MicroRNAs Add a New Dimension to Cardiovascular Disease

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Cardiovascular disease is the predominant cause of human morbidity and mortality in developed countries. Thus, extraordinary effort has been devoted to determining the molecular and pathophysiological characteristics of the diseased heart and vasculature with the goal of developing novel diagnostic and therapeutic strategies to combat cardiovascular disease. The collective work of multiple research groups has uncovered a complex transcriptional and posttranscriptional regulatory circuit, the integrity of which is essential for the maintenance of cardiac homeostasis. Mutations in or aberrant expression of various transcriptional and posttranscriptional regulators have now been correlated with human cardiac disease, and pharmacological modulation of the activity of these target genes is a major focus of ongoing research. Recently, a novel class of small noncoding RNAs, called microRNAs (miRNAs), was identified as important transcriptional and posttranscriptional inhibitors of gene expression thought to “fine tune” the translational output of target messenger RNAs (mRNAs).1,2 miRNAs are implicated in the pathogenesis of various cardiovascular diseases and have become an intriguing target for therapeutic intervention. This review focuses on the basic biology and mechanism of action of miRNAs specifically pertaining to cardiovascular disorders and addresses the potential for miRNAs to be targeted therapeutically in the treatment of cardiovascular disease.

miRNA Processing and Function

miRNAs originate from longer precursor RNAs called primary miRNAs that are regulated by conventional transcription factors and transcribed by RNA polymerase II. Primary miRNAs are hundreds to thousands of nucleotides long and are processed in the nucleus into an ~70- to 100-nucleotide hairpin-shaped precursor miRNA by the RNase III enzyme Drosha and the double-stranded RNA binding protein DGCR8. The precursor miRNA is then transported into the cytoplasm by the nuclear export factor exportin 5 and further processed into an ~19- to 25-nucleotide double-stranded RNA by the RNaseIII enzyme Dicer. This duplex miRNA is then incorporated into the RNA-induced silencing complex. One strand remains in the RNA-induced silencing complex and becomes the “mature” miRNA, whereas the other strand is often rapidly degraded and is called the “star” strand (miRNA*). On being loaded into the RNA-induced silencing complex, the mature miRNA associates with target mRNAs and acts as a negative regulator of gene expression by promoting mRNA degradation or inhibiting translation.3 Translational inhibition seems to be the predominant mechanism in mammals; however, target genes that are strongly downregulated on the protein level often show a reduced mRNA level,4 suggesting that mRNA destabilization is a major contributor to gene silencing.

A mature miRNA typically regulates gene expression via an association with the 3′ untranslated region (UTR) of an mRNA with complementary sequence, although emerging evidence suggests that miRNAs may also target 5′ UTRs or exons and may potentially even undergo base pairing with regulatory DNA sequences to regulate transcription. On miRNA binding to a 3′ UTR, the degree of mRNA degradation and/or translational repression is affected by multiple mechanisms, including the overall complementarity between the miRNA and target mRNA, the secondary structure of the adjacent sequences, the distance of the miRNA binding site to the coding sequence of the mRNA, and the number of target sites within the 3′ UTR.5 Complementarity between nucleotides 2 through 8 of the miRNA, called the “seed” region, appears to be essential for 3′ UTR identification. Therefore, miRNAs with high sequence homology and identical seed region are commonly grouped into miRNA families that are likely to target similar sets of mRNAs.6

Up to 1000 miRNAs are predicted to exist in the human genome, each of which could potentially target hundreds of miRNAs. Most 3′ UTRs contain potential binding sites for a large number of individual miRNAs, allowing redundancy or cooperative interactions between various seemingly unrelated miRNAs. Moreover, the targets of many miRNAs can modulate the expression of additional miRNAs or groups of miRNAs, generating positive or negative feedback loops. Finally, miRNA maturation seems to be posttranscriptionally regulated in a sequence-specific manner,7 potentially explaining why genetically clustered and cotranscribed miRNAs are often expressed at different levels.
Multiple miRNA target prediction tools are now available (summarized in the Table in the online-only Data Supplement). Generally, in silico target prediction algorithms use a standard scheme to identify and rank potential targets. Briefly, potential targets are ranked on the basis of the complementarity between miRNA and 3' UTR and the degree of conservation of the miRNA and the 3' UTR target sequence across species. A particular miRNA target is considered to be more meaningful if the sequence is evolutionarily conserved.

Identification and validation of miRNA targets remain a major hurdle in the study of miRNA function because many putative targets display little or no detectable regulation when tested in vitro. This is likely due, at least partially, to the relatively modest effect of any single miRNA on the translational output of the target mRNA. Therefore, many predicted miRNA binding sites are probably not true targets, and experimental validation is essential for confirming target genes.

A surprising number of published miRNA targets do not conform to the traditional rules of target prediction outlined above. For example, in several instances, cross-species conservation is not observed in the target sequence, even between rodents and humans. Of course, for a miRNA target to be therapeutically viable, the miRNA target sequence must be conserved in humans, not simply present in the model organism studied. Therefore, this review focuses primarily on published miRNAs that target 3' UTR binding sites that are conserved in humans.

A key insight into the mechanism of miRNA action has been that a large number of miRNAs apparently target multiple functionally related mRNAs. The coordinated regulation of multiple steps in a complex physiological process by 1 miRNA or a group of similarly expressed miRNAs is an important characteristic of miRNA biology that lends itself to therapeutic applications. In contrast to therapeutically modulating a single target with a conventional drug, miRNA biology can, in principle, modulate multiple levels of a pathological process by targeting a single nucleic acid molecule.

Expression of miRNAs Implicated in Cardiovascular Disease

As summarized in Figure 1, miRNA expression profiling has identified a subset of miRNAs expressed in the normal heart and modulated during cardiovascular disease. The 18 most strongly expressed miRNAs and miRNA families account for >90% of all miRNA expressed in the adult mouse heart (Figure 1), although the specific degree of expression likely varies between species and depends on the precise location of cardiac tissue harvested. In this respect, it is interesting that several of the most abundant miRNAs in the heart belong to families, with the let-7 family accounting for ≈14%, the miR-30 family for ≈5%, and the miR-29 family for ≈4% of all miRNAs expressed in the murine heart (Figure 1). Surprisingly, nearly all of the 18 most enriched miRNAs within the heart display altered expression during cardiac disease, indicating an extremely dynamic regulation of miRNAs in the adult heart and pointing toward the importance of miRNAs as modifiers of gene expression programs in cardiovascular disease.

Re-expression of a fetal cardiac gene program is a hallmark of various models of cardiovascular disease and human heart
failure. Upregulated genes include the fetal isoform of the myosin heavy chain gene (\(\text{MHC}\)) and the atrial and brain natriuretic factor genes, among others. Recently, it has been suggested that a fetal miRNA program also exists that is reactivated on cardiac stress and may contribute to the reactivation of the fetal gene mRNA program during cardiac disease.10

Although some of the miRNAs regulated during cardiovascular disease are not highly expressed outside the heart, a large number of such miRNAs are broadly expressed throughout the body. For example, miR-21, which typically displays dramatic alterations in expression after various cardiac or vascular stresses, is detectable in nearly every tissue analyzed. Inhibition of miRNAs that are specifically expressed, or highly enriched, in the cardiovascular system may circumvent side effects resulting from miRNA activity in additional organs.

miRNAs Experimentally Implicated in Various Cardiovascular Disease Settings and Regeneration

Myocardial Remodeling
Myocardial remodeling is typically characterized by cardiomyocyte hypertrophy, cardiomyocyte apoptosis, interstitial fibrosis, and aberrant cardiac conduction, which ultimately impair the electromechanical performance of the myocardium. The following sections review the potential roles of miRNAs in each of these processes (see Figure 2) and suggest potential therapeutic implications.

Cardiomyocyte Hypertrophy
Pathological hypertrophy is a maladaptive process that ultimately leads to reduced cardiac output and is an independent risk factor in heart failure.11 Pathological cardiac hypertrophy occurs primarily on pressure overload resulting from arterial hypertension or stenosis of the aortic valve, as well as inherited mutations in sarcomeric and cytoskeletal proteins. Myocardial infarction (MI) also frequently leads to hypertrophic growth of the remote myocardium as a means of compensating for lost contractile function.

The general importance of miRNAs for the homeostasis of cardiomyocytes was demonstrated by cardiomyocyte-specific deletions of Dicer12 and Dgcr8,9 two essential components of the machinery required to generate miRNAs. Embryonic deletion of Dicer in cardiomyocytes resulted in embryonic or early postnatal death, depending on the time point of Dicer deletion.13,14 Early postnatal deletion of Dicer induced fatal arrhythmias, whereas deletion of Dicer in adult mice led to the development of severe heart failure.15 Perinatal deletion of Dgcr8 also resulted in rapid development of severe heart failure and premature death.9 It is interesting to speculate that the downregulation of Dicer in patients with heart failure might contribute to the disease pathogenesis and progression.13

Several individual miRNAs are transcriptionally regulated during cardiac hypertrophy and heart failure (Figure 1). Some of them have been experimentally verified to play important roles in cardiac development and disease and are reviewed below.

