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MicroRNAs as Therapeutic Targets and Biomarkers of Cardiovascular Disease

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Abstract

MicroRNAs play central roles in cardiovascular disease, and their therapeutic manipulation raises exciting opportunities as well as challenges in the path toward clinical development.

INTRODUCTION

Despite the success of many widely prescribed drugs for cardiovascular disease, heart disease continues to increase in prevalence worldwide, highlighting the need for deeper insights into disease mechanisms and innovative therapeutic strategies. Recently, microRNAs (miRNAs) have emerged both as key regulators of cardiovascular disease and as potential drug targets. Numerous miRNAs display characteristic changes in expression during pathogenesis of the heart and blood vessels and may serve as markers for cardiovascular disease progression. Genetic deletion or transgenic overexpression of specific miRNAs in mice can also modify disease responses, either diminishing or exacerbating pathological phenotypes. Methods for selectively inhibiting miRNA function in vivo through the delivery of inhibitory oligonucleotides have raised hopes for new therapeutic modalities for treating cardiovascular disease. However, despite these advances, many questions remain regarding the mechanistic basis of miRNA actions, and numerous issues must be addressed on the path toward development of miRNA-based therapeutics. Several recent reviews have provided in-depth discussion of the roles for individual miRNAs in cardiovascular development and disease (1–3). This article highlights the opportunities and challenges that lie ahead in this field and considers important questions for the future.

MECHANISMS OF miRNA ACTION

miRNAs are single-stranded RNAs ~22 nucleotides in length that repress the expression of specific proteins by annealing to complementary sequences in the 3' untranslated regions (UTRs) of target mRNAs (Fig. 1). The association of miRNAs with their target mRNAs is mediated by Argonaute (AGO) proteins within the RNA-induced silencing complex (RISC) in the cytoplasm. There has been debate regarding the relative importance of translational inhibition versus mRNA decay as mechanisms of miRNA action, but both mechanisms likely contribute to the ultimate biological readout of miRNA activity (4). There have also been reports of miRNAs enhancing mRNA translation, but this seems to be a rare exception.

There are estimated to be up to 1000 miRNAs encoded by the human genome. Most miRNAs are transcribed as separate genes or as miRNA clusters that are generated from large precursor transcripts. However, roughly a third of miRNAs are encoded by introns of protein-coding genes and are generated after splicing of pre-mRNAs. In many cases, intronic miRNAs modulate, either positively or negatively, the same cellular processes as the host gene. The incorporation of miRNAs into protein-coding genes allows for coordinate control and alleviates the need for miRNAs to be independently regulated. An especially interesting example of such control is provided by myosin heavy-chain genes, which encode muscle-specific miRNAs within their introns that regulate myosin expression and muscle function (5).

Nucleotides 2 to 7 of an miRNA, known as the seed sequence, serve as the primary determinant of mRNA recognition (6). Individual miRNAs typically modulate the expression of dozens or even hundreds of mRNA targets. The ability of miRNAs to modulate broad collections of mRNAs offers opportunities to regulate complex biological processes that are otherwise resistant to modulation through single proteins. However, pinpointing the specific mRNA targets that evoke the biological effects of miRNAs and distinguishing primary versus secondary changes in the cellular proteome that may occur in response to up- or down-regulation of a miRNA represent vexing challenges, particularly with respect to understanding potential therapeutic outcomes of drugs that modulate miRNA functions. Compounding such uncertainties is the relatively modest inhibition that miRNAs impose on their targets, often inhibiting expression of individual proteins by less than twofold.

Determination of miRNA functions through genetic deletion in mice has also posed challenges. Despite the strong loss-of-function phenotype of mutations in *lin-4*, the founding miRNA in nematodes, most worms and mice with miRNA loss-of-function mutations do not display overt phenotypes under controlled laboratory settings. The minimal loss-of-function miRNA phenotypes likely reflect the fact that individual mRNAs are commonly targeted by multiple miRNAs, so that eliminating a single miRNA may have a relatively modest effect. Under conditions of homeostasis, the cellular proteome is likely able to rebalance in the absence of a single miRNA. However, under conditions of stress the functions of miRNAs are often revealed, pointing to their involvement as key components of disease mechanisms (7). Moreover, signaling pathways activated in response to stress profoundly alter the expression of numerous miRNAs and enhance their repressive activity.

Given the small size of miRNAs and the tolerance for nucleotide degeneracy in the recognition between miRNAs and mRNAs, it seems unlikely that single-nucleotide polymorphisms in miRNAs or their target sequences will contribute substantially to human disease. There are, however, some notable exceptions in the cardiovascular system. A rare, naturally occurring polymorphism outside the seed sequence of *miR-499*, a muscle-specific miRNA, has been reported to alter mRNA targeting, leading to subtle changes in the cardiac proteome, at least when overexpressed in mice (8). A human genetic variant within the 3'UTR of the atrial natriuretic peptide mRNA has also been correlated with enhanced expression of atrial natriuretic peptide and lowering of blood pressure. This A/G variant is contained within a binding site for *miR-425*, which binds to the A but not the G allele. Atrial

natriuretic peptide expression is elevated in individuals with the G allele, correlating with reduced blood pressure (9). These findings raise the possibility that inhibitors of *miR-425* might lower blood pressure by derepressing atrial natriuretic peptide expression.

miRNAS IN HEART DEVELOPMENT, DISEASE, AND REGENERATION

MicroRNAs have been implicated in virtually every cardiovascular disorder in which they have been examined, including heart failure, cardiac hypertrophy, remodeling after myocardial infarction, arrhythmias, atherosclerosis, atrial fibrillation, and peripheral artery disease (1–3). Virtually all of the basic cellular processes involved in cardiovascular development and disease, such as cell proliferation, differentiation, apoptosis, fibrosis, angiogenesis, and inflammation are subject to miRNA control (Fig. 2).

As is not uncommon in a new and rapidly evolving field, there are numerous conflicting reports regarding the functions of miRNAs in heart disease. For example, cardiac expression of *miR-25* has been reported to increase during heart failure in rodents, and blocking *miR-25* with short antisense oligonucleotides, referred to as antagomiRs or antimiRs, has been reported to prevent heart failure (10). In contrast, another group reported that *miR-25* decreases during heart failure, with inhibition of *miR-25* by an antimiR causing heart failure (11). