Heart Failure: A Growing Problem

With 550,000 new cases diagnosed annually and $37 billion spent per year, heart failure (HF) with reduced ejection fraction is one of the largest contributors to disease burden and healthcare expenditure in the United States. Despite significant progress in the treatment of HF, with medications, the prognosis of HF remains dismal, with a mortality rate of 42% at 5 years after diagnosis. Therefore, understanding the underlying molecular pathways in the transition from established cardiovascular disease to HF may spur the development of novel biomarkers and therapeutic targets.

The heart responds to stressors such as hypoxia (in myocardial infarction [MI]), increased wall stress (in valvular heart disease), and neurohormonal/metabolic stress (in diabetes mellitus and hypertension) by cardiomyocyte hypertrophy and fibrosis. Although initially compensatory for increased wall stress or myocyte loss, the molecular pathways that underlie pathological hypertrophy are ultimately maladaptive, recapitulating further hypertrophy, contractile dysfunction, apoptosis, and fibrosis. The progression to HF is associated with a characteristic cascade of altered intracellular signaling and gene expression, representing a final common pathway to ultimate decompensation. The various signaling pathways that underlie pathological hypertrophy and the progression to HF have been the subject of intense investigation and are summarized in multiple review publications. More recently, considerable attention has been paid to microRNAs (miRNAs), a novel biological control mechanism with the ability to regulate entire molecular networks by complex feedback and feed-forward mechanisms. Several reviews have summarized recent findings implicating miRNAs in cardiac development and disease. In the past few years, the discovery of circulating miRNAs has led to their investigation as biomarkers and mediators of cell–cell communication. This review focuses on recent developments detailing the role of miRNAs in the pathogenesis of HF, their potential role as biomarkers, and their use as possible novel therapeutic targets.

MicroRNAs: Novel and Potent Regulators of Gene Expression

The first miRNA gene was identified in the worm Caenorhabditis elegans. In less than 2 decades, this initial finding has blossomed into an expanding field. miRNAs are now known to be fundamental regulators of post-transcriptional gene expression. Much light has now been shed on both the highly regulated synthesis of miRNAs and their mechanism of post-transcriptional gene silencing, the details of which are beyond the scope of this review (Figure 1).

miRNAs are initially transcribed as primary miRNAs (pri-miRNAs) and processed to precursor miRNAs (pre-miRNAs) by the RNase enzyme Drosha, which acts as part of a complex of regulatory and other proteins in the so-called microprocessor complex. The complex cleaves the initial pri-miRNA transcript at the stem of a hairpin loop to release a 60- to 70-bp molecule, the pre-miRNA. The pre-miRNAs are then transported to the cytoplasm in a highly regulated manner by proteins related to the exportin-5 family of Ran-GTPase–dependent proteins.

In the cytoplasm they undergo a further cleavage step by a complex containing Dicer to become mature double-stranded miRNAs, in most cases with 1 functional miRNA and a second complementary nonfunctional miRNA (in some cases, both miRNAs are functional and are designated by 5p and 3p based on their positions within the pre-miR). Subsequently, the mature miRNA is incorporated into a complex called the RNA silencing complex that contains the Argonaute protein. This complex, with the miRNA presented to the target mRNA, is responsible for repression of the target mRNA via either degradation of the mRNA or inhibition of translation. Watson–Crick complementarity between the seed region of the miRNA (nucleotides 2–8) and the complementary sequence in the 3′-untranslated region of the target mRNA seems to be a critical determinant of miRNA–mRNA interaction although other regions of the miRNA have now been shown to further influence this interaction (recently reviewed by Dorn). In mammalian cells, mRNA translational repression rather than RNA-silencing seems to be the primary mode of miRNA action, and techniques to identify bona fide targets of miRNA are rapidly evolving. Large numbers of miRNAs have now been identified among all eukaryotic cells. Mirbase now lists >2000 known miRNAs in humans. Bioinformatics approaches to determining target mRNAs suggest that almost one third of all transcripts are miRNA targets, with single miRNA or members of a miRNA...
family regulating multiple components of pathways. A single miRNA may have tens to hundreds of targets in a given cell, and an individual mRNA may be targeted by multiple different miRNAs. Research to date has uncovered miRNAs in virtually all eukaryotic cells, and miRNAs have been implicated in biological processes ranging from embryogenesis to apoptosis, neoplasia to wound healing.

The roles of individual miRNAs in cardiac development, HF, and hypertrophy have been studied extensively. Animal models have implicated multiple miRNAs in critical processes such as cardiac hypertrophy, fibrosis, and apoptosis. The intriguing discovery of miRNAs circulating in the plasma has spurred multiple recent studies assessing their potential use as prognostic biomarkers. In this review, we summarize our understanding of the role of these novel and important molecules in both the pathogenesis of HF and their emerging clinical role as diagnostic and predictive biomarkers.

Involvement of MicroRNAs in Physiological and Pathological Adaptation: Hypertrophy, Fibrosis, and Failure

At the heart of maladaptive remodeling in response to mechanical and ischemic stress, the development and progression of pathological cellular and organ-level hypertrophy herald an inexorable decline in cardiac function before clinical HF. Reversing the process or slowing the transition to the decompensated state would be a major goal in treating the underlying pathological processes. A growing body of work has begun to shed light on the molecular mechanisms by which miRNAs affect pathological hypertrophy at the transition to HF. In this section, we discuss the evidence for the role of miRNAs as modulators of genetic networks responsible for pathological processes in HF progression, including hypertrophy, fibrosis, and apoptosis (see Figure 2 for important miRNAs involved in pathological and physiological cardiac remodeling).