miR-1
miR-1 is encoded by 2 genes (miR-1-1 and miR-1-2), each of which is coexpressed bicistronically with 1 copy of the 2 miR-133a genes. miR-1 expression is restricted to heart and skeletal muscle and is regulated by the transcription factors serum response factor and myocyte enhancer factor-2.
miR-23

There are 2 miR-23 genes that differ by only 1 nucleotide in the mature miRNA sequence. Each miR-23 gene is closely clustered with a miR-24 and a miR-27 gene, suggesting that they are transcribed as a common transcript. Accordingly, several groups found that miR-23, miR-24, and miR-27 are all upregulated in heart failure and murine cardiac hypertrophy (Figure 1). van Rooij et al\textsuperscript{27} showed that adenosivral overexpression of miR-23a induces hypertrophy of isolated cardiomyocytes. Lin et al\textsuperscript{28} showed recently that miR-23a expression in cardiomyocytes is regulated by nuclear factor of activated T cells (NFATc3) and that miR-23a promotes cardiomyocyte hypertrophy by downregulation of the muscle-specific RING-finger protein 1, an antihypertrophic protein. Knockdown of miR-23a by injection of a specific antagoniR attenuated isoproterenol-induced cardiac hypertrophy.\textsuperscript{28}

miR-133

The miR-133 family contains 3 mRNA genes, miR-133a-1, miR-133a-2, and miR-133b, which are each transcribed as bicistronic transcripts together with miR-1-2, miR-1-1, and miR-206, respectively. The expression of the 2 miR-1/133a clusters is regulated by MEF2 and serum response factor and is restricted to cardiac and skeletal muscle.\textsuperscript{29} The miR-206/133b cluster is expressed only in skeletal muscle.

Partial knockdown of miR-133 in mice with specific antagoniRs was shown to induce cardiac hypertrophy, suggesting that pharmacological elevation of miR-133 expression might prevent cardiac hypertrophy during cardiac disease.\textsuperscript{30} In contrast, Liu et al\textsuperscript{31} reported that mice with a genetic deletion of either miR-133a gene were phenotypically normal and showed a normal hypertrophic response to pressure overload of the left ventricle despite an \textasciitilde 50% decrease in miR-133 expression in the heart. Deletion of both miR-133a genes resulted in late embryonic or neonatal lethality because of ventricular-septal defects, accompanied by abnormalities in cardiomyocyte proliferation, apoptosis, and aberrant expression of smooth muscle genes in the heart.\textsuperscript{31} About a quarter of the double-knockout mice survived to adulthood, developed extensive myocardial fibrosis without evidence of cardiomyocyte hypertrophy, and ultimately died of heart failure or sudden death. Cardiac-restricted overexpression of miR-133a under the control of the \textbeta-microglobulin promoter resulted in embryonic lethality caused by inhibition of cardiomyocyte proliferation.\textsuperscript{31}

Many of the phenotypic abnormalities observed in miR-133a knockout mice such as ectopic expression of smooth muscle genes and aberrant cardiomyocyte proliferation could be ascribed at least partially to inappropriate expression of the miR-133 target genes serum response factor and cyclin D2.\textsuperscript{31} It is not clear why genetic deletion of miR-133 and knockdown by antagoniRs provoke different effects; however, a partial and transient knockdown with antagoniRs might have different consequences than a complete genetic deletion in which the gene is eliminated throughout the whole life. Perhaps long-term genetic deletion allows compensatory mechanisms that do not occur in response to transient miRNA knockdown. Further studies are required to determine
whether therapeutic modulation of miR-133 expression might represent an interesting target for the treatment of cardiac disease.

**miR-208a**

The cardiac-specific miR-208a is encoded by an intron of the αMHC gene. Thyroid hormone (T3) stimulates the expression of αMHC and miR-208a after birth while repressing the expression of the embryonically predominant βMHC isoform. Cardiac disease is associated with a reactivation of the fetal gene program with increased expression of βMHC and decreased expression of αMHC, although a corresponding decrease in miR-208a levels is not observed in short-term studies because miR-208a is very stable. The results of van Rooij et al demonstrated that miR-208a regulates the induction of βMHC expression on cardiac stress in adult mice. Callis et al further demonstrated that this result in the concomitant upregulation of miR-208b, which is encoded by an intron of the βMHC gene. Genetic deletion of miR-208a in mice resulted in blunted hypertrophy and reduced cardiac fibrosis in response to thoracic aortic constriction, whereas cardiomyocyte-specific overexpression of miR-208a induced cardiomyocyte hypertrophy. The regulation of cardiomyocyte growth by miR-208a might be due at least partially to the repression of thyroid hormone receptor-associated protein 1 and myostatin, 2 negative regulators of muscle growth and hypertrophy. Although initially protective against acute cardiac stress–induced remodeling, long-term deletion of miR-208a led to a decrease in cardiac contractility, possibly resulting from perturbations in the cardiac conduction system causing atrial fibrillation of miR-208a knockout mice. The latter seems to be caused by misregulation of GATA4, homeodomain-only protein, and connexin 40. It would be interesting to determine whether partial knockdown of miR-208 might prevent hypertrophy while circumventing the side effects of complete miR-208a knock-out, thereby enhancing the potential of miR-208 as a therapeutic target.

