signaling to reprogram cardiac gene expression. Since even a subtle shift towards β MHC reduces mechanical performance and efficiency of the adult heart^{44-46, 48}, it might be of therapeutic value to exploit miR-208 regulation to prevent an increase in β MHC expression during cardiac disease. The cardiac specificity, absence of overt maladaptive effects in the miR-208 mutant animals, and dedication of miR-208 to the cardiac stress response, but not to normal cardiac development, make miR-208 (and its down-stream effectors) an attractive therapeutic target for manipulating β MHC levels. However, as for all miRNAs, therapeutic modulation of miR-208 expression or function in vivo might affect targets in addition to β MHC.

Heart failure

All human data on miRNA function in heart disease thus far has come from heart failure patients^{19, 22, 23}. Heart failure is defined as the inability of the heart to pump sufficient blood to the organism and is a frequent and fatal outcome of hypertrophy developed under pathological circumstances. For obvious reasons, it is problematic to obtain human cardiac tissue during the hypertrophic phase, before the onset of heart failure. However, our own data and others have indicated that there is at least a partial overlap between the miRNAs regulated during hypertrophy and heart failure, and the miRNA expression pattern seems to dictate the disease state¹⁹. Northern blot analysis on both non-failing and failing human samples indicated that several miRNAs are regulated in a comparable manner as in hypertrophic mouse models²³. In addition to our data, Ikeda and coworkers compared miRNA expression in three different types of human heart disease (ischemic cardiomyopathy, dilated cardiomyopathy, and aortic stenosis) with normal heart. Among the 87 miRNAs detected in the heart, roughly half were differentially expressed in at least one disease group, while seven miRNAs were regulated in the same direction in all three disease states¹⁹. Although several studies already indicated miRNA expression to be regulated in human heart disease^{22, 23, 25, 37}, this study is the first to show commonalities in expression between distinct disease etiologies. These divergent miRNA expression patterns point to miRNAs as biomarkers for subtle phenotypic differences and disease progression and imply that they are active participants in the disease processes. As during hypertrophy, a hallmark of heart failure is the re-expression of a fetal gene program. A study by Thum et al. revealed similarities in the miRNA expression patterns of failing and fetal human heart, in that 353 mRNAs were found to be up-regulated > 2-fold in common in these two situations with respect to normal adult heart tissue²². In addition, microarray analysis indicated that a large fraction of the miRNAs tested were either up- or down-regulated in the same direction in fetal and failing heart with respect to normal adult cardiac tissue; the mRNAs that were up-regulated in failing heart contained binding sites mainly for the down-regulated miRNAs, and vice versa²². Thus, a shift towards fetal gene expression seems to be partly due to a change in cardiac miRNA levels occurring with hypertrophy and failure.

Angiogenesis

The formation of new blood vessels through neoangiogenesis is essential for cardiac repair following MI, when collateral vessels form at the site of the infarct and maintain blood flow to ischemic tissue⁴⁹. Myocardial vascularization following MI requires signaling by angiogenic growth factors, such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF)^{50,51}. miR-126 is an endothelial cell-specific miRNA that plays an essential role in neoangiogenesis following MI and in maintenance of vascular integrity in vivo⁵². The actions of miR-126 reflect, at least in part, its potentiation of MAP kinase signaling downstream of VEGF and FGF. Spred-1, an intracellular inhibitor of the Ras/MAP kinase pathway, serves as a key target for repression by miR-126^{52,53}. Thus, in the absence of miR-126, Spred-1 expression is elevated, resulting in repression of angiogenic signaling. Conversely, miR-126 over-expression relieves the repressive influence of Spred-1 on the signaling pathways activated by VEGF and FGF, favoring angiogenesis. A subset of miR-126

null mice dies during embryogenesis from vascular leakage, and those mutant mice that survive to adulthood are prone to cardiac rupture and lethality following MI, with defective neovascularization of the infarct⁵². These findings suggest that strategies to elevate miR-126 expression in the ischemic myocardium could enhance cardiac repair.