Two knockout mice illustrate the importance of miRNA synthesis and regulation to cardiac function. The first, developed by Rao et al., was a knockout for a gene called dger8 that functions in conjunction with Dicer to generate pre-miRNAs from pri-miRNAs. Mice with a conditional, cardiac-selective knockout developed ventricular dysfunction by ≈3 weeks of life, followed by progressive ventricular dilatation and fibrosis. Mice with a dicer knockout developed a similar phenotype; although initially more ventricular hypertrophy was noted in these mice, the end result was a dilated, hypofunctional ventricle. Conditional, cardiac-specific deletion of Dicer in neural crest cells at various times in development and adulthood led to a shared phenotype of lethal cardiomyopathy.

A second line of investigation by van Rooij et al. took the reverse process. miRNAs were measured in mice with pathological hypertrophy in response to either pressure-overload induced by transverse aortic constriction (TAC) or by constitutive calcineurin activation. Using a strategy of identifying miRNAs that were regulated in parallel in the TAC and calcineurin mice, the authors identified 11 miRNAs. At least 5 of these were upregulated in a similar manner in both mice and human cardiomyopathy tissue samples. Overexpression of these upregulated miRNAs (mir-23a, mir-23b, miR-24, miR-195, and miR-214) in primary cultured cardiomyocytes led to a dramatic hypertrophic response, whereas conversely, miRNAs that were downregulated in response to TAC or calcineurin (miR-150, mir-181b) led to a decrease in myocyte size suggesting a direct effect of these miRNAs on cardiac hypertrophy. The final proof of the causal role of miRNAs in cardiac hypertrophy was obtained using cardiac-specific overexpression of one of these miRNAs, miR-195 in transgenic mice, which led to cardiac hypertrophy because of marked cardiomyocyte enlargement. Interestingly, this hypertrophic response was followed by ventricular thinning, dilatation of the ventricular cavity, and deterioration of cardiac function. These studies demonstrated a functional role in the pathogenesis of HF in murine models for miRNAs implicated in human HF. An equally important conclusion from these early studies was that for some miRNAs, murine models of HF have relevance for developing mechanistic insight into human HF.

Additional work by several groups has expanded our understanding of the role of individual miRNAs in cardiomyocyte hypertrophy (see Figure 3 for a brief list of genes and downstream targets) and in modulating response of the heart to physiological and pathological stresses. The number of miRNAs that are expressed in the heart seems to play a functional role in cardiac health and disease is growing rapidly (see Figure 2 for genes up- and downregulated in response to pathological and physiological stress by specific miRNAs). Although a complete description of all such molecules is beyond the scope of this review, we will instead focus on...
key miRNAs that play an important role in disease processes known to be important in the pathogenesis of HF.

**miR Regulation of Cardiac Hypertrophy**

**Mir-1: Regulation of Multiple Hypertrophy-Associated Genes**

Mir-1 was first discovered in 2002 in a screen for muscle-specific miRNAs in mouse. In the mouse heart, 1 miRNA, mir-1, accounted for ≈40% of all miRNA transcripts in the cell. Zhao et al. found that targeted deletion of mir-1 in mice led to the in utero death of 50% of the mice, and many of the remaining mice died of heart defects within a few months of birth. Sayed et al. first demonstrated a role for miR-1 in cardiac hypertrophy, by subjecting mice to TAC (leading to pressure overload–induced hypertrophy). Comparison of miRNA expression profiles in sham and TAC-treated mice using a microarray approach that allowed for simultaneous screening of all 334 contemporary mouse miRNAs demonstrated a multitude of miRNAs that were either up or downregulated after TAC. miR-1 emerged as the predominantly expressed miRNA in cardiomyocytes, consistent with the data of Rao et al. In addition, it was downregulated early (1 day after TAC), and in a sustained manner for the 14-day duration of the study. The authors went on to show that miR-1 inhibited hypertrophy in neonatal mouse cardiomyocytes in response to serum-enriched media. Further work by several groups has identified several genes known to be involved in cardiac hypertrophy as downstream targets of miR-1.

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**Figure 2.** In response to pathological (pressure overload, ischemia–reperfusion), or physiological (exercise) stresses different microRNA (miRNA) expression patterns are observed. CsA indicates cyclosporin A; PE, phenylephrine; and TAC, transverse aortic constriction.