**Cardiomyocyte Apoptosis and Regeneration**

Because the adult heart has only limited regenerative capacities, an excessive loss of cardiomyocytes after myocardial ischemia or infarction can significantly decrease cardiac performance. Some miRNAs seem to play important roles in the regulation of cardiomyocyte apoptosis in vivo and are discussed below. MiRNAs are certainly crucial regulators of cell fate determination and differentiation of stem cells; however, it is currently unclear whether they play a role in the regeneration of the adult heart.

**miR-195**

miR-195 belongs to a family including miR-15a, miR-15b, miR-16-1, miR-16-2, miR-424, and miR-497 (the miR-15 family). The miR-15 family was consistently found to be strongly upregulated in cardiac ischemia and heart failure (Figure 1). Cardiomyocyte-specific overexpression of miR-195 resulted in cardiac hypertrophy and rapid progression to fatal dilated cardiomyopathy. The exact function of the miR-15 family in the heart is not clear, but studies in other cell types suggest that the miR-15 family might induce apoptosis by downregulation of the antiapoptotic factor Bcl-2. In this respect, antagoniR-induced knockdown of the miR-15 family might be a means to prevent ischemia-induced cardiomyocyte apoptosis.

**miR-199a**

The miR-199 family contains 3 miRs, miR-199a-1, miR-199a-2, and miR-199b, that are all encoded by the antisense strand of an intron of a dynamin gene (Dnm2, Dnm3, and Dnm1, respectively). Furthermore, miR-199a-2 is cotranscribed with miR-214. The transcriptional regulation of miR-199 in the heart is unknown, but Ran et al reported recently that miR-199a was rapidly downregulated in cardiomyocytes on hypoxia conditions, most likely via a posttranscriptional mechanism because the expression level of the miR-199a precursor was unaffected. Downregulation of miR-199a derepressed the expression of hypoxia-inducible factor-1α, the most important transcription factor for the induction of gene expression on hypoxia. miR-199a downregulation also resulted in the derepression of Sirtuin 1, which was responsible for downregulation of prolyl hydroxylase 2, the enzyme that hydroxylates hypoxia-inducible factor-1α to induce its degradation. The results of this study demonstrated that knockdown of miR-199a during hypoxia induced apoptosis, whereas knockdown of miR-199a before hypoxia surprisingly mirrored preconditioning and protected cardiomyocytes against hypoxic damage.

**miR-320**

In contrast to the upregulation of the miR-15 family, miR-320 expression was decreased on ischemia/reperfusion injury. Transgenic mice with cardiac-specific overexpression of miR-320 exhibited increased apoptosis and infarct size after ischemia/reperfusion injury. Conversely, administration of miR-320 antagoniRs reduced infarct size, probably at least partially by derepression of the cardioprotective heat-shock protein 20. It is early to speculate, but targeting miR-320 with antagoniRs after MI could be a new treatment option to decrease cardiomyocyte loss.

**Cardiac Conduction**

Membrane excitability, a special characteristic of cardiomyocytes, is regulated via ion channels. Specifically, Na+, Ca2+, and K+ channels and gap junction proteins such as connexin 43 are important regulators of cardiomyocyte polarization and depolarization during contraction and relaxation, respectively. Several miRNAs, including miR-1 and miR-133, are predicted to target ion channels and might therefore play important roles in cardiac conduction and the onset of arrhythmias during cardiac disease. Zhao et al reported that adult miR-1-2 knockout mice showed several ECG alterations such as reduced heart rate, shortened PR interval, and widened QRS complexes and died as a result of cardiac arrhythmia. The ECG alterations were presumably due at least partially to elevated expression of the miR-1 target Irx5, a transcription factor that regulates the expression of Kcnd2, a potassium channel important for normal cardiac repolarization. Further, Yang et al showed that miR-1 is upregulated in coronary artery disease. Overexpression of miR-1 exacerbated and antagoniR-induced
knockdown relieved arrhythmogenesis on MI, most likely via regulation of connexin 43 and the K\(^+\) channel Kir2.1 subunit.\(^{37}\) Additionally, miR-1 regulated Ca\(^{2+}\) signaling via targeting of the B56a regulatory subunit of the protein phosphatase PP2A.\(^{38}\) Downregulation of B56a by miR-1 resulted in hyperphosphorylation of L-type calcium channels and ryanodine receptor 2 by disrupting localization of PP2A to these channels.\(^{38}\) These studies suggest that miRNA-1 and potentially other miRNAs might be promising targets to treat cardiac arrhythmia.

