MicroRNAs in Cardiovascular Biology and Heart Disease

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Abstract—MicroRNAs play important roles in many cellular and biological functions via the regulation of mRNA target translation. In the cardiovascular field, microRNAs are now acknowledged as fundamental in regulating the expression of genes that governs physiological and pathological myocardial adaptation to stress. Here, we review recent progress in the understanding of microRNA functions and their involvement in heart disease. (Circ Cardiovasc Genet. 2009;2:402-408.)

Key Words: cardiovascular diseases • genes • myocardium • microRNA • heart failure • cardiac hypertrophy • antagonirs

MicroRNAs (miRs) are endogenous, single-stranded, short RNA sequences (∼22 nucleotides) that regulate target gene expression by base pairing with specific binding sites located in the 3′ untranslated region (UTR) of target mRNAs.1 The first discovered miR was lin-4 which, during the developmental phase of Caenorhabditis elegans, was shown to downregulate protein levels of its target, lin-14.2 Since then, multitudes of miRs have been discovered, and, with the last miRBase 12.0 database release, ∼1000 miRs have been reported in the human genome.3–5

miRs act in a complex functional network in which each miR might control hundreds of distinct target genes, and the expression of a single coding gene can be regulated by many different miRs.6–7 In line with this, recent evidence shows their important role in the regulation of a multitude of physiological functions, such as stem cell differentiation, neurogenesis, hematopoiesis, immune response, and skeletal and cardiac muscle development and stress.8–14 Furthermore, a variety of diseases, such as cancer, diabetes, and heart hypertrophy and failure, have been related to aberrant expression of miRs. Here, we present an overview of recent relevant findings on the role of miRs in the cardiovascular field.

Biogenesis and Target Recognition

Approximately half of all miRs are clustered in polycistronic units that generate large primary transcripts containing more than one miR. In addition, a number of miRs can be transcribed from multiple units within the genome.15 Although they do not encode proteins, miRs are transcribed by RNA polymerase II as part of longer molecules called pri-miRs (which are ∼2 kb in length; Figure 1). Pri-miRs are cleaved in the nucleus by the RNase III-type enzyme Drosha, which is associated with the double-stranded RNA-binding protein DGCR8 (DiGeorge syndrome critical region gene 8)/Pasha, to yield a pre-miR, which has a short stem-loop structure consisting of ∼650 nucleotides. Subsequently, pre-miR is exported to the cytoplasm, via exportin-5, and further processed by the ribonuclease Dicer, a highly conserved protein of ∼200 kDa, and its cofactors, protein activator of protein kinase PKR and human immunodeficiency virus transactivating response RNA-binding protein, to form a double-stranded RNA molecule. Subsequently, one strand of the duplex called the “passenger strand” is peeled away to leave a single-stranded miR, which is incorporated into the miR-induced silencing complex. Here, EIF2C2, or Ago2A in humans,16 a member of the argonaute endonuclease family, contributes to form the core component of miR-induced silencing complex, which is indispensable for miR-mRNA binding. Target recognition relays on the specific interaction between the miR and miR recognition elements (seeds) located in the 3′ UTR of target messenger RNAs. Finally, translational inhibition or, in some cases, mRNA degradation, contributes to the downregulation of the corresponding target protein.3,17–20

Five widely used bioinformatic tools for mammalian target prediction include DIANA-microT,21 miRanda,22 TargetScan,23 TargetScanS,6 PicTar,24 and PITA.25 Collectively, these algorithms rely on several criteria for prediction of targets, including conservation among species, seed complementarity, ΔG of target mRNA binding site, and multiple miR binding sites in the 3′UTR (cooperativity). For interested readers, an exhaustive discussion about miR target binding recognition rules can be found in a recent excellent review.26 However, among the several hundreds of identified miR targets so far, only a few have been validated and confirmed as true targets. Nevertheless, these validations are sufficient
MicroRNAs and Cardiac Development

Systematic analysis of the spatial-temporal expression of miRs has shown that many of these tiny regulators have strong tissue specificity together with tight temporal expression regulation starting from early phases of embryogenesis. This fine modulation has been shown to play an important role in cell lineage commitment and embryonic tissue development by temporal activation or inactivation of specific targets. In line with this observation, interference of miR biogenesis through deletion of Dicer results in embryonic lethality partly due to defects in cardiogenesis.