Cardiac injury, in addition to activating the migration and proliferation of endothelial cells, which give rise to collateral vessels in the ischemic myocardium, also promotes the homing of circulating hematopoietic progenitor cells to sites of ischemia and their contribution to cardiac repair^{54, 55}. miR-126 is expressed in hematopoietic stem cells, and might therefore contribute to the regenerative functions of this cell population^{56, 57}. Numerous other miRNAs are expressed in endothelial cells and have been implicated in various aspects of vascular biology, including inflammation and atherosclerosis. These studies are beyond the scope of this review and have recently been reviewed in detail⁵⁸.

Taken together, these data strongly implicate miRNAs in the different aspects of heart disease and highlight their potential as targets and/or agents for novel therapies (Fig.1).

miRNA modulation as a therapeutic approach

Some lessons learned from existing antisense technology and gene therapy approaches can be adapted to manipulate miRNA levels *in vivo*. To date, there are several tools available to selectively target miRNA pathways (Fig. 2). Modified antisense oligonucleotides targeting the mature miRNA sequence, antimiRs, can reduce the levels of pathogenic or aberrantly expressed miRNAs¹¹. Conversely, miRNA mimics can serve to elevate the levels of miRNAs with salutary functions⁴¹. It is important to note that because miRNAs typically act as inhibitors of gene expression, the effect of adding specific miRNA mimics to a system is to decrease the expression of the mRNAs controlled by the miRNA. Conversely, the effect of inhibitors of specific miRNAs is to relieve the inhibition of the genes normally targeted by the miRNA. Thus, the primary effect of a miRNA inhibitor is activation of gene expression and a miRNA mimic is suppression of gene expression (Fig. 2A).

Antisense miRNA oligonucleotides

For miRNAs whose up-regulation in a disease state plays a causal role in the disease, specific reduction of the miRNA would be therapeutically desirable. Inhibition of miRNA activity can be achieved through the use of chemically modified single-stranded reverse complement oligonucleotides (RCOs). The synthetic RCO approach can theoretically act at multiple levels to affect miRNA levels; (1) by binding to the mature miRNA within the RISC and acting as a competitive inhibitor, (2) by binding to the pre-miRNA and preventing its processing or entry into the RISC, (3) by interfering with the processing or export of the pre- or pri-miRNA from the nucleus. In any case, the net result is a reduction in the concentration of a specific miRNA-programmed RISC. This approach is similar in concept to traditional antisense targeting of mRNAs, except that whereas there are a very large number of potential targeting sites on a mRNA, the number of targeting sites for a miRNA is very limited. Although conceptually comparable, to date only a handful of modifications have been employed to achieve inhibition of targeted miRNA.

In 2004 Hutvagner and colleagues first reported on the successful use of 2' O-methyl, antisense oligonucleotides to knockdown let-7 function in *Drosophila*⁵⁹. A year later, Krutzfeldt et al. reported on the first mammalian *in vivo* study using these so-called antagomiRs, to inhibit miR-122, a liver-specific miRNA¹¹. These chemically modified oligonucleotides are complementary to the mature miRNA sequence and are conjugated to cholesterol to facilitate cellular uptake. Systemic delivery via intravenous injection appears sufficient to efficiently reduce the level of the miRNA of interest in multiple tissues for an extended period of time

⁶⁰. Inhibition of miR-122 resulted in up-regulation of genes involved in cholesterol biosynthesis and, more importantly, led to a reduction in serum cholesterol levels of 44% ¹¹. However, the mRNAs that were up-regulated in response to miR-122 inhibition were not predicted targets of miR-122, suggesting that miR-122 modulates their expression through secondary mechanisms. Care et al. showed this approach also to be applicable for the heart. *In vivo* inhibition in mice of miR-133 appeared sufficient to induce significant hypertrophic growth of the heart with induction of fetal gene expression compared with saline-treated mice ²⁵. Using a comparable approach for miR-29, a miRNA regulating fibrosis-related genes, we also demonstrated efficient cardiac inhibition *in vivo* ³⁴. However, since miR-29 was essentially completely inhibited in the liver, where most drugs are metabolized, the gene regulatory effects were most pronounced in that tissue. The molecules have been dosed to animals at quite high concentrations (80 mg/kg/day for multiple days) and have demonstrated rather widespread effects in a variety of tissues. Remarkably, a single intravenous bolus injection of an antagomir is sufficient to inhibit the function of its target miRNA for weeks. The mechanistic basis for such long-term effects remains to be defined. Nevertheless, these findings point to the potential of appropriately modified RCO's for treatment of chronic diseases.