**Fibrosis**

Fibroblast activation and proliferation during cardiac disease lead to inappropriate secretion of extracellular matrix proteins and concomitant interstitial fibrosis. Fibrosis results in impaired cardiac contractility and alters the electromechanical characteristics of the myocardium, often leading to arrhythmias, an important cause of mortality in heart disease. The expression of several miRNAs is altered after MI or other fibrotic pathologies, some of which have been shown to play both direct and indirect roles in the regulation of cardiac fibrosis.

As mentioned above, adult miR-133a double-knockout mice develop severe fibrosis and heart failure.\(^{31}\) In line with these data, Duisters et al\(^{39}\) showed that miR-133 could target connective tissue growth factor. Downregulation of miR-133 during cardiac disease might therefore result in increased expression and secretion of connective tissue growth factor from cardiomyocytes, which consecutively stimulates extracellular matrix synthesis in fibroblasts.

The fibroblast-enriched miR-21 is upregulated in failing and hypertrophic myocardium, possibly as a consequence of fibroblast proliferation. Thum et al\(^{22}\) demonstrated that miR-21 increases fibroblast survival and fibrosis possibly via inhibition of sprouty homolog 1 and consecutive extracellular receptor kinase–mitogen-activated protein kinase activation. Knockdown of miR-21 with antagoniRs attenuated interstitial fibrosis and cardiac remodeling after aortic banding.\(^{22}\) miR-21 is also upregulated in the border zone, a fibroblast-rich region adjacent to the infarct, after MI and was shown to promote MMP-2 expression via repression of phosphatase and tensin homolog.\(^{40}\)

van Rooij et al\(^{41}\) found that all members of the miR-29 family downregulated after MI, particularly in the border zone. miR-29 is predicted to target myriad genes that are involved in fibrosis such as collagens, fibrillins, and elastin and is a prime example of the ability to modulate a large portion of a particular pathology by pharmacologically targeting 1 miRNA. Knockdown of miR-29 by intravenous injection of an antagoniR resulted in the upregulation of collagens in the heart, suggesting that miR-29 indeed acts as an inhibitor of cardiac fibrosis.\(^{41}\) In the future, it will be interesting to determine the effect of miR-29 replacement strategies on the outcome of fibrotic pathologies such as MI.

**miRNAs in the Vascular System and Vascular Disease**

Vessel injury is characterized by profound phenotypic changes in molecular and physiological identity; in particular, vascular smooth muscle cells (VSMCs) undergo a program of dedifferentiation and become more proliferative and migratory after injury. These changes contribute to neointimal thickening during proliferative vascular diseases such as atherosclerosis, hypertension, and restenosis. Expression profiling has identified a signature pattern of miRNA expression in VSMCs and endothelial cells of the major vessels that are specifically altered in vessel injury (Figure 1).

**Restenosis**

**miR-21**

Several groups have demonstrated roles for various miRNAs in SMC phenotypic modulation and the response of the vasculature to injury. Antisense-mediated knockdown of miR-21, which is normally increased after vessel injury (Figure 1), blunted the formation of a neointimal lesion in response to balloon angioplasty of the carotid artery.\(^{42}\) The results of Ji et al\(^{42}\) demonstrated that miR-21 promoted SMC proliferation after vessel injury via inhibition of phosphatase and tissue homolog and the subsequent activation of the PI3K/Akt signaling pathway, which is partially blocked on knockdown of miR-21 (Figure 3).

**miR-145**

Recently, the SMC-enriched miR-143/miR-145 gene cluster, which is downregulated in the carotid artery after mechanical injury, has been implicated in the regulation of SMC contractility and the stress response to vessel injury. Mice harboring deletions of miR-143, miR-145, or both reveal a role for these miRNAs in the acquisition of a contractile SMC phenotype; vessels of knockout mice are thin and distended, and null VSMCs appear to have acquired a “synthetic” phenotype based on reduced actin stress fiber formation and increased rough endoplasmic reticulum production.\(^{43,44}\) Furthermore, miR-143/miR-145–null mice display decreased vascular tone and a reduction in systolic blood pressure. However, mutant mice were viable and appeared to have functional SMCs, albeit shifted slightly toward the synthetic phenotype. This is in contrast to in vitro analyses that demonstrated a critical role for miR-145 in the differentiation of cultured SMCs and embryonic stem cells,\(^{43,45}\) likely via repression of Klf4 and Klf5, transcriptional repressors that have previously been shown to inhibit a differentiated state.

