Emerging Role of MicroRNAs in Cardiovascular Biology

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Abstract—The heart is among the most conserved organs of the body and is susceptible to defects more than any other organ. Heart malformations, in fact, occur in roughly 1% of newborns. Moreover, cardiovascular disease arising during adult life is among the main causes of morbidity and mortality in developed countries. It is not surprising, therefore, that much effort is being channeled into understanding the development, physiology, and pathology of the cardiovascular system. MicroRNAs, a newly discovered class of small ribonucleotide-based regulators of gene expression, are being implicated in an increasing number of biological processes, and the study of their role in cardiovascular biology is just beginning. Here, we briefly overview microRNAs in general and report on the recent findings regarding their importance for the heart and vasculature, in particular. The new insights that are being gained will permit not only a greater understanding of cardiovascular pathologies but also, hopefully, the development of novel therapeutic strategies. (Circ Res. 2007;101:1225-1236.)

Key Words: microRNA ■ heart development ■ hypertrophy ■ heart failure ■ arrhythmias ■ cardiovascular disease

The events that brought to the discovery of animal microRNAs started in the early 1980s, when a loss-of-function mutation of lin-4, a heterochronic gene, capable of controlling the timing of development of nematodes, was reported to lead to an increased number of molts and continued synthesis of larval-specific proteins in Caenorhabditis elegans.1 Several years later, lin-4 was demonstrated to function as an inhibitor of 2 other heterochronic genes, lin-14 and lin-28, which halt the larva-to-adult switch, thus relieving their inhibition on lin-29 and permitting continuation of development.2 Subsequently, deletions within the 3’ untranslated region (UTR) of lin-14 mRNA were described to result in gain-of-function mutants, with abnormal accumulation of the assumed LIN-14 protein in the later larval stages.3 The authors speculated that the lin-4 gene product bound to the 3’ UTR of lin-14 negatively regulating it. Two independent studies, published in the same issue of the journal Cell, then suggested that transcription could be regulated not by protein–RNA interactions but rather by RNA–RNA interactions.4,5 In fact, these authors reported that lin-4 does not code for a protein but produces small RNA transcripts, complementary to the 3’ UTR of lin-14 mRNA. Thus, translation of lin-14 could be inhibited by the interaction of small lin-4 RNA transcripts with the 3’ UTR of lin-14 mRNA. It was not until 7 years later that a second heterochronic switch gene, let-7, was also found to have a complementary sequence to the 3’ UTR of lin-14, as well as to other mRNAs.6 At the time, these transcripts were called small temporal (st)RNAs and were considered to be restricted in importance. However, other groups soon obtained evidence showing a more general role of stRNAs in the regulation of genes. In fact, researchers from 3 different laboratories isolated a new class of RNA, referred to as microRNAs (miRs), with similar characteristics to lin-4 and let-7, ie, capable of regulating gene expression at the posttranslational level by base pairing at the 3’ UTR of their mRNA targets.7–9

Since then, a multitude of miRs have been found, some of which are highly conserved, reflecting their extreme value. Importantly, a distinct tissue-specific distribution of some miRs has led to the idea that these small RNA molecules must be involved in tissue differentiation.10 Moreover, bioinformatics analyses have predicted that each miR may regulate hundreds of targets, thus suggesting that miRs may play a role in almost every biological process.11

Characteristics of MiRs

Structure and Biogenesis

MiRs are 21- to 26-nucleotide (nt), single-stranded RNA molecules, produced from hairpin-shaped transcripts. In mammals, a large fraction of these transcripts derive from miR genes that are found as part of introns of either protein-coding or noncoding genes. A small number are also found within exons,12,13 whereas many others are present in genomic repeats or have an unknown origin. Moreover, a significant number of miRs are expressed in clusters in which 2 or 3 miRs are generated from a common parent mRNA.

The genes of MiRs differ from other genes in that they do not have the canonical TATA box and they do not contain...
miR genes may be transcribed by RNA polymerase III. Recent study has shown that a significant number of human small interfering (si)RNAs (see below). The pre-miR is then processed into a 60- to 70-nt transcript, termed pre-miR. On the basis of both computational and biochemical analyses, Han et al have proposed that this processing occurs through a single-stranded RNA–double-stranded RNA junction-anchoring model. In this configuration, a protein complex containing the enzyme Drosha, a nuclear ribonuclease III, is responsible for cropping pri-miR into pre-miR. The pre-miR is exported out of the nucleus by exportin-5 (Exp5)/RanGTP, and hydrolysis of RanGTP to RanGDP releases the pre-miR in the cytoplasm. Here, the miR processing pathway converges with that of the small interfering (si)RNAs (see below). The pre-miR is then processed into a 18- to 22-nt miR duplex by another RNase III, called Dicer, which is associated to another dsRBP. The duplex is probably unwound by a helicase activity, and 1 strand, the so called “passenger” strand (or miR*), is de-graded, whereas the other strand, called the “guide” strand, accumulates as a mature miR. The miR is then handed over to Argonaute, which binds to the 3′ end of the miR, with its RNA binding domain, PAZ (piwi–argonaute–zwille) (reviewed elsewhere). The association of Dicer, Argonaute, and a miR forms a ribonucleoprotein (RNP) called the miR-induced silencing complex (miRISC), which, after binding with an mRNA target, accumulates in cytoplasmic foci known as processing bodies (P-bodies) and stress granules (reviewed elsewhere). Recently, a pathway for miR biogenesis has been reported that is partially distinct from the above-described canonical pathway in that it does not necessitate Drosha processing. Pre-miR–sized genes, termed mirtrons, have been located within the introns of genes from Drosophila melanogaster and C. elegans. When spliced of the host gene, mirtrons form branched intermediates (lariats), which after debranching, are folded to form a pre-miR hairpin. From here on, this pathway converges onto the canonical miR biogenesis pathway. Interestingly, the mirtron pathway has been speculated to represent the ancestral pathway of miR biogenesis from which the canonical biosynthetic pathway evolved after the emergence of Drosha. Only few pre-miR–sized introns have been found within higher animal species. To date, no mirtron has been demonstrated in mammals, but their presence has not been formally excluded yet.