Another approach has used the widely employed 2'-O-methoxyethyl phosphorothioate modification and demonstrated effective inhibition of microRNA activity in the liver ¹⁰. This class of molecules is being examined as an antisense mRNA targeting agent in several clinical trials. Additionally, oligonucleotides employing the locked nucleic acid (LNA) phosphorothioate chemistry have recently demonstrated excellent activity in targeting miRNAs, again particularly in the liver ⁶¹. The LNA derivatives have recently been evaluated in non-human primates ⁶² and are being evaluated in the first human clinical trials of miRNA inhibition. Although there are individual beneficial characteristics to these different approaches they have all been shown to efficiently inhibit the target miRNA *in vivo* (Fig. 2A).

miRNA mimics

In situations in which a reduction in miRNA level causes a disease state, an increase in the concentration of the specific miRNA in question would be a beneficial therapeutic approach. Instead of delivering the single-stranded oligonucleotide equivalent of the mature miRNA, an increase in the effective concentration of a reduced miRNA can be achieved through the use of synthetic RNA duplexes in which one strand is identical to the native miRNA. In this case, short double stranded oligonucleotides are designed in which one strand is the mature miRNA sequence (guide strand) and a complimentary or partially complementary strand is complexed with the mature miRNA sequence (passenger strand). The double stranded structure is required for efficient recognition and loading of the guide strand into the RISC complex ⁶³. Care must be taken in the design of such species to eliminate the potential of the passenger strand to act as a new miRNA and confound interpretation of the experimental results. Bioinformatic and chemical modification approaches can be employed to ensure that only one strand is employed ⁶⁴.

It is interesting to note that this type of construct is analogous to the siRNA molecules commonly employed in gene silencing experiments. In fact, it is likely that siRNA-mediated gene silencing is so effective because it co-opts the fundamental miRNA machinery or a highly analogous cellular machinery. Although to date, miRNA mimics have not yet been demonstrated efficacy *in vivo*, this approach represents an attractive means of enhancing miRNA levels for those that are down-regulated during disease (Fig. 2A).

Sponging, target occupiers and erasers

In addition to tools available to directly target a miRNA or reconstitute reduced miRNA levels there are several approaches possible to target a miRNA pathway. One way of interfering with miRNA function is by scavenging away the miRNA and thereby preventing it from binding its mRNA targets. An expression-based approach for the reduction of miRNA levels, referred to as “miRNA sponges”, was reported by Sharp and colleagues in 2007⁶⁵. In this technique, a series of either perfectly or imperfectly paired binding sites for a specific miRNA are introduced into an expression cassette in the 3' untranslated region of a reporter gene. The multiplexed binding sites serve as competitive inhibitors and occupy the specific native miRNA-programmed RISC complexes in the cell. This effectively reduces the concentration of the programmed RISC available for binding to its native targets and thereby relieves the inhibitory effect of the specific miRNA on mRNA targets⁶⁵ (Fig. 2B). This approach is extremely effective for inhibiting the function of miRNA families. Instead of separately targeting single miRNA family members, this approach scavenges all members at once since they recognize the same binding sequence.

The association of a miRNA with a specific mRNA target can also be perturbed using an oligonucleotide with perfect complementarity to the miRNA target sequence in the 3' UTR of the mRNA, which thereby masks the binding site and prevents association with the miRNA⁴¹ (Fig. 2B). A theoretical advantage of this approach is its specificity. Since a miRNA has multiple targets, directly inhibiting a miRNA will influence all downstream targets, which may increase the probability of off-targets effects, while target occupation can modulate the interaction of a miRNA with one specific target. A third approach to inhibit miR function involves so-called “erasers”, in which expression of a tandem repeat of a sequence perfectly complementary to the target miR inhibits endogenous miRNA function²⁷. While this approach is comparable to the method described by Ebert et al., the eraser employs only 2 copies of the perfectly complementary antisense sequence of the miRNA, while a sponge contains multiple antisense copies of a miRNA designed for bulged or perfect pairing to the miRNA. Although ‘sponging’, ‘masking’ and ‘erasing’ provide interesting opportunities to interfere with miRNA function, their *in vivo* efficacy has yet to be demonstrated.