Adenovirus-mediated overexpression of miR-145 at the onset of carotid artery injury blunted the phenotypic modulation of VSMCs and reduced neointima formation in response to angioplasty.\(^{46}\) Surprisingly, Xin et al\(^{43}\) described a diminution of neointima formation in miR-145–null mice after ligation of the carotid artery. This disparity might suggest that a fully differentiated state at baseline may be essential for appropriate phenotypic modulation of the VSMC state. It is also possible that the reduced vascular tone in miR-145–mutant mice impinges on the responsiveness of the vessel wall to vascular injury and that the phenotype reflects an inability to “sense” the injury as opposed to an inability to “respond” to injury. Finally, miR-143/miR-145 targets a disproportionate number of genes involved in actin cytoskeletal rearrangements and SMC migration (Figure 3), and it is possible that deficiencies in stress-induced migration account
for the protection from neointima formation in mutant mice. Additional studies should be undertaken to determine the viability of pharmacological modulation of miR-145 as a therapy for restenosis.

miR-221

miR-221, although not VSMC specific, is induced in VSMCs on platelet-derived growth factor stimulation. Activation of the platelet-derived growth factor signaling pathway results in the switch of VSMCs from a fully differentiated, contractile state to a less differentiated, synthetic state typified by increases in proliferation and cell migration, contributing to the formation of a neointimal lesion after arterial injury. Indeed, miR-221 expression is moderately elevated after carotid artery angioplasty (Figure 1). In vitro data suggest that miR-221 contributes to SMC phenotypic switching by affecting multiple cellular responses: miR-221 targets cKit for repression, resulting in a decrease in SMC differentiation, and inhibits p27Kip1, thereby increasing SMC proliferation. Therefore, knockdown of miR-221 after vessel injury may block neointima formation. Further examination in animal models should be forthcoming to ascertain its therapeutic potential.

miR-221 and miR-222

Studies using human umbilical vein endothelial cells demonstrated that miR-221 and miR-222 regulate angiogenesis in response to stem cell factor, presumably by directly repressing the levels of cKit and attenuating cell survival, migration, and endothelial tube formation. Although it was not clear from this study whether the expression of miR-221/miR-222 is altered during the physiological process of capillary formation in response to stem cell factor, it is clear from overexpression studies that modulation of miR-221/miR-222 affects endothelial tube formation and represents a potential avenue for therapeutic modulation of angiogenesis.

miR-210

miR-210 expression is elevated during hypoxic conditions and has been shown to possess proangiogenic properties in vitro. Overexpression of miR-210 in endothelial cells resulted in accelerated tube formation under normoxic conditions and enhanced vascular endothelial growth factor–dependent migration. Conversely, knockdown of miR-210 blocked capillary formation in response to hypoxia. This study demonstrated ephrin-A3 as an important target of miR-210, the inhibition of which is a major contributor to miR-210–mediated cell survival, migration, and tube formation in response to hypoxia (Figure 3). A recent study suggests that cytoprotection afforded by cardiac ischemia/reperfusion may be partially mediated by the induction of miR-210, which promoted mesenchymal stem cell survival by blocking caspase-8–associated protein-2. The results of these studies suggest that miR-210 should be further examin-
miR-126

miR-126 has been implicated in the maintenance of vascular integrity and promotion of vessel growth as a proangiogenic factor both in vitro and in vivo. Genetic deletion of miR-126 resulted in profound vascular defects, phenotypes that were previously ascribed to the host gene Eglf7, because of inadvertent deletion of miR-126 in the Eglf7-null mouse. Although a significant fraction of miR-126-null mice died embryonically because of vessel leakage, those mice that escaped neonatal lethality were particularly susceptible to vascular rupture after MI as a result of a deficit in neovascularization of the infarcted tissue. The proangiogenic effect of miR-126 was attributed, at least in part, to the repression of Spred-1, an intracellular inhibitor of vascular endothelial growth factor– and fibroblast growth factor–mediated angiogenesis and phosphatidylinositol-3-kinase regulatory subunit PIK3R2 (p85β) (Figure 3). Interestingly, miR-126 is also expressed in hematopoietic progenitor cells, a stem cell population that might contribute to post-MI cardiac regeneration. It is tempting to speculate that miR-126 may also influence cardiac repair by affecting the homing ability of circulating hematopoietic progenitor cells. In addition to a direct role in angiogenesis, miR-126 may play a role in the process of leukocyte infiltration and vascular inflammation. Vascular cell adhesion molecule expression is modulated by miR-126, thus affecting tumor necrosis factor-α–stimulated leukocyte adherence to endothelial cells and vessel inflammation. Taken together, these studies suggest that pharmacological elevation of miR-126 levels may be a viable therapeutic strategy to enhance neoangiogenesis and cardiac repair in ischemic myocardium.