**Nomenclature**

To avoid confusion, a distinction must be made between miRs and siRNAs. MiRs are short, endogenous RNAs derived from single-stranded precursor RNAs fashioned with an imperfectly base paired hairpin segment. SiRNAs, on the other hand, are similar in length but are derived from longer, perfectly complementary double-stranded RNA precursors of mainly exogenous origin. Both mature through a duplex intermediate, with unpaired 3′ extensions and a 5′ phosphate. Functionally, miRs tend not to be exactly complementary with their targets, because of the presence of mismatches and bulges, whereas siRNAs are usually exactly complementary. This seems to be important for their mechanism of action, as is discussed below. However, if a miR is perfectly matched with its target, it can act as an siRNA and, similarly, an imperfectly matched siRNA can act as if it were a miR. Thus, miRs and siRNA cannot be distinguished by mechanism but only through their origins and biogenesis.

Bioinformatics approaches have been developed to predict putative miRs present in the genome of different organisms, based on the fact that they are usually highly conserved between related species and produced from precursor transcripts of similar size and structure. The first miR search algorithm to be developed was miRNAScan (http://genes.mit.edu/mirscan), whereas other algorithms such as PromiR II (http://cbit.snu.ac.kr/~ProMiR2) and PalGrade were specifically designed to be used for humans (reviewed elsewhere). Newly identified miRs are denoted with sequential numbers unless they are orthologs (homologous miRs in different organisms), in which case the same number is used. When a miR is produced from more than 1 locus, the 2 family members are differentiated by numerical suffixes, as in the...
Mechanisms of Action

MiRs negatively modulate gene expression at the posttranscriptional level by base pairing at sites contained in the 3′ UTR of target mRNAs. The binding specificity of individual miRs for their target mRNAs has been presumed to be dictated by only ≈6 to 7 of the 22 to 26 nt that compose a miR. This sequence, located at the 5′ end of the miR molecule, contributes disproportionately to target RNA binding, and is called the “seed” sequence, a term intended to suggest that this region nucleates binding and that the more 3′ region subsequently zippers up with the target RNA. Based on this notion, computational predictions of miR targets have revealed that a single miR has the potential to inhibit up to ~200 mRNAs. Moreover, binding of a single miR alone may not be sufficient to measurably block translation and several miRs may need to bind to the same target to have any effect.22

The exact mechanisms through which miRs regulate gene expression are still subject of debate, but a simplified notion may be that, depending on the overall degree of complementarity with the target, miRs will either inhibit translation or induce degradation of mRNA. Usually, the interaction of a miR and its target mRNA is characterized by extensive mismatches and bulges, which result in a reduced efficiency of translation rather than a decrease in mRNA abundance. The exact point of repression, ie, before or after initiation, is, however, still controversial; inhibition of eIF4E-dependent initiation,43,44 elongation,45 and cotranslational nascent protein degradation46,47 have all been reported. Moreover, when miRs bind with more precise complementarity to their target mRNA, they promote mRNA degradation like their siRNA counterparts, with the induction, however, of an exonuclease activity after deadenylation and decapping steps rather than endonucleolytic cleavage of mRNA at the site of complementarity, reminiscent of siRNA-mediated silencing.48–51

After inhibition of the translation machinery, miRISC and the bound target localize to P-bodies.24 These cytoplasmic foci contain enzymes important in the normal pathway of mRNA degradation, such as RXN1, a 5′→3′ exoribonuclease.52 Once within the P-bodies, translationally repressed mRNA is either sequestered in storage structures or can be processed for degradation. Although P-bodies play a role in the silencing process, it has been shown that miRs can function even in the absence of these cytoplasmic structures, thus providing the evidence that miR-dependent mRNA degradation in P-bodies may be the result rather than the cause of repression.25,53

Novel Mechanisms and Inhibition of Endogenous MiRs

Posttranslational gene silencing within the cytoplasmic compartment of the cell, whether via reduced translation efficiency or degradation of the targeted mRNA, is the classic mechanism of action. As more is learned about this class of small RNAs, new and ever more fascinating modes of action are uncovered (Figure 1). For example, miRs containing a terminal hexanucleotide motif relocate back into the nucleus, where they may be responsible for some sort of transcriptional control.54 Moreover, exosomes have recently been demonstrated to contain a cargo of various RNAs, including miRs.55 This led to the extraordinary hypothesis that cells can exchange genetic material through cell–cell interactions, delivering specific sets of miRs to cells that would otherwise not synthesize them.