Challenges for therapeutic targeting of miRNA-based mechanisms

The apparent importance of miRNAs and the ability to manipulate them *in vivo* provides a unique opportunity to exploit miRNAs therapeutically. However, miRNA-based therapeutics pose a different set of challenges from those associated with classical drugs (Table 1). Whereas specificity for a single cellular target (generally a receptor or enzyme) is paramount in classical drugs, miRNAs have numerous molecular targets, which raises the possibility that the targeting of a miRNA may perturb multiple cellular functions, some pathological and others beneficial. Issues around pharmacokinetics, biodistribution and cell penetration also represent potential obstacles to therapeutic miRNA manipulation strategies using synthetic nucleic acids. For example, since native nucleic acids are rapidly degraded by a variety of nucleases and phosphodiesterases in blood and other biological environments, synthetic nucleotide derivatives require specific chemical modifications to alter their biophysical properties. Fortunately, several such modifications that increase the stability of the oligonucleotides, including phosphorothioate, 2' O-methyl, and 2'-fluoro substitutions, among many others, may find utility in the field⁶⁶.

Methods of delivery

Currently, there is an intense effort to identify agents capable of targeted delivery of nucleic acids to tissues and cells. Delivery approaches can be broadly divided into two categories, conjugation and formulation. Conjugation strategies include direct attachment of targeting and

cell-penetrating peptides, antibodies and other bioactive molecules to the oligonucleotide. Formulation approaches vary broadly and include complex lipid emulsions from natural sources, synthetic liposomes, polyplexes, polymers, and nanoparticles. In order to enter mammalian cells, the RCO needs to be able to cross the lipid bilayer of the cell membrane. This can be achieved by packaging the oligonucleotide into liposomes or nanoparticles, which facilitate endocytosis. Alternatively, the oligonucleotide can be linked to a lipophilic moiety or receptor ligand, such as cholesterol, an approach taken by Soutchek et al. that appeared to greatly enhance cellular uptake ⁶⁶. Combinatorial chemistry also yielded a novel class of “lipidoids” that may allow for the development of new classes of delivery reagents ⁶⁷.

Despite significant advances in systemic delivery technology, most nucleic acid delivery agents developed to date have only demonstrated efficacy in delivery to the liver. The expansion of effective delivery approaches, especially to the heart, is a primary requirement for the use of synthetic nucleic acids as therapeutics for cardiovascular disease. The majority of nucleic acid therapeutic approaches that target intracellular mechanisms (antisense, siRNA) currently are focused on local or compartmentalized delivery (intravitreal injection, inhalation) or conditions in which the molecular target is in the liver. Part of the strategy in going after these conditions is driven by the pharmacokinetics and biodistribution of the molecules. Heart failure affords an interesting opportunity to expand the potential for local delivery through the use of catheters. Additionally, adding specific targeting components, such as cell surface receptor ligands, to nucleic acids, can enhance target binding to the tissue or cell type of interest.

Future perspectives and concluding remarks

It is remarkable to consider that miRNAs were first shown to function in mammals less than a decade ago, and the concept of miRNA manipulation *in vivo* to regulate disease-related processes is already becoming a feasible future therapeutic approach. Moreover, the rapidly expanding number of miRNAs makes it likely that the relatively few miRNAs studied to date represent only a subset of the miRNAs of interest in human disease.

While much remains to be learned about the biology of miRNAs and the optimal chemistry and delivery systems to allow for their efficient manipulation as therapeutics, the first human trials using an antisense approach to regulate miRNA function are underway, with many more undoubtedly on the horizon. Rarely has an opportunity arisen to advance such a new biology so rapidly toward novel therapeutics. Given the obvious involvement of miRNAs in numerous facets of heart disease, and the many sophisticated techniques for delivery to the heart, diseases of the heart will likely be among the first to yield to miRNA-based therapies. Indeed, there is sense in antisense.