A number of miRs have been shown to be involved in cardiogenesis, such as miR-1, miR-133, miR-126 to 3p, miR-30c, miR-26a, and miR-208. miR-1 and miR-133, which are encoded by the same duplicated bicistronic genetic locus (miR-1 to 1/miR-133a-2 and miR-1 to 2/miR-133a-1), play important key roles in muscle proliferation and differentiation. miR-1 has been shown to control the balance between differentiation and proliferation during cardiogenesis mainly by inhibiting regulatory protein expression in the heart (Table). In fact, overexpression of miR-1 in *Drosophila* resulted in embryonic lethality because of disrupted myoblast patterning as a result of an insufficient number of cardioblasts. On the other hand, miR-1 to 2 deletion in mice resulted in common and often fatal sepal abnormalities together with thickened ventricular walls caused by continued cell proliferation. Similar to miR-1, the coexpressed miR-133 has been shown to be involved in the regulation of developmental phases and demonstrated to be necessary for atrioventricular canal development in zebrafish.

MicroRNAs and Cardiac Disease

**Myocardial Hypertrophy, Remodeling, and Heart Failure**

In response to physiological stimuli or pathological states, such as hypertension, ischemic myocardial injury, and valvular disease, the myocardium reacts with changes in the gene expression profile. This microadaptation usually results in cardiac remodeling, which is characterized by severe structural alterations of myocardial tissue, modification of the extracellular matrix, and reshaping of left ventricle geometry and performance. All of these consequences are associated with poor prognosis and high mortality in the long term. Under pathological conditions, cardiac hypertrophy (defined as an increase in ventricular mass caused by increased cardiomyocyte size) is characterized by an initial compensatory process that helps the heart to sustain the cardiac output. However, this process is only an initial “adaptive” response, and chronic exposure to stress signals, neuroendocrine activity, and re-expression of fetal type genes lead to impaired systolic and diastolic function, characterizing heart failure. Recently, several reports have revealed important roles of miRs in cardiac hypertrophic growth and heart failure. During the adaptive response of the heart to stress stimuli, microarray analyses have shown upregulated, downregulated, or unchanged miR expression, when compared with normal heart. Moreover, differential regulation of subsets of miRs has been shown to characterize various etiologies. For example, Sucharov et al observed different miR expression patterns in patients affected by dilated cardiomyopathy and ischemic cardiomyopathy. Furthermore, juvenile and adult Dicer conditional knockout mice were characterized by aberrant myocardial morphology and function.

An impressive similarity has been found between the miR expression pattern occurring in human failing hearts and that observed in the hearts of 12- to 14-week-old fetuses. Approximately, >80% of the analyzed miRs have been found to be similarly regulated both in failing adult and fetal human hearts compared with that of heart tissue from normal adults. The most consistent changes were upregulation of miR-21, miR-29b, miR-129, miR-210, miR-211, and miR-423 and downregulation of miR-30, miR-182, and miR-526. In addition, studies of pressure-overloaded mice, transgenic calcineurin mice, and human heart failure patients have identified additional miRs involved in cardiomyocyte hypertrophy. Among 186 miRs analyzed, miR-23a, miR-23b, miR-24, miR-195, miR-199a, and miR-214 were found to be upregulated. Interestingly, miR-24, miR-125b, miR-195, miR-199a, and miR-214 were also upregulated in patients with end-stage failing hearts, and transfection of neonatal cardiomyocytes with these miRs resulted in significant hypertrophy. The concordant data derived from these expression profiles indicate that miRs may be involved in common pathways mediating the hypertrophic response.