Increasingly intricate regulatory mechanisms are also being continuously documented. For example, a miR may have ubiquitous expression of the pre-miR but tissue restriction of the mature species because of specific cell-selective inhibition at the processing step.56 Extratranscriptional miR regulation might also profoundly alter miR action. This can occur by adenosine-to-inosine editing through the action of adenosine deaminases.57 Indeed, new miR isoforms may be created, which then interact with a differently recognized set of mRNAs. Similarly, subtle editing of the 3′ UTR of miRNAs might be a mechanism whereby mRNAs are recoded for differential miR recognition, but experimental proof is still lacking.59

Interestingly, at least in plants, cells may regulate miR function through the synthesis of noncleavable, nonprotein coding RNAs that interact stably with complementary miRs, thus repressing their action (a process termed target mimicry).60 This mechanism resembles similar approaches that have already been applied in the laboratory setting to knockdown miR function, highlighting an instance of scientific innovation preceding biological discovery. Specifically, anti-miRs,61 antiagomirs and decoys,62 and miR sponges63 have been synthesized and successfully used experimentally. Inhibition of specific endogenous miRs has been achieved by the administration of either synthetic or transcribed expressed competitive inhibitors. Anti-miR oligonucleotides64 are antisense to the miR itself and can be used to compete with mRNA for an overexpressed endogenous miR, similarly to the target mimicry described in plants.60 Antagomirs,64 anti-miR oligonucleotides synthesized with cholesterol moieties to permit entry into the cell, also can be used to bind with unwanted miRs and have been shown to be capable of silencing miR expression in the heart of mice.65 In addition, selective suppression of endogenous miRs can be achieved by genetically expressing complementary “decoy” sequences that, placed at the 3′ UTR of a reporter gene, act as molecular miR-traps.62 MiR sponges are based on this same approach: with the introduction of a bulge in target miR seed sequences or an increase of the number miR-binding sites in the decoy
sequence, the genetically encoded miR sponge can be used to inhibit the miR seed family. 63

Target Prediction
Based on the existence of the 5' end–restricted complementarity to mRNA targets, it has been predicted that miRs regulate a large number of genes. However, the number of targets verified to have biological relevance is still very small in animals compared with plants, because of their small size and the tolerance for mismatches of the animal miRs. Several algorithms based on different criteria have been developed, such as Diana-MicroT (http://www.diana.pcbi.upenn.edu/cgi-bin/micro_t.cgi), PicTar (http://pictar.bio.nyu.edu), miRanda (http://www.microrna.org/miranda_new.html), and TargetScan (http://www.targetscan.org) (reviewed elsewhere65). Not all complementary sequences contained in the 3' UTR are necessarily bona fide binding sites; thus, most of these algorithms predict a large number of targets, not all of which are necessarily true. Reliability in the identification of animal mRNA targets seems to have been improved by the additional evaluation of the energy states of sequences flanking the miR target (ΔG) and the presence or absence of stabilizing/destabilizing elements in target mRNA. 65 Zhao et al reported, in fact, that virtually all miR-binding sites are located in “unstable” regions and hypothesized that miRs target 3' UTR regions with a less complex secondary structure because more accessible. This notion has been used to correctly identify targets in cardiomyocytes (see below). More recent reports have further dissected the complexities behind miR/target binding. Sfold, a software for computing folding and design of nucleic acids (http://sfold.wadsworth.org/index.pl), 66 has been used to model this interaction and elaborate a 2-step hybridization reaction based on energy potentials67: an initial nucleation step, which occurs through access of the miR to an accessible site on the 3’ UTR, is then followed by disruption of local secondary structure, hybrid elongation, and formation of a stable duplex. These authors report that a block of at least 4 open nucleotides seems to be sufficient for nucleation and that targeting is not dependent on the seed sequence as nucleotides also in the 3’ region of the miR can be used for this aim. Features of site configuration have also been described that boost site efficacy68: (1) an AU-rich nt composition near the miR binding site impacts mRNA secondary structure destabilization; (2) proximity of coexpressed miR sites probably leads to beneficial effects by as yet unknown mechanisms of having more than 1 bound miRISC; (3) Watson and Crick pairing of a 3’ core of nts (at position 13-16) of the miR probably decreases the dissociation rate of the miRISC, leading to a more stable complex; and (4) positioning of the binding site(s) at the 3’ and 5’ extremities rather than at the center of the 3’ UTR, with at least 15 nt from the stop codon for the latter, probably improves accessibility or a needed proximity with the translation machinery and reduces interference with translating ribosomes, respectively. A resource containing these determinants has been made available at http://www.targetscan.org. A further structural requirement for miR processing and function has been recently put forward by a study on single nucleotide polymorphisms (SNPs) associated with 227 known human miRs. 69 Duan et al reported, in fact, that an SNP at nt position 8 of mature miR-125a critically compromises the recognition of mRNA targets. Notably, the same SNP was shown to be determinant for the correct processing of the pri-miR to pre-miR. These data suggest the relevance of SNPs that could intervene in the regulation miR biogenesis and alter target selection.

MiR in Cardiac Muscle
It is thought that up to one-third of a complete genome may be regulated by miRs. Studies have indicated that they are implicated in a variety of basic biological processes, such as metabolism, 70,71 proliferation, 65,72 stress, 73 apoptosis, 71,74,75 neural development, 76 hematopoiesis, 77 and oncogenesis, acting as either tumor suppressors or oncogenes. 78 However, in most of these studies, the actual role of the miR in these processes is fairly unclear. Only during the last year or so have elegant experimental studies started to unravel the important role played by miRs in cardiovascular pathophysiology. Various miRs are expressed in a tissue-specific manner and, thus, may regulate tissue-specific functions. Currently, a number of miRs have been found to be striated-muscle specific (such as miR-1 and miR-133), whereas only miR-208 has been found to be purely cardiac specific, to date.