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Abbreviations

miRNA / miR	microRNA
RISC	RNA Induced Silencing Complex
MI	Myocardial infarction
ANP	atrial natriuretic peptide
BNP	B-type natriuretic peptide
MHC	β-myosin heavy chain
VEGF	vascular endothelial growth factor
FGF	

fibroblast growth factor

RCOs

reverse complement oligonucleotides

antagomiRs

2' O-methylated, antisense oligonucleotides

MOE

2'-O-methoxyethyl phosphorothioate

LNA

locked nucleic acid

UTR

untranslated region

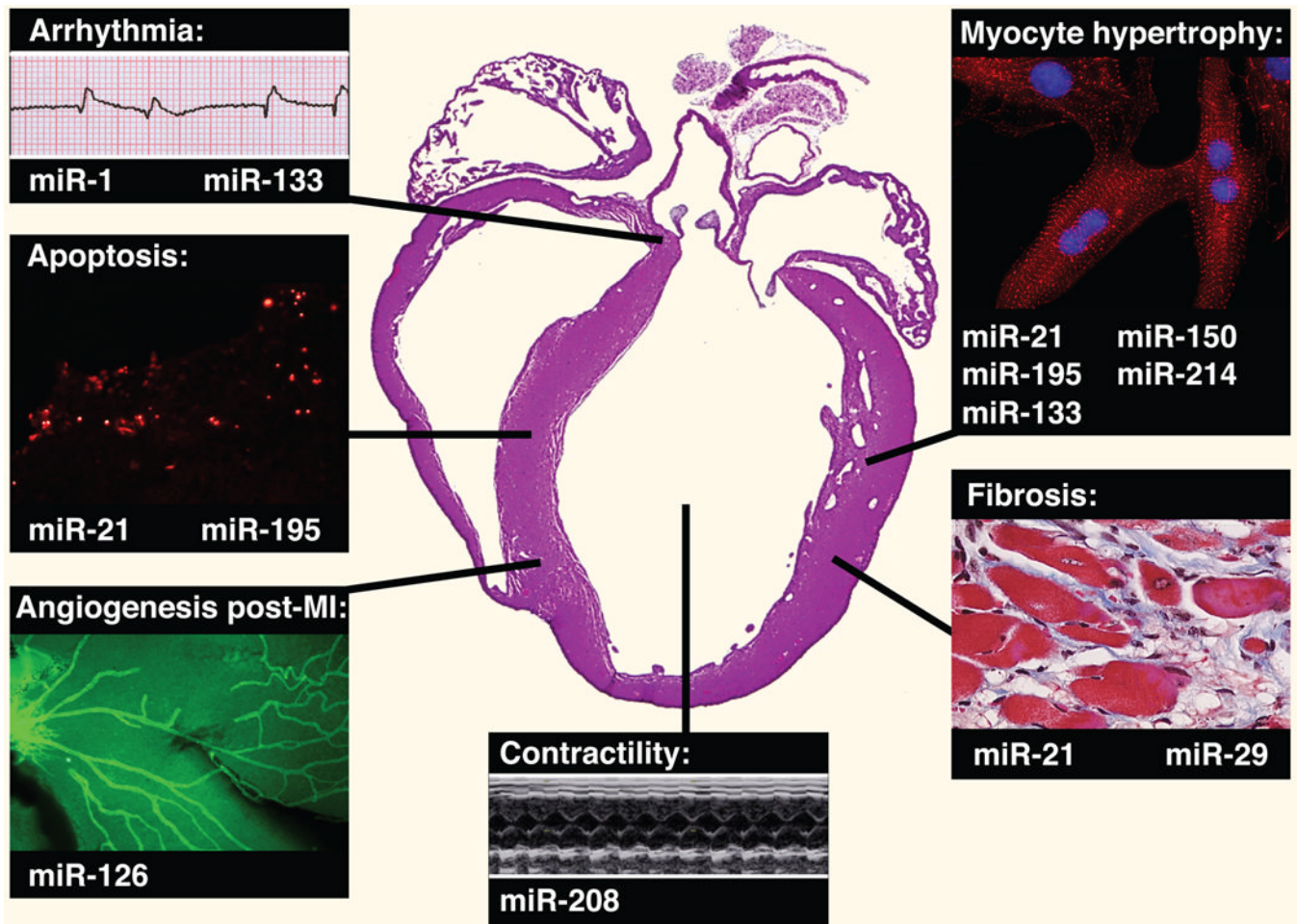


Figure 1. miRNA function during heart disease

Cardiac remodeling involves numerous disease processes. It is becoming increasingly clear that miRNAs fulfill specific functions during this process. While miR-21, -133, -150, -195 and -214 influence cardiomyocyte hypertrophy, miR-1 and -133 have additionally been implicated in cardiac arrhythmogenesis. In addition miR-21 and miR-195 also appear to influence apoptosis. miRNA-208 influences cardiac contractility by regulating myosin content and by so doing secondarily influences hypertrophy and fibrosis. The process of cardiac fibrosis is regulated by miR-21 and -29, both of which are highly expressed in cardiac fibroblasts. Neoangiogenesis post-MI relies on miR-126.