miR-17-92 Cluster

Members of the miR-17-92 cluster were demonstrated to either positively or negatively influence angiogenesis, depending on the cellular context. The endothelial cell–enriched miR-92a, which is upregulated after ischemia, is a negative regulator of vessel growth. In vivo knockdown of miR-92a with antisense oligonucleotides resulted in improved recovery from ischemic damage because of accelerated vessel growth. The mechanism for the antiangiogenic role of miR-92a was attributed to repression of the proangiogenic factor integrin α5 (ITGA5; Figure 3). Conversely, other members of the miR-17-92 cluster (particularly miR-18 and miR-19) suppressed antiangiogenic factors in tumor cells and promoted angiogenesis and the vascularization of tumors in vivo. These studies demonstrated the importance of miRNAs in the modulation of angiogenesis in a pathological setting and highlighted the potential for pharmacological intervention by inhibition of an miRNA with an antisense approach.

miRNAs as Therapeutic Targets in Cardiovascular Disease

miRNAs are rapidly becoming an intriguing pharmacological target in the treatment of cardiovascular disease. The development of antisense oligonucleotide-mediated (antimiR) knockdown and miRNA-mimic–mediated overexpression techniques might soon allow the regulation of any given miRNA in cardiovascular disease.

Antisense-Mediated miRNA Knockdown

AntimiRs are antisense oligonucleotides with the reverse complementary sequence of the target miRNA. They can be conjugated to a cholesterol moiety to increase cellular uptake and often contain a modified sugar backbone that increases stability. On cellular uptake, antimiRs bind to the mature miRNA, thereby inhibiting its activity. Because miRNAs typically act as repressors of gene expression, inhibition of an miRNA should result in the derepression of miRNAs that are directly targeted by the miRNA. Thus, the primary effect of an miRNA inhibitor is activation of gene expression. Krützfeldt et al reported the first mammalian antimiR knockdown using cholesterol-conjugated, 2'-O-methyl-modified antimiRs, called antagomiRs, that resulted in derepression of putative target miRNAs. In a later study, they showed that systemic antagomiR delivery via intravenous injection efficiently reduced the level of a given miRNA in multiple tissues over an extended period of time. Several animal model studies have now demonstrated the value of antagomiRs in downregulating the expression of specific miRNAs in the heart and thereby influencing myocardial remodeling.

Besides antagomiRs, other antimiRs with different modifications are now being developed that display distinct pharmacokinetics and possibly mechanisms of action. For example, antimiRs with 2'-O-methoxymethyl phosphorothioate substitutions have proven useful for the inhibition of miR-122 in the liver. A very promising new approach might be the use of locked nucleic acid chemistry, which has already been demonstrated in the downregulation of miRNAs in nonhuman primates and is currently being tested in the first human clinical trial of miRNA inhibition.

miRNA Mimics

miRNA mimics are synthetic RNA duplexes in which 1 strand is identical to the mature miRNA sequence (guide strand) and is designed to “mimic” the function of the endogenous miRNA. The other strand (passenger strand) is often only partially complementary to the guide strand and typically linked to cholesterol to enhance cellular uptake. The double-stranded structure is required for efficient recognition and loading of the guide strand into the RNA-induced silencing complex. The use of miRNA mimics would be particularly useful for enhancing the expression of miRNAs that are downregulated in cardiovascular disease; however, their in vivo efficacy has not yet been experimentally validated in a pathological setting. It is important to note that the use of miRNA mimics also results in their uptake in tissues that do not normally express the miRNA and may result in unanticipated side effects, which could be overcome by tissue-specific targeting strategies.

Intravenously injected antagomiRs and miRNA mimics have been the primary method of systemic delivery to date; however, they are preferentially targeted to the liver, kidney, and spleen. Therefore, a major challenge will be the devel-
opment of strategies for the enrichment of antimiRs or miR mimics in the cardiovascular system. One possible approach would be a conjugation strategy with the nucleic acid linked to targeting molecules such as peptides, antibodies, or other bioactive molecules, which may promote homing of the antimiR/miRNA mimic to the heart. Alternatively, the antimiR/miRNA mimic could be encapsulated into a lipid-based formulation that enhances cardiac uptake. Finally, the specific application of the antimiR/miRNA mimic to the heart, eg, into the coronary vessels during cardiac catheterization on MI, might improve cardiac uptake.