Regulation of Heart Development
As pointed out above, miRs were first described as elements involved in the regulation of nematode development, acting as molecular “switches.” Consequently, subsequent investigations into the role of miRs in other species focused above all on this aspect of cell biology. In fact, 2 miRs, miR-1 and miR-133, were identified originally as having important roles in the control of differentiation and proliferation of muscle cells.

The MiR-1 Family
MiR-1 is among the most highly conserved miRs and is found in nematodes, flies, and all vertebrates. 79-81 The miR-1 family is comprised of the miR-1 subfamily and miR-206, which is not expressed in the heart. The miR-1 subfamily consists of 2 closely related transcripts, miR-1-1 and miR-1-2, encoded by distinct genes found on chromosomes 2 and 18, respectively. In flies, transcription of miR-1 is activated in a broad pan-mesodermal domain before gastrulation, whereas the 2 mouse miR-1 genes are first detected later, at the 18, respectively. In flies, transcription of miR-1 is activated in a broad pan-mesodermal domain before gastrulation, whereas the 2 mouse miR-1 genes are first detected later, at the beginning of muscle differentiation, and then become progressively more expressed. MiR-1-1 is first expressed in the inner curvature of the heart loop and in atria, during mammalian development, but becomes ubiquitously expressed in the heart as development continues; on the other hand, miR-1-2 is prevalent in the ventricles. 65 The differences in the spatiotemporal occurrence of miR-1 reported between flies and mice, and in zebrafish, seems to imply that miR-1 has evolved as a mesodermal/muscle-specific miR early in animal evolution but then has been integrated into a hierarchy of muscle transcription networks. Therefore, the role of a given miR may be slightly different from 1 species to the next. 82 In mammals, miR-1 cardiac expression is controlled by serum response factor (SRF), which recruits a coactivator, myocar-
This is different to that occurring in skeletal muscle, where miR-1 expression requires the myogenic transcription factors, MyoD (myogenic differentiation 1) and Merf2 (myocyte enhancer factor 2).

In mammals, miR-1 is responsible for the inhibition of cardiomyocyte progenitor proliferation: this has been shown to occur via inhibition of translation of Hand2, a transcription factor known to regulate ventricular cardiomyocyte expansion.65 In fact, overexpression of miR-1 in a transgenic mouse model resulted in a phenotype characterized by thin-walled ventricles, attributable to premature differentiation and early withdrawal of cardiomyocytes from the cell cycle. In contrast, adult miR-1-2 knockout mice presented with thickened chamber walls attributable to hyperplasia that had continued into adult life, whereas many of the embryos from these mice often had septal defects, further demonstrating the fundamental role of miR-1 for heart development.84 The effect of miR-1 in the heart is consistent with its function in skeletal muscle in that overexpression of miR-1 in myoblasts decreases proliferation and promotes skeletal muscle differentiation.72 Moreover, the expression of predicted miR-1 mRNA targets was found reduced after differentiation into myotubes, when this miR is functional, indicating that miRs may destabilize preexisting mRNAs, permitting a more vigorous transition toward myogenic differentiation.85 Analysis of the expression of target mRNAs in flies also revealed that mRNAs with miR-1 target sites are expressed largely in nonmuscle tissues.86 Thus, it seems that miR-1, and similar miRs, might confer robustness to tissue-specific gene expression by blocking potentially large sets of mRNAs that are expressed inappropriately in tissues in which their presence would be detrimental, and thus act in a “fail-safe” mechanism.84

The MiR-133 Family

The miR-133 family (comprised of miR-133a-1, miR-133a-2, and miR-133b) is expressed from bicistronic units together with miR-1. An ancient genomic duplication is thought to have resulted in 2 distinct loci for the miR-1/miR-133 cluster in vertebrates, with identical mature sequences derived from the duplicated loci.72,87 The resulting mature products are either identical or have only 1 base of difference. MiR-133 is expressed in heart and skeletal muscle, and microarray analysis has revealed an increased expression in developing mouse hearts from embryonic day (E)12.5 through to at least E18.5.62 Similar to miR-1, muscle-specific expression of miR-133 is regulated by SRF. Moreover, a negative regulatory loop is responsible for the repression of SRF by miR-133 itself. Functionally, miR-133a inhibits differentiation and promotes proliferation of myoblasts and, therefore, has opposite effects to miR-1. Nevertheless, both miR-133 and miR-1 increase in expression with development, a fact coherent with them deriving from a common mRNA polycistron, demonstrating the complex mechanisms involved in the functioning of miRs (Figure 2).
they found miR-18b to be the most upregulated miR. Differences also exist between the dysregulated miRs after TAC in adult mice hearts and those found in neonatal rat cardiomyocytes treated in vitro, but a number of them (miR-18b, miR-20b, miR-21, miR-106a, and miR-125b) were common to both models.

Thus, these articles, together with a fourth by Sayed et al., have demonstrated that a complex array of miRs is dysregulated in disease-related hypertrophy of the postnatal heart. The pattern of miRs found during end-stage heart failure has been documented to be extraordinarily similar, and probably not unexpectedly so, to that of 12- to 14 week-old fetuses.92

In fact, Thum et al found 67 miRs upregulated and 43 miRs downregulated >1.5-fold in end-stage heart failure, and ≈87% and ≈84% of the analyzed miRs were regulated in the same direction in fetal heart. Upregulated genes were also found to have a significant number of putative binding sites for downregulated miRs. It is known that with hypertrophy, a number of fetal genes are reactivated, probably in attempt to circumvent negative effects associated with chronic stress. The miRs repressed during heart failure appear, therefore, to be involved in the reduced suppression of the upregulated mRNAs and, so, contribute to the creation of a fetal-type transcriptome.