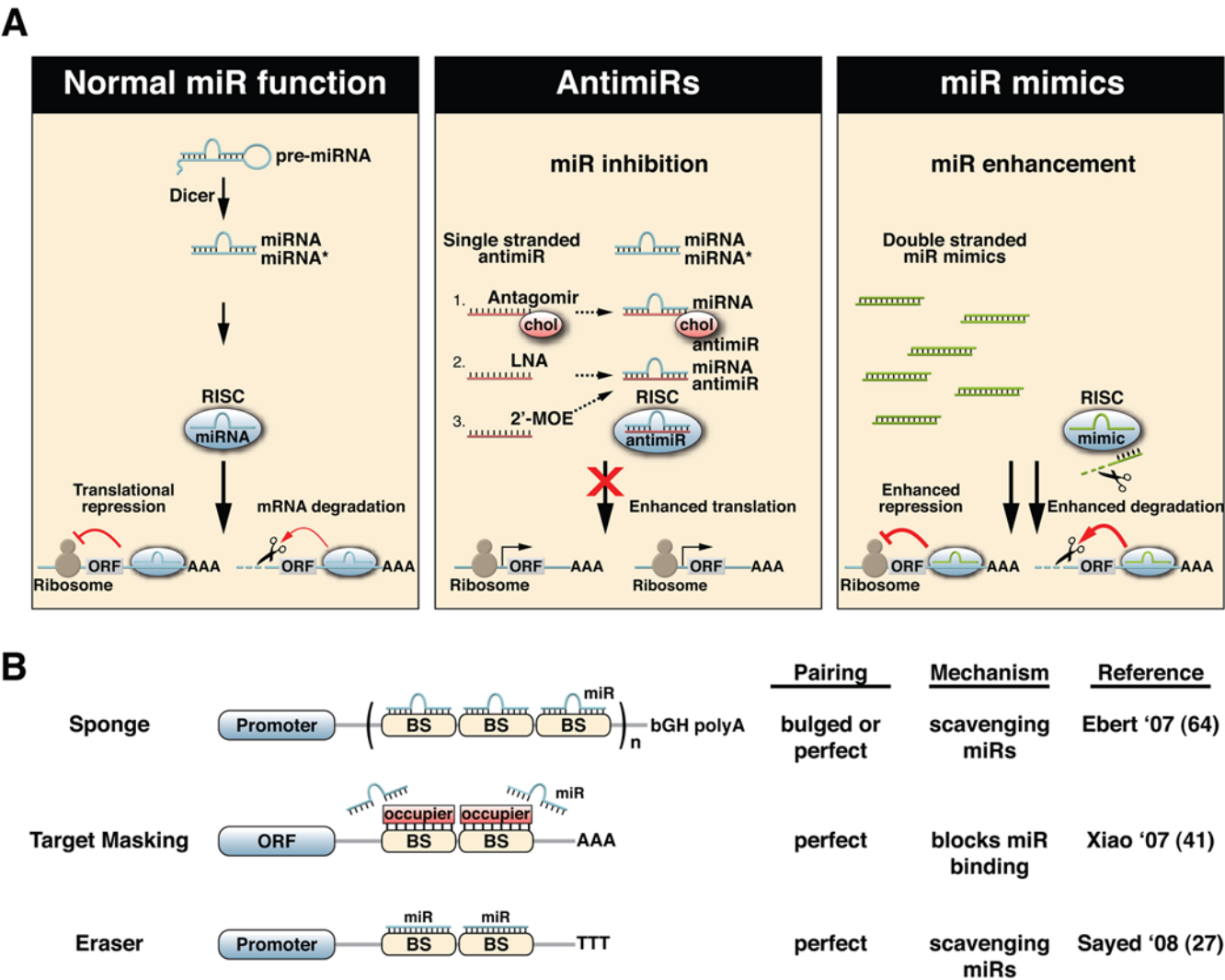


Figure 2. RNAi based technologies to regulate miRNA function *in vivo*

A) miRNAs are generated from pre-miRNAs by Dicer, giving rise to a duplex containing the mature miRNA and a partially complementary strand, referred to as miRNA*. AntimiRs are single stranded, antisense oligonucleotides designed to inhibit miRNA function. The perfect sequence complementarity allows the antisense oligonucleotide to bind to the miRNA and interfere with miRNA function (indicated in red). Although the exact mechanism of action remains undefined, this approach has been proven to be efficacious *in vivo* in preventing repression by the miRNA. One class of anti-miR, the antagomir, is conjugated to cholesterol to facilitate cellular uptake (1). Other approaches use the oligonucleotides employing the locked nucleic acid (LNA) phosphorothioate chemistry (2) or the widely employed 2'-O-methoxyethyl phosphorothioate (MOE) modification (3). miR mimics are short double stranded oligonucleotides in which one strand is identical to the mature miRNA sequence (guide strand) and a complimentary or partially complementary stand is complexed with the mature miRNA sequence (passenger strand). The double stranded structure is required for efficient recognition and loading of the guide strand into the RNA Induced Silencing Complex (RISC) complex, after which it can function like the endogenous miRNA to increase the level of the miRNA of interest and more potently block targeted gene expression (indicated in green). B) In addition to tools available to directly target a miRNA or reduce miRNA levels, there are several potential