In addition to the miRs mentioned earlier, Sayed et al demonstrated suppressed miR-1 expression from 1 day to 1 week after transverse aortic constriction in mice. Furthermore, miR-1 expression has been shown to be inversely correlated with cardiac hypertrophy (Table) and to be associated with regulation of hypertrophy-associated genes such as calmodulin. Similarly, Care et al observed impaired expression of both miR-1 and miR-133 in patients with hypertrophic cardiomyopathy and atrial dilatation as well as in 3 different murine models of cardiac hypertrophy, i.e., endurance-trained rats, transverse aortic constriction-operated...
mice, and Akt E40K transgenic mice, which overexpress an active cardiospecific Akt kinase. In the same study, in vitro overexpression of miR-133 resulted in suppressed protein synthesis and inhibition of hypertrophic growth induced by the hypertrophic agonists phenylephrine and endothelin-1. In addition, buffering of endogenous miR-133 with a targeted 3'UTR decoy resulted in marked cell hypertrophy, significantly increased protein synthesis, increased fetal gene expression, and perinuclear expression of atrial natriuretic factor. In vivo, the administration of an antisense RNA oligonucleotide conjugated to cholesterol (antimir-133), capable of silencing endogenous miR-133 function, induced significant myocardial hypertrophy associated with reinduction of the fetal gene program. Cdc42 and Rho-A, both GTG-GDP-binding molecules involved in cell growth, myofibrillar organization, and contractility regulation, were identified and validated as targets of miR-133. Another validated target is WHSC2/NELF-A, a nuclear factor involved in the development, hypertrophy, and function. Additional studies are required to unravel its multiple mechanisms of action. The discrepancy between the 2 studies might be explained by the 2 different models used, ie, antagonist treatment has a transient effect as compared with a genetic deletion where the gene is eliminated throughout the life of the organism. Furthermore, antagonist treatment might not result in a complete loss of the targeted miR and may also unspecifically target other miRs. Together, however, the 2 articles indicate a key role of miR-133 in myocardial development, hypertrophy, and function. Additional studies are required to unravel its multiple mechanisms of action.

Another miR involved in cardiac hypertrophy is miR-208, a cardiac-specific miR encoded by an intron within the α-major histocompatibility complex (MHC) gene. miR-208 knockout mice are viable and do not show any obvious cardiac phenotype, but they fail to undergo stress-induced cardiac remodeling, hypertrophic growth, and β-MHC up-regulation following transverse aortic constriction (Table). The identified mechanism implicates the involvement of miR-208 in regulating β-MHC by repressing thyroid hormone receptor-associated protein 1, a cofactor of the thyroid hormone receptor. Its repression affects the activity of the thyroid receptor-associated protein complex, thereby blocking β-MHC expression, which is regulated by thyroid response elements.

miR-21 is another miR involved in cellular growth and fetal gene reactivation, although its role in hypertrophy is not completely understood. Two studies have reported upregulation of miR-21 following pressure overload in mice. Cheng et al reported a blunted hypertrophic response and absent fetal gene re-expression after agonist stimulus following oligonucleotide-mediated knockdown of miR-21 in vitro. In contrast, Tatsuguchi et al reported a blunted increase in cell size and increased fetal gene expression following antisense knockdown of miR-21 in cultured neonatal cardiomyocytes. miR-21 has also been identified as an oncogene, promoting cell proliferation, and was recently identified as a modulator of myocardial fibrosis. In this regard, Thum et al reported progressive upregulation of miR-21 during late stages of heart failure, with an expression profile restricted exclusively to cardiac fibroblasts (Table). Upregulation of miR-21 was shown to be responsible for increased extracellular signal-regulated kinase/mitogen-activated protein kinase signaling through inhibition of its target, spry1 (sprouty 1), an inhibitor of the Ras/map-erk kinase (MEK)/extracellular signal-regulated kinase pathway. The same authors proposed a mechanism whereby miR-21 is responsible for increased fibroblast survival and interstitial fibrosis independent of cardiomyocyte loss. Likewise, Van Rooij et al recently demonstrated that downregulation of the fibroblast-enriched miR-29 family in fibrotic areas surrounding a cardiac infarct is responsible for the regulation of miRNAs that encode a multitude of proteins involved in fibrosis such as collagens, fibrillins, and elastin (Table). In addition, Creemers’ group identified a role of miR-30 and miR-133 in myocardial matrix remodeling. More specifically, downregulation of these miRs during cardiac disease was inversely correlated with the upregulation of collagen production and fibrosis by directly targeting connective tissue growth factor.