**Figure 3.** Multiple microRNAs (miRNAs) have been observed to modulate the hypertrophic process. Several of these are shown in the figure along with known mRNA targets. CTGF indicates connective tissue growth factor; ERK, extracellular signal-regulated kinase; IGF1, insulin-like growth factor 1; and IGFR, IGF receptor 1. Reprinted with permission from Nishimura et al. Authorization for this adaptation has been obtained both from the owner of the copyright in the original work and from the owner of copyright in the translation or adaptation.
Perhaps most compelling, Karakikes et al. recently demonstrated that the adenoviral delivery of miR-1 to TAC-treated mice was able to reverse the hypertrophy and ventricular dysfunction–associated TAC, with improvement in fractional shortening, reversal of ventricular dilatation, and decreased fibrosis. On a gene expression level, a regression of the pathological hypertrophic profile was seen. In humans, Cañé et al. examined myocardial biopsies that were subjected to hypertrophic stimuli (atria in patients with mitral stenosis and myomectomy specimens from patients with hypertrophic cardiomyopathy undergoing surgical resection) and observed a decrease in miR-1 levels, suggesting a similar role in human hypertrophy. These studies suggested miR-1 as a plausible therapeutic target in HF, leading to further investigation of the pathways and mRNAs targeted by miR-1. Multiple studies have identified downstream targets of miR-1. In various studies, MEF2a, calmodulin, GATA4, insulin-like growth factor-1, and twinfilin have been shown to be downregulated by miR-1. All these molecules are known to be essential for the development of cardiac hypertrophy, providing a putative mechanism of miR-1 action.

miR-133: the Most Abundant Human miRNA

miR-133 is the most abundant miRNA in human myocardium and was initially identified by microarray analysis as a muscle-specific miRNA. The 3 related miRNAs, miR-133a-1, miR-133a-2, and miR-133b, are cotranscribed with miR-1 to 2 and miR-1-1. miR-133 and miR-1 were initially reported to be downregulated in skeletal muscle after functional overload, suggesting a role in skeletal muscle hypertrophy and growth. Subsequently, several groups have confirmed the role of miR-133 in cardiac hypertrophy. Interestingly, a downregulation of miR-133 was noted in murine models of pathological TAC-driven by the β-heart driven by the β-myoosin heavy chain (MHC) promoter. The investigators also showed downregulation of miR-133 in hypertrophied human ventricles. The molecules NFATc4 and calcineurin, both central regulators of cardiac hypertrophy, and Rac and Cdc42, 2 regulators of the prohypertrophic mitogen-activated protein (MAP) kinase pathways, have been identified as miR-133 targets. Work by other groups suggests a more complex role for miR-133 in the regulation of cardiac hypertrophy. Although knockout of both 133a isoforms in the embryo is lethal because of heart defects, miR-133 overexpression in the developing heart driven by the β-myoosin heavy chain (MHC) promoter did not seem to have any appreciable effects on LV function or dimensions. However, after TAC in the miR-133–overexpressing mice, the authors noted a significant decrease in myocardial fibrosis and improved ventricular compliance without appreciable cardiomyocyte hypertrophy. Interestingly, a different group demonstrated downregulation of miR-133 in both a rat model of hyper-reninemic hypertension (Ren2 rats) and humans with aortic stenosis, in concert with increased levels of connective tissue growth factor. The investigators demonstrated that miR-133 could directly downregulate connective tissue growth factor, suggesting a role for miR-133 both intramyocardially in regulating hypertrophy and potentially having paracrine downstream effects on the cardiac interstitium (perhaps by acting on fibroblasts). In sum, these studies suggest that downregulation of miR-133 may be deleterious in both cardiac hypertrophy and development and hypertrophy, but that forced overexpression of this miR may be cardioprotective in certain models of pathological hypertrophy. These studies demonstrate the complex, context-dependent nature of miRNA regulation of cardiac phenotype: the role of the miRNA being investigated is often dependent on the exact model of hypertrophy and the nature of miRNA manipulation (eg, transgenic overexpression versus virally mediated gene delivery). Careful attention to these details is, therefore, necessary in elucidating a role for these miRNAs in cardiac disease.

miR-208a: Regulation of MHC Expression

miR-208a is a cardiac-specific miRNA embedded within an intron of the α-MHC gene and hence is subject to the same transcriptional regulation as the α-MHC gene. A related miRNA, miR-208b, is encoded within the β-MHC genes. During embryogenesis, there is a switch from fetal β-MHC to α-MHC with a concurrent increase in the expression of miR-208a and reduction in miR-208b.

Interestingly, the overexpression of miR-208a leads to upregulation of β-MHC in mice and is sufficient to induce cardiac hypertrophy. The authors found that although the overexpressing mice had homogeneously increased expression of miR-208a in the heart, the areas of hypertrophied myocytes were restricted to certain focal areas, and these areas were surrounded by increased interstitial fibrosis. These results suggest that although miR-208a clearly mediates some aspects of hypertrophy, other cellular factors or conditions may be necessary for it to exert its downstream effects. miR-208a likely targets repressor proteins that may eventually contribute to β-MHC regulation with the thyroid hormone nuclear receptor (ThraP1) and myostatin being targets, but the exact mechanisms remain far from clear. Further, in cultured cardiomyocytes, transfection with miR-208a resulted in clear enlargement of the cells, and although knockdown of 208a with an antisense oligonucleotide resulted in diminished β-MHC levels, cell size was not affected at baseline, suggesting that miR-208a regulation of MHC is likely only 1 of many factors regulating cell size and hypertrophy.

Nonetheless, these experiments led to the exploration of miR-208a as a possible target in pathological hypertrophy and HF. Indeed, genetic ablation of miR-208a in mice led to an absence of hypertrophic response to both TAC and calcineurin overexpression. In contrast to wild-type mice who undergo LV hypertrophy, with fibrosis and cardiomyocyte (CM) apoptosis after TAC, the miR-208a–/– mice displayed almost no increase in cardiac mass or LV size; as expected, there was no increased expression of β-MHC in response to TAC, but several other genes associated with hypertrophy (ANP [atrial natriuretic peptide], BNP [B-type natriuretic peptide]) were
found to be increased, again suggesting that miR-208a may be a 1 of several key regulators of hypertrophy.42 Montgomery et al44 evaluated the role for of miR-208a in salt-sensitive hypertensive rats, which develop cardiomyopathy, fibrosis, and diastolic dysfunction in response to a high-salt diet. They found that inhibiting miR-208a blunted the increase in β-MHC in response to salt-induced hypertension, with concurrent amelioration of hypertrophy, intramyocardial fibrosis, and an improvement in diastolic function.

Mir-378 Regulation of Hypertrophic Signaling

miR-378 is a muscle-enriched miRNA that is expressed in cardiomyocytes but not in fibroblasts. It was first identified in the heart as a miRNA that is expressed at significantly higher levels in 7-day-old neonatal mice compared with 16-day fetal hearts.45 miR-378 is expressed in both cardiac and skeletal muscle and was shown to directly downregulate expression of the insulin-like growth factor receptor 1 by binding to its mRNA. In neonatal cardiomyocytes, miR-378 overexpression leads to decreased insulin-like growth factor receptor 1 and inhibition of its downstream effectors PI-3K (phosphatidylinositol 3-kinase) and Akt.46 These are important antiapoptotic signals under conditions of stress, and in fact inhibiting miR-378 was cardioprotective against apoptosis in response to hydrogen peroxide or hypoxic stress.

In contrast, there was a decrease in miR-378 in the heart after TAC, and maintenance of miR-378 levels by genetic overexpression of miR-378 decreased hypertrophy and improved LV function.46 The authors found miR-378 levels to be decreased in both a mouse TAC model and in a β-adrenergic receptor overexpression model, as well as in human patients with dilated cardiomyopathy, and that miR-378 directly suppresses insulin-like growth factor receptor 1, Ksr1, Grb2, and MAP kinase 1, all components of the MAP kinase signaling cascade known to be involved in cardiac hypertrophy. A second group working similarly identified miR-378 as a negative regulator of hypertrophy in mouse TAC and isoproterenol infusion models, as well as in a rat aorta-caval volume overload model in addition to human dilated cardiomyopathy hearts. The authors identified and confirmed by luciferase assay that Grb2 was a target of miR-378.46,47 These experiments again underlined a recurrent theme in miRNA biology, namely that the role of the miRNA seemed to be specific to the type of disease model and type of stressor investigated and cautioned against generalization of results obtained from any particular model.

miRNA Regulation of Fibrosis

miR-29: Regulation of Profibrotic Genes

miR-29 was identified by a microarray screen looking at miRNA species that are differentially regulated in a rat model of MI.48 miR-29 was noted to be dramatically downregulated in the border zone of an induced left anterior descending territory infarction compared with noninfarcted myocardium from the same heart. These findings were reproduced in explanted human hearts at the time of transplantation, where levels of miR-29 were significantly lower in the border zones of hearts that had suffered MIs relative to nonfailing hearts. The investigators demonstrated that miR-29 targeted genes involved in cardiac remodeling and fibrosis, including elastin, fibrillin 1, collagens type I and III. miR-29 seemed to be downregulated by transforming growth factor-β, a regulator of cardiac fibrosis.48 In an interesting study, Zhou et al49 implicated miR-29 in myoblast transdifferentiation into myofibroblasts in vitro, consistent with its role as a mediator of tissue fibrosis. In the mdx mouse model of Duchenne muscular dystrophy, the skeletal and cardiac muscles are gradually replaced by fibrotic tissue in a manner parcelling the human disease. miR-29 levels are decreased in mdx myoblasts and restoration of miR-29 levels inhibited this fibrotic process by inhibiting myoblast transdifferentiation.50

The role and regulation of miR-29 are emerging as both crucial to the regulation of cardiac fibrosis and to cardiac adaptation to a variety of stimuli. During exercise training of Wistar rats, downregulation of the antihypertrophic miRNAs, miR-1, miR-133a, and miR-133b, was observed with concurrent upregulation of miR-29 and downregulation of its known target connective tissue miRNAs. The authors hypothesized that upregulation of miR-29 may underlie the improved diastolic function seen with exercise training although this is yet to be proven by manipulation (overexpression or inhibition) of miR-29 levels.51

These experiments suggested that miR-29 is a central regulator of cardiac fibrosis and may play an important role in LV remodeling after stressors. In the heart under normal conditions, the fibrotic process itself can be both adaptive and maladaptive. After MI the transdifferentiation and matrix-synthetic pathways are essential for the rapid formation of a protective fibrotic scar to replace ischemic, necrotic myocardium; however, the overproduction of fibrotic tissue in the heart can lead to diastolic and systolic dysfunction and adverse hemodynamic consequences. Future manipulation of miR-29 may need to take into account these complex dynamics. The upregulation of miR-29 under exercise conditions thus represents a favorable adaptation that improves hemodynamics under high cardiac workloads, but whether upregulation of miR-29 after MI is beneficial needs to be established.