Besides ascertaining whether a particular miR or sets of them are up- or downregulated in a given condition, the fundamental aspect in understanding the role of individual miRs is correlation with specific targets. To date, only a few miRs have been studied in this regard (Table 2).

**MiR-1/MiR-133**

Very recently, we reported an inverse correlation between miR-1 and miR-133 with cardiac muscle hypertrophy; decreased miR-1/miR-133 expression was found in the ventricular tissue of mice subjected to TAC and in cultured cardiomyocytes treated with phenylephrine (PHE), a hypertrophic stimulus. Interestingly, miR-1/miR-133 expression was also decreased in atria and ventricles from Akt-overexpressing transgenic mice and exercise-trained wild-type mice, 2 physiological hypertrophic models. Thus, irrespective of whether the underlying cause is pathological or physiological in nature, miR-1 and miR-133 seem to be implicated in the increase of cardiomyocyte size. Importantly, a significant reduction in miR-1 and miR-133 expression was found in myectomies originating from hearts of cardiomyopathic patients, revealing the pertinence of the finding for human pathology.

To determine the functional significance of reduced miR-133 expression during hypertrophy, gain-of-function and loss-of-function studies were performed. Transduction of cardiomyocytes with a miR-133a-2–expressing viral vector blunted the hypertrophic response of cardiomyocytes to PHE treatment in vitro. Moreover, in vivo administration of antagonomir-133, an antisense RNA oligonucleotide capable of silencing miR-133 function, was responsible for the induction of spontaneous hypertrophy. MiR-133 targets were then studied. Of the possible targets predicted, expression of Cdc42 and Rho-A, both GTP-GDP binding molecules, and Wolf–Hirschhorn syndrome complex 2 (WHSC2/NELF-A), a nuclear factor involved in heart genesis, were found to correlate with hypertrophy. Rho-A and Cdc42 are involved in cell growth, myofibrillar rearrangements, and regulation of contractility.93,94 WHSC2/NELF-A is a repressor of transcription, probably operating at the RNA elongation step.95 Notably, in Wolf–Hirschhorn syndrome, a congenital condition characterized by mental retardation, cardiovascular abnormalities are typical features among others. Interestingly, transduction of cardiomyocytes in vitro and

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**Table 1. MiRs Reported Dysregulated in Array Analyses to Date**

<table>
<thead>
<tr>
<th>Downregulated MiRs</th>
<th>Upregulated MiRs</th>
<th>No Change</th>
<th>Species/Model or Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>1, 7d, 10a/b, 26a/b, 29a/c, 30a-3p/a-5p/b/c/d/e/f, 139, 149, 150, 151, 155, 185, 194, 218, 378</td>
<td>15b, 21, 23a/b, 24, 27a/b, 31, 103, 107, 125b, 127, 140b, 195, 199a/a'/b, 214, 221, 222, 351, let-7b/c</td>
<td>133a/b</td>
<td>mouse/TAC91</td>
</tr>
<tr>
<td>29c, 30e, 93, 133a/b, 150, 181b</td>
<td>10b, 19a, 21, 23a/b, 24, 25, 27a/b, 125b, 126, 154, 195, 199a/a', 210, 214, 217, 218, 330, 351</td>
<td>mouse/TAC and CnA Tg98</td>
<td></td>
</tr>
<tr>
<td>29a/b/c, 30e, 125-5p, 133a/b, 149, 150, 185, 451, 486</td>
<td>21, 27a/b, 146, 214, 341, 424</td>
<td>mouse/TAC98</td>
<td></td>
</tr>
<tr>
<td>30b/c, 150</td>
<td>17-5p, 18b, 19b, 20b, 21, 23a, 25, 29a, 106a, 125b, 140, 142-3p, 153, 184, 200a, 208, 210, 211, 221, 222</td>
<td>mouse/TAC98</td>
<td></td>
</tr>
<tr>
<td>187, 292-5p, 373, 466</td>
<td>18b, 20b, 21, 23a, 106a, 125b, 133a</td>
<td>25, 29a</td>
<td>rCM/PHE90</td>
</tr>
<tr>
<td>16, 17-5p, 19b, 22, 23b, 24, 27a, 30a-5p/b/c/e-5p, 107, 126, 130b, 135a, 136, 148a, 150, 182, 186, 192, 199a*, 218, 299-5p, 302*, 302c*, 325, 339, 342, 452a, 494, 495, 497, 499, 507, 512-5p, 515-5p, 520d/h, 520, 523, 526b/b*</td>
<td>1, 7a/b/c/d/e/f, 10b, 106b, 17-3p, 21, 26a, 28, 29a/b/c, 32, 34b, 98, 125a, 126a, 129-3p, 130a, 132, 196a, 199b, 200c, 204, 205, 208, 210, 211, 212, 213, 215, 292-3p, 294, 295, 296, 297, 300, 302a, 320, 322, 330, 331, 333, 340, 341, 343, 365, 367, 372, 373, 377, 381, 382, 423, 424, 429, 432, 500, 520c, 529*</td>
<td>Human/HF92</td>
<td></td>
</tr>
</tbody>
</table>