Arrhythmia
Cardiac remodeling and heart failure are associated with an increased risk of fatality. This complication is characterized by important changes in ion channel function and expression, collectively named electrophysiological remodeling, in both atria and ventricles. Although the clinical relevance of miRs in regard to this phenomenon is not well established, recent evidence supports their role in the induction of arrhythmia. For example, transduction of miR-1 in rat hearts after myocardial infarction resulted in a significant enlargement of the QRS complex, prolongation of the QT interval, and an increased incidence of arrhythmias (Table). Conversely, a low incidence of fatal arrhythmias was obtained when miR-1 was knocked down with antisense oligonucleotides. In the same study, the authors identified GJA1 and KCNJ2 as validated
targets of miR-1. GJA1 encodes connexin 43 (CX43) and is critical for intercellular conductance whereas KCNJ2 encodes the K^+ channel subunit Kir2.2, which is relevant for the setting and maintenance of the membrane potential.

Studies by Srivastava’s group corroborated the involvement of miR-1 in electrophysiological remodeling. More specifically, miR-1 to -2 knockout mice that survived until birth had a high incidence of electrophysiological abnormalities that often resulted in sudden death. This phenotype was related to miR-1 targeting the transcription factor, Irx5, which represses KCND2, a potassium channel subunit involved in the transient outward K^+ current (I_{to}). Further evidence for a role of miR-1 in heart failure comes from a recent study in which it was found to be up to 3-fold overexpressed in left ventricular tissue from patients with end-stage cardiac disease.51 Similarly to miR-1, the coexpressed miR-133 has also been shown to be involved in electrophysiological remodeling. In particular, downregulation of miR-1 and miR-133 in hypertrophic hearts has been associated with a concomitant increase in protein levels of HCN2/HCN4, 2 important ion channels that, when upregulated, enhance automaticity and the development of arrhythmia as observed in heart failure.34,62 Moreover, in a model of diabetic cardiomyopathy, overexpression of miR-133 has been shown to result in downregulation of the ERG (ether a-go-go-related gene) with consequent QT prolongation, responsible for arrhythmias (Table).44 Recently, further evidence suggesting the involvement of miR-133 in electrophysiological remodeling has come from a study in which the pore forming unit of the L-type calcium channel was shown to be a target of miR-133.63

Vascular Angiogenesis

Neoangiogenesis results from the balance between proliferation and apoptosis of vascular smooth muscle cells and is an important repair mechanism that is recruited, for instance, for the maintenance of adequate blood flow after ischemic injury.64 miRs have recently been shown to play a role in the modification of this delicate process.

Dicer knockout mice die early during embryonic development because of thinning of vessel walls and severe disorganization of the network of blood vessels. In these mice, upregulation of vascular endothelial growth factor and its receptor, KDR, has been associated with dysregulation of miR biogenesis.65 Similarly, transfection of endothelial cells with small interfering RNA targeting Dicer mRNA resulted in significantly decreased migration rate and tube-forming activity.65 Similar results were by obtained with RNA interference of Dicer in human endothelial cells.66 Together with a decrease in endothelial cell growth, several key regulators of endothelial biology and angiogenesis, such as TEK–Tie-2, Tie-1, endothelial nitric oxide synthase, and interleukin-8, were found to be affected.66