miR-21: Regulation of MAP kinase Profibrotic Signaling

Roy et al52 first described miR-21 as a miRNA increased in mouse hearts subjected to ischemia–reperfusion in the infarct zone. Using laser microdissection they were able to show that the upregulation was restricted to infarcted and reperfused myocardium and not to surrounding tissues. In situ hybridization demonstrated that miR-21 expression was restricted to fibroblasts in the infarct zone. The authors went on to demonstrate that miR-21 is a direct inhibitor of PTEN (phosphatase and tensin homolog; a phosphatase that dephosphorylates PIP2 [phosphatidylinositol 4,5-bisphosphate]), thereby leading to akt activation and production of the matrix metalloproteinase MMP-2 in fibroblasts.

Contemporaneously, miR-21 was also shown to be upregulated in microarray screen in a β-adrenergic receptor transgenic mouse model of HF. Thum et al53 demonstrated that miR-21 levels were increased selectively in fibroblasts of failing mouse hearts. The authors showed that Sprouty2 was a target of miR-21 in cardiac fibroblasts, and that inhibition of
Sprouty2 led to extracellular signal-regulated kinase (ERK)1/2 activation. The consequence of this was decreased fibroblast apoptosis and increased FGF (fibroblast growth factor) secretion, suggesting that miR-21 led to a profibrotic program in the heart. Importantly, inhibition of miR-21 by an antagonist protected mice subjected to TAC from fibrosis, hypertrophy, and LV dilatation. Relevance to human heart disease was provided subsequently on examination of plasma and biopsy samples in 75 patients with aortic stenosis. Levels of miR-21 were increased in patients with aortic stenosis, but decreased after surgical correction of the valvular disease, and importantly, the level of miR-21 correlated with myocardial collagen level. Consistent with findings of prior groups, miR-21 expression was restricted to interstitial cells in the biopsy samples.

Whether miR-21 also plays a role in cardiomyocyte biology remains unclear. Although the overexpression did not induce hypertrophy, Suget et al found that overexpression of miR-21 in neonatal CMs led to Sprouty2 suppression and induced slender outgrowths that promote cell-to-cell connection and gap junction formation. Whether this has any relevance to in vivo models or is an artifact of viral overexpression in a cell type where the miRNA is not usually expressed (thereby leading to off-target effects) has not been fully explored.

miR-24: Regulation of Cardiomyocyte and Fibroblast Function

miR-24 was originally identified as a miRNA species that was differentially regulated after TAC in mice and was shown to be downregulated in a murine model of MI. Suppression of miR-24 was maximal 1 week after infarction, with levels returning to normal by 2 weeks after infarction. The authors observed concurrent upregulation of collagen, transforming growth factor-β, and fibronectin. Mice with adenoviral-driven miR-24 expression (via direct adenovirus injection into myocardium) before infarction induced by left anterior descending ligation exhibited significantly reduced infarct scar size. The effects of miR-24 seemed to be mediated by inhibition of fibroblast proliferation. The authors identified the protease furin as a miR-24 target by bioinformatics analysis and showed that inhibition of furin was sufficient to inhibit downstream transforming growth factor-β signaling.

The role of miR-24 in cardiomyocytes was elucidated in a different study, which identified the protein junctophilin-2 as a putative miR-24 target. Junctophilin-2 is a protein involved in structurally coupling the T-tubules to the sarco/plasmic reticulum in cardiomyocytes. Overexpression of miR-24 in cultured cardiomyocytes led to structural changes in the T-tubules, which were shown to translate into a decreased efficiency of excitation–contraction coupling and worsened cardiomyocyte function. In a follow-up study, the inhibition of miR-24 by antagonir administration after TAC did not prevent the development of cardiac hypertrophy but ameliorated the transition of hypertrophied hearts to decompensated HF. Cardiomyocytes isolated from mice subjected to miR-24 inhibition did not exhibit the loss of E–C coupling seen in the nontreated animals. These experiments again highlighted the recurrent theme we outlined previously, namely that the same miRs may play different roles depending on the type of cell and stressor examined. As discussed above, overexpression of miR-24 in the MI model may promote beneficial LV remodeling by inhibiting fibroblast proliferation, whereas the opposite, miR-24 inhibition may be beneficial in the TAC model of pressure-overload–induced HF.

It is evident from the discussion above that miRNAs are potent mediators of the cardiac remodeling. miRNAs have been found to mediate apoptosis, fibrosis, and hypertrophy in response to various exogenous stressors. The number of identified miRNA species continues to grow, and without a doubt the number of physiological and pathological processes mediated by miRNAs will increase accordingly. A complete list is beyond the scope of this review. For additional recently discovered miRNAs and a brief discussion of their role in the heart along with relevant references, we refer the reader to the Table.

### microRNAs as Therapeutic Targets in HF: Promise or miRage?

It is evident from the discussion above that microRNAs are able to modulate entire networks of gene expression, and in the process modulate entire programs of tissue adaptation and response to stress. In addition to the aforementioned effects on cardiac hypertrophy and fibrosis, miRNAs are known to be involved in a plethora of human diseases, from cancer to macular degeneration.