Bold indicates miRs reported validated by Northern blots; italics, miR reported validated by PCR; CnA Tg, calcineurin A transgenic mice; rCM, neonatal rat cardiomyocytes; PHE, phenylephrine; HF, end-stage heart failure. †Expression found variable.
in vivo with an adenosinergic vector containing a Wshc2 transgene resulted in decreased protein synthesis but induced the fetal gene program.62

Downregulation of miR-133a/b was reported also in the study by van Rooij et al,88 whereas no mention of miR-1 nor miR-133a/b, but not miR-1, are listed in the group of >30% dysregulated miRs by Cheng et al.89 In a previous report, Sayed et al91 performed a time-course analysis of miR expression after TAC using a gene chip approach and found more than 50 miRs with expression that progressively changed during development of pressure overload–induced cardiac hypertrophy. These authors identified downregulation of miR-1 as 1 of the most prevalent features but reported that miR-133a/b expression remained unchanged. Northern blot analysis revealed that reduced expression of the mature transcript of miR-1 is an early event, detectable after only 1 day of TAC: expression reached a minimum after 7 days and then returned to near normal levels by day 14. Importantly, in silico–predicted targets of miR-1 were found to be pro-apoptotic with validated targets belonging to the heat shock proteins family (HSP60 and HSP70). Conversely, an antiapoptotic function was attributed to mir-133, which has caspase9 as a validated target.75

**MiR-208**

The miR-208 sequence is encoded in intron 27 of the human and mouse α-myosin heavy chain (MHC) genes. This miR seems to control regulation of β-MHC in conditions of stress but not during normal development.90 In samples of idiopathic cardiomyopathy, a correlation was found with the expression of pre-miR-208 and not of the mature transcript because of the extended half-life of miR-208. The knockout of miR-208 in mice produced viable animals with no obvious cardiac phenotype apart from the upregulation of fast skeletal muscle contractile proteins and stress proteins. These genes, however, were not miR-208 targets. Interestingly, though, miR-208 knockout produced a phenotype with blunted hypertrophic and fibrotic responses to TAC. Moreover, upregulation of stress markers (such as atrial natriuretic peptide and brain natriuretic peptide) were increased in hearts of these mice, as predicted, but the increase in β-MHC was absent. In contrast, α-MHC was increased, rather than reduced. The protein product of a predicted miR-208 target, thyroid hormone receptor (TR)-associated protein 1 (THRAP1), the TR coregulator, was found to be increased in miR-208 knockout mice. This fact was used to develop a model whereby stress stimuli, responsible for the reduction of α-MHC transcription, consequentially also reduce the level of the miR-208 transcript, which, in turn, relieves transcriptional repression on its target mRNA, **thrap1**. The resulting increase in THRAP1 protein influences the TR-regulated expression of α- and β-MHCs, which are inversely affected through a

<table>
<thead>
<tr>
<th>MiR</th>
<th>MiR Dysregulation</th>
<th>Target(s)</th>
<th>Function</th>
<th>Species/Model or Disease</th>
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<td>miR-191</td>
<td>↓</td>
<td>Rheb</td>
<td>Facilitate translation</td>
<td>m/TAC, h/idiopathic CMP</td>
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<td></td>
<td></td>
<td>RasGAP</td>
<td>Mediate signaling for various growth factor receptors</td>
<td></td>
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<td></td>
<td></td>
<td>Cdk9</td>
<td>Facilitate transcription elongation (cardiac growth)</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Fibronectin</td>
<td>Component of the extracellular matrix</td>
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<td>miR-175</td>
<td>↑</td>
<td>HSPO60</td>
<td>Sequestration of Bax</td>
<td>H9c2/oxidative stress</td>
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<td></td>
<td></td>
<td>HSP70</td>
<td>Inhibitor of JNK and Apaf-1</td>
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<tr>
<td>miR-133</td>
<td>↑</td>
<td>Kir2.1</td>
<td>Repolarization and diastolic activity</td>
<td>m/LAD, h/ CAD</td>
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<td></td>
<td>Connexin 43</td>
<td>Intercellular conductance</td>
<td></td>
</tr>
<tr>
<td>miR-133</td>
<td>↓</td>
<td>Rho-A</td>
<td>Cardiac growth</td>
<td>m/TAC, m/training</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NELF</td>
<td>Transcription repressor</td>
<td>m/Akt-Tg, h/idio- pathic CMP</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cdc42</td>
<td>Cardiac growth</td>
<td></td>
</tr>
<tr>
<td>miR-133</td>
<td>↑</td>
<td>HERG</td>
<td>Plateau termination</td>
<td>rb/ADM, h/DM</td>
</tr>
<tr>
<td>miR-208</td>
<td>↓</td>
<td>THRAP-1</td>
<td>Activator of downstream caspases</td>
<td>H9c2/mir overexpression</td>
</tr>
<tr>
<td>miR-21</td>
<td>↑</td>
<td>PTEN</td>
<td>Proliferation</td>
<td>rt/ballon injury of</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bcl-2</td>
<td>Apoptosis</td>
<td>carotid artery</td>
</tr>
<tr>
<td>miR-221/222</td>
<td>↑</td>
<td>c-kit</td>
<td>Angiogenesis</td>
<td>h/HUVEC</td>
</tr>
</tbody>
</table>

↓ Indicates downregulated; ↑, upregulated; m, mouse; h, human; rb, rabbit; rt, rat; H9c2, rat ventricle embryonic cells; CMP, cardiomyopathy; LAD, left anterior descending coronary artery ligation; CAD, coronary artery disease; DM, diabetes mellitus; ADM, alloxan-induced diabetes mellitus; HUVEC, human umbilical cord vein endothelial cell.
positive and negative TRE, respectively. Interestingly, other TR-regulate genes, such as phospholamban (PLN), sarcoplasmic reticulum calcium ATPase (SERCA), and the glucose transporter Glut4, were not affected, and, thus, different TR isoforms or factors may confer specificity. Thus, miR-208 is an important miR regulating gene expression of β-MHC in response to stress.