Ji et al (Table) reported aberrant expression of multiple miRs in the vascular wall after angioplasty. More specifically, miR array analysis revealed differential expression of 133 of the 140 artery miRs 1 week after balloon injury (60 miRs were upregulated and 53 were downregulated), with a decreasing number of miRs upregulated after 2 and 4 weeks. These data indicate the involvement of multiple miRs in neointimal lesion formation, which is a common feature of atherosclerosis, coronary artery disease, and balloon angioplasty treatment.

miR-21, the most upregulated miR in the vascular wall of balloon-injured rat carotid arteries, may also play a central role in angiogenesis. Notably, inhibition of miR-21 results in a significant decrease in neointimal formation after angioplasty, mainly because of decreased cell proliferation and cell apoptosis. PTEN and Bcl-2, which are involved in proliferation and apoptosis, respectively, were identified as target genes for miR-21 in vascular smooth muscle cells.38

Recently, miR-126 has been identified as an endothelial cell-specific miR that plays an important role in neoangiogenesis.
genesis following ischemic injury, such as myocardial infarction (Table). miR-126 overexpression in mice induces eliminating the repressive modulation of the signaling pathways activated by vascular endothelial growth factor and FGF by Spred-1, an intracellular inhibitor of the Ras/mitogen-activated protein kinase pathway. Ablation of miR-126 results in high embryonic lethality because of vascular leakage, whereas mutant mice that survive to adulthood are prone to cardiac rupture and lethality following infarction as a result of poor neovascularization of the infarcted area. In a zebrafish study, miR-126 was demonstrated to regulate the endothelial cell response to vascular endothelial growth factor by targeting Spred-1 and PIK3R2, both regulators of the vascular endothelial growth factor pathway.

MicroRNA Modulators as Potential Therapeutics in Cardiovascular Diseases

The biological activity of miRs highlights relevant potentials for the development of possible therapeutic agents (Figure 2). Indeed, several strategies have been used for manipulating target miR levels in vivo, including both loss-of-function (modified antisense oligonucleotides) and gain-of-function (ie, miR mimic) approaches.

Antisense miR oligonucleotides (AMOs) are fully complementary to target miRs, and they abolish miR action through a mechanism that is still not completely understood. Antagomirs, on the other hand, are chemically modified AMOs conjugated with a cholesterol base at the 3′ end. This latter modification increases antagomir efficiency for 2 main reasons. First, the binding to apolipoproteins, in particular high-density lipoprotein, facilitates systemic delivery of AMOs. Second, it allows cellular uptake of the AMO through the low-density lipoprotein scavenger receptor. Once in the cell, antagomirs bind to mature miRs before, during, or after RNA-induced silencing complex loading and effect miR degradation. Antagomirs have been successfully used in vivo and have been proven to be efficient and specific silencers of endogenous miRs in the mouse heart.

Using this approach, Krutzfeld et al obtained a marked reduction of the corresponding miR (miR-122, a liver-specific miR) in different organs with a silencing effect observed for an extended period of time. The first in vivo application of this approach in the heart was applied to miR-133. In that study, antagomir treatment resulted in a significant hypertrophic response following myocardial infarction, thickened ventricular wall, and attenuation of cardiac dysfunction after TAC.