Strategies to inhibit or mimic miRNAs to modulate these disease pathways have therefore attracted a lot of attention. Most current effort has focused on the use of RNA-based miRNA inhibitors, termed antagonirs or anti-miRs. Several chemical modifications to miRNAs enhance their stability in serum and enhance cell membrane permeability (thereby increasing delivery into cells), among these phosphorothioate linkages and locked nucleic acids (LNA; comprehensively reviewed by Burnett and Rossi). These modified miRNA inhibitors can be delivered intravenously, and even subcutaneously, and a single administered dose can maintain therapeutic levels with inhibition of target miRNA for several weeks.

The design of such inhibitors is critical to their function, as illustrated by recent work looking at LNA-based miR-21 inhibitors. Thum et al. used an LNA miR-21 inhibitor in vitro to show that inhibition of miR-21 leads to CM hypertrophy. In contrast, the use of a shorter LNA that targeted only the 6- to 8-bp seed region of miR-21 failed to show any effect. This discrepancy was shown to be because of the rapid clearance and effects of the short LNAs from the myocardium, with a correspondingly brief duration of action. There were additional concerns from in vivo models that some of the miR-21 anti-miR effects may result from off-target effects: high levels of circulating anti-miRs may not only inhibit the target miRNA in the intended tissue but may also inhibit the target miRNA in other tissues. Moreover, the nonphysiological level of circulating anti-miR may also target other miRNAs or miRNAs with similar seed regions, which may not be targets at baseline levels. Thus, there is much to still be learned about the optimal design and delivery of endogenous and LNA if they are to become reliable and useful therapies. Nonetheless, the promise in this technology is being borne out by clinical trials of miRNA molecules and miRNA inhibitors that are already underway. Preclinical trials of miR-208 inhibition for the treatment of chronic HF have been initiated by MiRagen.
### Table. miRNAs Involved in Cardiac Remodeling

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<th>Function/Phenotype</th>
<th>Target</th>
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<td><strong>Inhibitors of hypertrophy</strong></td>
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<td>Decreases KCNQ1 levels</td>
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<td>Improved LV function, decreased remodeling after MI in mouse model</td>
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<td>Downregulates CTGF; downregulated in human (aortic stenosis) and mouse (TAC) models of LVH</td>
<td>CTGF</td>
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<tr>
<td>miR-101a/b</td>
<td>Levels decreased in peri-infarct zone after LAD ligation in rats</td>
<td>FOS</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td>Overexpression improves cardiac function after MI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-214</td>
<td>Knockout mice develop more fibrosis after ischemia–reperfusion in mice, possibly secondary to increased NCx expression and Ca++ overload</td>
<td>NCx</td>
<td>74</td>
</tr>
<tr>
<td>miR-29</td>
<td>Upregulated in borderzone of mouse and human heart</td>
<td>Fibrillin 1</td>
<td>48–51</td>
</tr>
<tr>
<td></td>
<td>Inhibits fibrosis in mdx mouse</td>
<td>Collagen I, III</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Causes myoblast transdifferentiation to myofibroblasts downregulated by TGF-β</td>
<td></td>
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</tr>
</tbody>
</table>

ANP indicates atrial natriuretic peptide; BNP, B-type natriuretic peptide; CTGF, connective tissue growth factor; ERK, extracellular signal-regulated kinase; IGF1, insulin-like growth factor 1; IGFR, IGF receptor 1; LAD, left anterior descending; MI, myocardial infarction; miRNA, microRNA; PE, phenylephrine; PI-3K, phosphatidylinositol 3-kinase; PTEN, phosphatase and tensin homolog; TAC, transverse aortic constriction; and TGF, transforming growth factor.
therapeutics, and trials of a miR-122 inhibitor for the treatment of Hepatitis C are currently in phase II,\textsuperscript{79} and several other miRNAs are currently in the preclinical stage (reviewed by Wahid et al.).\textsuperscript{80}

Strategies to deliver miRNA mimics have lagged behind, but recently several delivery systems have been developed for use in animal models. The use of adeno-associated viruses has been well described in animal models and both can efficiently deliver pre-miRNA–encoding vectors into cells.\textsuperscript{81} Nonviral delivery systems developed to date include nanoparticles, cationic lipids, and carbon nanotubes. Cationic polymers, such as in vivo jetPEI made by Life Technologies, have been used to deliver small interfering RNA in animals. Atelocollagen,\textsuperscript{82} a derivative of calf type I collagen, has been used similarly in multiple mouse models to successfully deliver small interfering RNA with a demonstrated therapeutic effectiveness.\textsuperscript{83–85} Nanoparticles, composed of complexes of cationic liposomes, have similarly demonstrated efficacy in animal models. One of these is a monolamellar liposome termed SNALP (stable nucleic acid lipid particles), by Tekmira pharmaceuticals, can efficiently deliver miRNAs in multiple in vivo models, including a primate model, where demonstrated suppression of apolipoprotein B levels was shown.\textsuperscript{86} None of these have yet translated to human trials, but continue to evolve in that direction.