**MiR-21**

A number of studies have reported miR-21 upregulation during cardiac hypertrophy. Cheng et al\(^89\) found miR-21 as the most upregulated cardiac-specific miR 7 days after TAC. The expression of this miR decreased from then on and was found back at normal levels after 21 days, the point at which hearts were overtly failing. Tatsuguchi et al\(^90\) also described upregulation of this miR at 14 days after TAC, with subsequent reduction after 28 days, whereas Sayed et al\(^91\) and van Rooij et al\(^88\) reported maintained upregulation to 14 and 21 days of TAC, respectively. Uregulation of miR-21 was also reported in calcineurin-overexpressing transgenic mice.\(^88\) Evidence for the involvement of miR-21 in hypertrophy was obtained also with in vitro studies where treatment with hypertrophy inducers, such as angiotensin II and PHE, was found to increase miR-21 expression. Knockdown with a miR-21 antisense oligonucleotide was shown to be sufficient to blunt this hypertrophic growth.\(^89\) Expression of this miR has been reported to be both normal\(^88\) and increased\(^92\) in tissue obtained from end-stage heart failure patients. MiR-21 is upregulated in some human cancers, where it may play an antiapoptotic role. In this regard, Fas ligand and transforming growth factor-β receptor (TGF-βR) are considered potential targets of miR-21, but both await validation. In heart failure, the compensatory mechanisms operant during the initial stages, such as the activation of antiapoptotic pathways, eventually fail, causing increased cell death and fibrosis.

**Role in Conduction Pathophysiology**

Membrane excitability is a fundamental characteristic of the cardiomyocyte. Not surprisingly, this aspect of cardiac biology also has been reported to be regulated by miRs. In fact, an important role for miRs in the physiological distribution of K⁺ channels has been described.\(^97\) KCNQ1 and KCNE1 are 2 subunits that assemble in the heart to form the slow delayed rectifier K⁺ current (I\(_\text{Kr} \)) It is well established that an important spatial patterning exists for this channel at the protein level in the normal heart, such as apex to base, and epi-/endocardium to midmyocardium gradients. Interestingly, these authors found that miR-1 and miR-133 expression was also spatially heterogeneous and, intriguingly, specular in many aspects to that of I\(_\text{Kr} \). Thus, in many of the areas where I\(_\text{Kr} \) is more densely expressed, miR-1 and miR-133 are less abundant. Computational predictions, however, did not evidence that KCNQ1 or KCNE1 were targets of either miR-1 or miR-133, but a careful analysis of the 3’ UTRs by these authors revealed putative binding sites for miR-1 on KCNE1 and for miR-133 on KCNQ1. Their data support the important notion that spatial differences in the expression of miRs can exist within an adult organ and this is responsible for modulating, at least in part, the expression pattern of target proteins.

These authors have also implicated dysregulation of miRs in the altered cardiac electrical mechanisms of pathological states. They have reported overexpression of miR-133 and of its transactivator, SRF, in the hearts of rabbits rendered diabetic with alloxan and in ventricular samples from diabetic patients.\(^98\) In this form of cardiomyopathy, repolarization slowing and QT prolongation occur. The target of miR-133 was found to be ERG (ether-a-go-go–related gene), which codes for the rapid delayed rectifier K⁺ current (I\(_\text{Ks} \)), and, coherently, this has been reported downregulated in diabetic hearts.

Furthermore, Wang and colleagues have also described upregulation of miR-1 in individuals with coronary artery disease.\(^99\) Importantly, overexpression of miR-1 in normal rat hearts was found to widen the QRS complex and prolong the QT interval, indicating slowing of cardiac conduction, and cause membrane depolarization through a defective inward rectifier K⁺ current, I\(_\text{Ks} \). Ablation of miR-1 with an antisense inhibitor was sufficient to relieve arrhythmogenesis of infarcted rat hearts. Two targets of miR-1 were found downregulated in mice after myocardial infarction and in samples from coronary artery disease patients: KCNJ2 (which encodes the K⁺ channel subunit, Kir2.1, responsible for I\(_\text{Ks} \)) and GJA1 (which encodes for connexin 43, involved in intercellular conductance).

Abnormal propagation of cardiac electrical activity was also a feature reported by Srivastava and colleagues in the miR-1–2 knockout mice that survived to adulthood.\(^100\) Many of these mice presented with apparently normal anatomy and function but had a slowed heart rate, a shortened PR interval, and a broadened QRS complex, indicative of bundle-branch block associated with sudden death. They identified 1 possible target of miR-1 in the adult mice as Irx5 (Iroquois family of homeodomain-containing transcription factor), which regulates cardiac repolarization by repressing transcription of a key potassium channel, Kcnq2.