approaches to target a miRNA pathway. One way of interfering with miRNA function is by scavenging away the miRNA and thereby preventing it from binding its mRNA targets, called sponging. In this technique, a series of either perfectly or imperfectly paired binding sites for a specific miRNA are introduced into an expression cassette in the 3' untranslated region of a reporter gene. The multiplexed binding sites serve as competitive inhibitors and occupy the specific native miRNA-programmed RISC complexes in the cell. The association of a miRNA with a specific mRNA target can also be perturbed using an occupier, an oligonucleotide with perfect complementarity to the miRNA target sequence in the 3' UTR of the mRNA, which thereby masks the binding site and prevents association with the miRNA. A theoretical advantage of this approach is its specificity. Since a miRNA has multiple targets, directly inhibiting a miRNA will influence all downstream targets, which may increase the probability of off-targets effects, while target occupation can modulate the interaction of a miRNA with one specific target. A third approach to inhibit miR function involves so-called erasers, in which expression of a tandem repeat of a perfect complementary sequence of the target miR inhibits endogenous miRNA. Although 'sponging', 'masking' and 'erasing' provide interesting opportunities to interfere with miRNA function, to date no studies have been published that prove their *in vivo* efficacy.

Table 1

Advantages and potential challenges of miR-based therapeutics compared to conventional drug approaches.

miR-based Therapies	
Advantages	<ul style="list-style-type: none">- miRNAs regulate multiple components of the same pathway / cellular process- long-lived, sustained effects- the target miRNA is defined- effective <i>in vivo</i> regulation of the miRNA
Potential challenges	<ul style="list-style-type: none">- delivery of the miRNA modulator- off-target effects of unintended, or unidentified targets of a miRNA- a single miRNA can have both beneficial and pathogenic effects- toxicity of the miRNA modulator