**MicroRNAs: Circulating Biomarkers or Secreted Signaling Molecules?**

More recently, microRNAs have been detected in bodily fluids, including urine, saliva, and plasma.\textsuperscript{87} Extracellular miRNAs seem to be over prolonged period of time and resistant to freeze/thaw cycles, making them attractive candidates for disease biomarkers.\textsuperscript{87,88} Extracellular RNAs circulate in the plasma, both as passengers in microparticles and exosomes,\textsuperscript{89,90} and components of other complexes, such as with high-density lipoprotein\textsuperscript{90} and Argonaute.\textsuperscript{91} Their stability has been hypothesized to be a consequence of their incorporation into secreted cellular extracellular microvesicles and exosomes. However, whether extramembrane vesicle (EMV)-associated miRNAs are causally related to disease pathogenesis or simply markers of pathology is not evident.

Some recent studies suggest an intriguing role for miRNA-containing EMVs in disease pathogenesis. EMVs and their associated proteins and miRNAs seem to play an important role in cancer neoangiogenesis and metastasis\textsuperscript{92} by mediating horizontal transfer of biologically active proteins and genetic information\textsuperscript{93,94} between cell types. In the cardiovascular system, 2 recent studies suggest that EMVs can be released from endothelial cells\textsuperscript{95} and cardiomyocytes,\textsuperscript{95} and the associated miRNAs/miRNAs can alter gene expression in other cell types such as smooth muscle cells and fibroblasts. The possibility of miRNA-mediated communication between CMs and other cells in the cardiac interstitium or other tissues would represent a novel and important mechanism of cardiac adaptation and is the focus of active investigation. However, these circulating miRNAs at the minimum represent disease reporters and have been exploited for their potential as biomarkers in human diseases.

**Circulating microRNAs as Biomarkers in Human HF: An Evolving Field**

In comparison with the burgeoning literature documenting a pathogenic role for miRNAs in the progression toward HF,\textsuperscript{11,96–101} there has been relatively little research investigating the use of miRNAs as instruments in diagnosis, prognosis, and predicting reversibility in HF. Indeed, the miRNA arena is ripe for novel biomarker discovery, with recent studies in failing myocardium documenting hundreds of miRNAs with differential expression by HF pathogenesis without well-characterized role in remodeling or HF.\textsuperscript{102} Furthermore, distinct miRNA profiles have emerged in patients treated with mechanical circulatory support, clustering more closely with normal patients than with decompensated advanced HF.\textsuperscript{99} These results suggest that further developments investigating the clinical role of circulating miRNAs in LV remodeling/function and prognosis in parallel with research in molecular mechanisms of HF progression may be fruitful. In this section, we will extend the molecular implications of miRNA expression discussed thus far to elaborate some of the seminal work involving circulating miRNAs in HF diagnosis and cardiac structure.

Although a diversity of candidate circulating miRNAs have been studied in HF, a unifying theme in circulating miRNA research in HF is a consistent association with cardiac structure, HF pathogenesis, and markers of disease severity. Tijsen et al\textsuperscript{100} used a SD-validation study design based on 16 candidate miRNAs derived from screening 12 men with moderate LV systolic dysfunction and 12 healthy controls and applied to a cohort of 39 normal volunteers without HF and 50 patients with dyspnea (of whom 30 had a diagnosis of HF). Among a host of other miRNAs increased in patients with HF (miR18b, 129-5p, 1254, 675, HS_202.1, 622), the authors found that miR-423-5p strongly discriminated HF cases from healthy controls (C-statistic 0.91) and from non-HF dyspnea (C-statistic 0.83; both statistically significant). miR-423-5p was modestly associated with N-terminal pro-B-type natriuretic peptide (\(r=0.43; P=0.002\)) and LV ejection fraction (\(r=0.34; P=0.02\)). Furthermore, there were significant relationships between selected miRNAs and functional class and HF pathogenesis.\textsuperscript{97} In a study of 45 patients with dilated cardiomyopathy and 39 age- and sex-matched controls, a candidate miRNA approach involving miRs 423-5p, 126, 361-5p, 155, and 146a was undertaken. Again, miR-423-5p was higher in dilated cardiomyopathy and was associated with B-type natriuretic peptide (\(r=0.43; P=0.003\)) and had a modestly strong discrimination for HF diagnosis (C-statistic, 0.67).\textsuperscript{103} The association with natriuretic peptides and functional status in HF has been replicated for several other miRNAs in the literature.\textsuperscript{104} For example, miR210 in peripheral mononuclear cells was noted to be higher in patients with more destabilized HF (by New York Heart Association functional class) in a study. Furthermore, although miR210 was not associated with B-type natriuretic peptide, those patients with an improved natriuretic peptide level on subsequent hospitalization (suggesting better compensation and less neurohormonal activation) had a lower miR210 level.\textsuperscript{105}

Importantly, the ability of miRNAs to forecast risk and provide insight into LV remodeling seems to depend on the stage of
HF progression. In parallel studies, in 246 patients studied after index MI with serial echocardiograms performed during index hospitalization, 3 months, and 1-year post-discharge, both miR-133 and miR-423-5p rose over time post-MI. However, these miRNAs were not associated with LV remodeling, function, or natriuretic peptide levels, in contrast to results reported by Bauters et al in manifest HF. Nevertheless, in more subtle forms of HF (eg, HF with preserved LV ejection fraction), miRNAs may continue to play an important role as a reflection of underlying physiology: in a small study of patients with LV diastolic dysfunction (with normal LV ejection fraction), echocardiographic markers of LV diastolic dysfunction (eg, tissue Doppler and transmitral indices) were strongly associated with selected miRNAs (454, 500, 1246), without an association with LV ejection fraction. Given the emerging role of miRNAs in myocardial hypertrophy and fibrosis—and the importance of preclinical detection and therapies for HF—the use of circulating miRNAs as probes into disease stage, physiology, and reversibility is clearly warranted.