**Angiogenesis**

Tissue specificity is a peculiar feature of miRs and has relevance also during angiogenesis, where highly coordinated multistep processes are required. Dicer knock-out mice showed, in fact, a lethal phenotype early during embryonic development, with a characteristic thin and severely disorganized blood vessel network.\(^100\) In addition, these mice presented with a significant upregulation in the expression of vascular endothelial growth factor (VEGF) and its receptor, KDR, and downregulation of the angiopeitin receptor, Tie-1. Kuehbach et al demonstrated that siRNA silencing of Dicer and Drosha significantly reduced the capillary sprouting of endothelial cells and tube-forming activity both in vitro and in vivo.\(^101\) Interestingly, silencing of Dicer negatively affected endothelial migration as well as in vivo angiogenesis, whereas Drosha siRNA had no effect. A subsequent screening analysis performed by the same authors revealed that members of the let-7 family, miR-21, miR-126, miR-221, and miR-222 are highly expressed in endothelial cells, and silencing of Dicer and Drosha led to a reduction of let-7
and mir-27b levels. In a third study, in vitro knockdown of Dicer in endothelial cells was reported to affect important regulators of angiogenesis, such as TEK/Tie-2, KDR/VEGFR2, endothelial NO synthase, and interleukin-8. An expression profile identified 25 highly expressed miRs, and in vitro experiments determined the role of miR-221/222 in the control of endothelial NO synthase protein levels. Another report from Rainaldi and colleagues focused on the direct effect of miRs on endothelial cells. Here, a large-scale analysis of miR expression in human umbilical vein endothelial cells revealed that the 15 highly expressed miRs have angiogenic factor receptors as putative targets. Specifically, miR-221 and miR-222 were found to affect angiogenic properties by targeting c-kit expression. The involvement of these miRs in the control of erythropoiesis has also been documented. In addition, selective depletion of miR-221 and miR-222 in vitro was shown to critically affect the properties by targeting c-kit expression. The involvement of these miRs in the control of erythropoiesis has also been documented. In addition, selective depletion of miR-221 and miR-222 in vitro was shown to critically affect the specific endothelial cell miR-expression pattern: 9 miRs were upregulated, whereas 23 were found downregulated, thus predicting the presence of a complex miR network involved in transcription factor control. Of note, the same authors showed that the muscle-specific miR, miR-133a, was also overexpressed, thus anticipating a potential role of this miR even in the control of the vascular system.

In addition, Ji et al documented the selective expression of 140 miRs in the vasculature, 49 of which were highly expressed. Of interest, the study focused on the effect on neointimal formation, which is a common pathological lesion in diverse cardiovascular diseases such as atherosclerosis, coronary heart diseases, postangioplasty restenosis, and transplantation arteriopathy. Balloon injury of rat carotid arteries revealed misexpression of 113 of the 140 artery miRs, with 60 miRs upregulated, and 53 miRs downregulated 7 days after injury, and 55 upregulated and 47 downregulated after 20 days. This study revealed an important role for miR-21 in proliferation of the intima of vessels: inhibition of miR-21 with an antisense oligonucleotide was sufficient to inhibit neointimal formation in vivo and had antiproliferative and proapoptotic effects in vascular smooth muscle cells grown in vitro. These effects were attributable to 2 targets involved in proliferation and apoptosis, ie, PTEN and Bcl-2, respectively.

Concluding Remarks
Correct temporal patterning of miR expression is fundamental for normal development and function of the cardiovascular system, similarly to the rest of the organism. This is underlined by the continually growing number of studies that are reporting dysregulation of miRs as a cause of congenital defects and disease of the heart. What is more, mutations that modify the susceptibility of mRNAs to miRs can produce abnormal muscle phenotypes, such as documented for Texel sheep in which creation of a target site for miR-1 and miR-206 in the 3' UTR of myostatin gene contributes to the occurrence of muscular hypertrophy. It will not come as a surprise if this type of alteration is found to be at the basis of many other pathologies. However, differences in some of the data obtained by the various groups studying the role of miRs in cardiac disease still need to be clarified to gain a clearer understanding of the mechanisms involved. For example, discrepancies in data, such as for miR-1, miR-133, miR-208, and miR-21 demonstrate that temporal patterning of miR expression should not be overlooked and should be more precisely reported and possibly linked to age and the functional state at the time of assay. Moreover, the importance of spatial expression within the adult organ has also been highlighted and needs further elucidation. Importantly, many studies have shown that pathological characteristics, such as hypertrophy, can be blunted by the use of gain-of-function or loss-of-function mutants, depending on whether the miR under question is downregulated or upregulated during disease. Unfortunately, adverse functional effects, such as the generation of arrhythmias in the case of miR-1 and miR-133, secondary to grossly modulating miR expression, might be produced. Nevertheless, miR-based therapy holds great promise for future strategies aimed at treating and preventing cardiovascular diseases. On this point, miR mimics can be individually designed on the 3' UTR of specific genes: this approach has been successful in regulating expression in cardiomyocytes of the cardiac pacemaker genes HCN2 and HCN4, without disturbing other genes that would be otherwise affected with the use of an endogenous miR, such as miR-1 or miR-133. In addition to their use as drugs, miRs can also be targeted for therapy. In fact, antisense oligonucleotide therapy has already begun to timely filter through to the clinical setting, ie, Vitrvane for cyclosporins retinitis, and a number of other antisense oligonucleotides are currently under clinical trials. However, the mechanisms behind oligonucleotide-mediated miR silencing has not yet been fully elucidated, and more work still needs to be conducted to understand the role of miRs in cardiovascular physiopathology before miR-based therapy becomes a reality.

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Disclosures
None.

References