There are several difficulties to overcome to promote miRNAs as a viable therapeutic target. As stated above, results obtained from pharmacological knockdown or overexpression by administration of antimiRs and miR mimics often differ from those observed with genetic mouse models. Several possibilities might explain these findings. First, miRNAs often have hundreds or even thousands of predicted mRNA targets, but at physiological expression levels, a miRNA most likely targets only a small fraction of them. Forced overexpression of a miRNA, however, might result in the regulation of physiologically irrelevant targets. Second, antimiRs similarly may potentially have considerable off-target effects that in some cases might be more responsible for the observed effects than the actual knockdown of the miRNA. Third, because systemic delivery of antagonimiRs and miRNA mimics affects miRNA expression globally, effects observed in the heart could also be secondary to effects in extracardiac tissues, eg, as a result of altered blood pressure or a change in the level of circulating hormones. Finally, a short-term and partial knockdown with antagonimiRs might have different consequences than a complete genetic deletion in which the gene is eliminated from embryogenesis to adulthood; genetic deletion may result in embryonic phenotypes that may complicate the analysis of adult disease models. Furthermore, compensatory mechanisms could be activated in genetic knockout mice that account for mild phenotypes compared with transient knockdown. Nevertheless, the most valuable model to study the function of a miRNA is genetic deletion, which should specifically derepress only those miRNAs that are physiologically repressed by the miRNA. In summary, it is apparent that miRNA biology must be examined through the use of a combination of genetic models and pharmacological manipulation.

Concluding Remarks

The demonstration that miRNAs play a crucial role in cardiovascular disease and can be easily regulated in vitro and in vivo by antimiRs and miR mimics has tremendously accelerated miRNA research and has nourished hopes that the drugs used and verified in animal models could be used in humans. Currently, therapeutic applications of miRNA biology focus only on affecting translational regulation. Besides the use of miRNAs as therapeutic targets, they might serve a valuable diagnostic and prognostic function for various cardiovascular pathologies because cardiac damage results in detectable levels of cardiac miRNAs in the blood.67 miRNAs represent a relatively young field of basic biological and translational research into new and innovative therapeutic applications. However, the rapid advancement toward viable miRNA-driven therapeutic options increases the odds that the next “small RNA step” will be a “giant leap” for the treatment of cardiovascular disease.

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References

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### Supplemental Table 1. MicroRNA target prediction resources

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<th>Algorithm</th>
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<td>TargetScan</td>
<td><a href="http://www.targetscan.org/">http://www.targetscan.org/</a></td>
<td>Short seed; conservation</td>
<td>1</td>
</tr>
<tr>
<td>miRanda</td>
<td><a href="http://www.microrna.org/">http://www.microrna.org/</a></td>
<td>Thermodynamic stability; conservation</td>
<td>4</td>
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<tr>
<td>MicroInspector</td>
<td><a href="http://bioinfo.uni-plovdiv.bg/microinspector/">http://bioinfo.uni-plovdiv.bg/microinspector/</a></td>
<td>Allows for user inputs of UTR sequence and thermodynamic cutoffs</td>
<td>6</td>
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<tr>
<td>miTarget</td>
<td><a href="http://cbit.snu.ac.kr/~miTarget/">http://cbit.snu.ac.kr/~miTarget/</a></td>
<td>Weights position 4, 5, and 6 in seed. SVM classifier</td>
<td>8</td>
</tr>
<tr>
<td>PITA</td>
<td><a href="http://genie.weizmann.ac.il/pubs/mir07/mir07_prediction.html">http://genie.weizmann.ac.il/pubs/mir07/mir07_prediction.html</a></td>
<td>Scans user defined miRNA or 3’UTR for target sites</td>
<td>9</td>
</tr>
<tr>
<td>miRTarget2</td>
<td><a href="http://mirdb.org/miRDB/index.html">http://mirdb.org/miRDB/index.html</a></td>
<td>Target prediction by SVM learning machine</td>
<td>10, 11</td>
</tr>
<tr>
<td>miRecords</td>
<td><a href="http://mirecords.umn.edu/miRecords/">http://mirecords.umn.edu/miRecords/</a></td>
<td>Convenient integration of major target prediction programs. Includes validated targets.</td>
<td>12</td>
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</tbody>
</table>
Supplemental References