Table. In Vivo Modulation of MicroRNAs: Cardiovascular Phenotypes

<table>
<thead>
<tr>
<th>MiR</th>
<th>Modulation</th>
<th>Phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Knockout</td>
<td>Electrophysiological abnormality, embryonic lethality, thickened ventricular wall</td>
<td>35</td>
</tr>
<tr>
<td>1</td>
<td>Mimic</td>
<td>Increased arrhythmia</td>
<td>36</td>
</tr>
<tr>
<td>1</td>
<td>AMO</td>
<td>Fatal arrhythmia following myocardial infarction</td>
<td>36</td>
</tr>
<tr>
<td>21</td>
<td>Antagomir</td>
<td>Inhibition of interstitial fibrosis and attenuation of cardiac dysfunction after TAC</td>
<td>37</td>
</tr>
<tr>
<td>21</td>
<td>‘2’oMe</td>
<td>Decreased neointimal formation, cell proliferation, and apoptosis</td>
<td>38</td>
</tr>
<tr>
<td>29</td>
<td>Antagomir</td>
<td>Enhancement of the fibrotic response</td>
<td>39</td>
</tr>
<tr>
<td>126</td>
<td>Morpholino</td>
<td>Loss of vascular integrity and hemorrhage during embryonic development (zebrafish model)</td>
<td>40</td>
</tr>
<tr>
<td>126</td>
<td>Knockout</td>
<td>Lethal embryogenesis due to vascular leakage; defective cardiac neovascularization following myocardial infarction in survival adults</td>
<td>41</td>
</tr>
<tr>
<td>133</td>
<td>Antagomir</td>
<td>Cardiac hypertrophy</td>
<td>42</td>
</tr>
<tr>
<td>133a1</td>
<td>Knockout</td>
<td>No phenotype</td>
<td>43</td>
</tr>
<tr>
<td>133a2</td>
<td>Knockout</td>
<td>No phenotype</td>
<td>43</td>
</tr>
<tr>
<td>133a1/133a2</td>
<td>Knockout</td>
<td>Embryonic-postnatal lethality, ventricular septal defect, atrial and right ventricular enlargement, dilated heart, increased fibrosis</td>
<td>43</td>
</tr>
<tr>
<td>133a2</td>
<td>Transgenic</td>
<td>Embryonic-postnatal lethality, impaired cardiomyocyte proliferation</td>
<td>43</td>
</tr>
<tr>
<td>133</td>
<td>Mimic</td>
<td>Long QT, arrhythmia</td>
<td>44</td>
</tr>
<tr>
<td>208</td>
<td>Knockout</td>
<td>No basal phenotype, failure to undergo stress-induced cardiac remodeling after TAC</td>
<td>45</td>
</tr>
</tbody>
</table>

Targeted miRs is more strongly suppressed than when using separate inhibitors because an entire miR family is affected. Recently, the possibility of developing a new class of miR sponge that targets multiple miRs has been explored and applied to a new generation of AMOs. miR masking can also be used to reduce the function of miRs: with this technique, however, oligonucleotides are designed in such a way as to be antisense to a specific 3′ UTR mRNA binding site, without producing target cleavage. Occupation of the binding site by a miR mask precludes attachment of the cognate miR and, thus, translational repression or degradation of the targeted mRNA is inhibited.

As described previously, many pathological conditions are characterized by downregulation of specific miR levels. In this case, development of miR-mimic techniques represents an attractive tool for preventing and treating cardiovascular disease. MiR-mimics are synthetic double-stranded RNA designed to mimic the action of mature endogenous miRs. Recently, administration of miR mimic of miR-29 has been
shown to capable of blunting fibrosis during hypertrophy and after myocardial infarction, through the targeting of collagen I, II, and II gene translation. In that study, miR mimics consisted of a double-stranded chemically modified RNA molecule with a cholesterol-conjugated 3’ end.

Conclusions

miRs play important roles in modulating many biological functions, and their dysregulation under pathological conditions has recently been described in a number of studies. Although our knowledge of miR biology and function is still partial, a growing list of identified validated tissue-specific miR targets is emerging.

In vivo modulation of miR activity has provided further insights into the regulatory mechanisms involved in cardiac biology and disease (Table). Moreover, because of the important pathogenic implication of miRs in many diseases, intervening at this level might represent a novel therapeutic strategy. However, further studies are required to provide further insights into the underlying mechanisms of miR action, to optimize delivery techniques, and to clarify specificity, reversibility, and potential toxicity of miR modulators.

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Disclosures

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