From a diagnostic perspective, the complementary molecular targets of miRNAs have been used to craft a multimarker approach to HF diagnosis, similar to studies involving standard biomarkers in HF. In a study of 186 candidate circulating miRNAs profiled in 30 patients with HF and 30 healthy controls, miRs 423-5p, 320a, 22, and 92b were elevated and were combined into a predictive score for the diagnosis of HF. This multimarker approach yielded powerful discrimination for HF diagnosis (C-statistic 0.90), with high sensitivity 90% and specificity 90%. In addition, the multimarker predictive score was associated with BNP (P = 0.002), wide QRS (P = 0.009), LV remodeling (by increased LV end-diastolic dimension P = 0.03), and left atrial size (P = 0.01). These results suggest the multifaceted use of a multimarker approach in HF diagnosis and to provide a noninvasive, rapid surrogate of LV structure and function.

Despite this emerging literature on the use of circulating miRNAs for diagnosis, there has been little investigation on the prognostic use of miRNAs or their reversibility with therapy. In a study involving a hypertensive rat model, a subset of miRNAs upregulated in pressure-overload HF (miR-16, miR-20b, miR-93, miR-106b, miR-223, and miR-423-5p) seemed to mitigate after treatment with angiotensin-converting enzyme inhibitor. In addition, miRNA levels increased progressively over time with pressure overload, again hinting at the possibility of using miRNAs as an early barometer of cardiac injury before clinical HF (stage A and B HF). In total, the limited balance of evidence using miRNAs in the clinical arena calls for more epidemiological investigation to translate their molecular implications into clinically applicable biomarkers of disease severity, diagnosis, and ultimately prognosis.

Conclusions

miRNAs, potent modulators of gene expression, are emerging as critical regulators of cardiac remodeling and all aspects of HF pathogenesis. The cardiac miRNA species discussed above are able to directly regulate the expression of cardiac signaling proteins, contractile proteins, and transcription factors (Figure 3) and play a critical role in the transition from adaptive changes in the heart to maladaptation and HF. miRNAs identified thus far in animal models modulate the processes of hypertrophy, fibrosis, and apoptosis. Work from several groups demonstrating the ability of exogenously administered miRNA inhibitors to modulate these pathological processes, thereby ameliorating the progression to HF is promising and potentially opens the door for novel therapies for human HF. However, the translation of these approaches to larger animal models and ultimately to human trials remains a daunting task complicated by the possibility of off-target effects of systemically administered oligomers. Nonetheless, the use of miRNA-based treatment for hepatitis C is an encouraging beacon of light that speaks to the potential of this approach.

Reassuringly, many of the miRNAs characterized in murine models of HF are also altered in human HF; perhaps speaking to evolutionary conserved pathways in these fundamental processes. However, this enthusiasm has to be tempered by repeated observations that the functional role of the miRNA seems to depend both on the exact cell type and the exact disease model studied, and generalizations about the role of any particular miRNA should be avoided.

Considerable work remains to be done in this field. There are multiple miRNAs identified thus far that seem to be able to inhibit hypertrophy in the TAC model, and there are many miRNA species that are differentially regulated in response to TAC whose role has not yet been characterized. Some of these (miR-1, miR-133) are known to be bicistronically encoded and cotranscribed. However, the complex interplay of the other known and hitherto unknown miRNAs in the signaling networks that are integrated within the heart during adaptation and maladaptation has yet to be defined. Current paradigms of second messenger and downstream signaling pathways suggest integration and cross-talk between canonical pathways at various levels, and it is possible that similar principles govern the expression and the miRNA networks in the heart. The recent role of miRNA binding sites in other non-coding RNAs (such as pseudogenes) that act to titrate miRNA levels in the cell, thereby constituting a novel regulatory role for miRNA, has been described elegantly by Salmena et al. However, the role of these types of mechanisms has not yet been explored in the heart. The heart has long been known to be able to respond differently to different stimuli, undergoing concentric and eccentric hypertrophy, in response to pressure and volume overload stressors, respectively. It is likely that multiple miRNA species, regulated in subtly different manners, allow the heart to respond in a fine-tuned manner to the spectrum of stressors to which it is exposed over the lifetime of an individual.

The role for circulating microRNAs as biomarkers in human HF is also in its earliest stages. Although levels of circulating miRNAs do seem to correlate with certain stages of HF, their role as prognostic biomarkers has yet to be worked out, and larger studies with longer follow-up will be needed to better define their role as diagnostic and prognostic biomarkers. As larger human studies are conducted, further work on microRNA regulation, signaling is worked out and the feasibility of using miRNAs for therapeutics in larger animals and humans is developed, we will have the good fortune of seeing a nascent field of biology, barely 2 decades old, potentially takes a critical and central role in our therapeutic arsenal.
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References


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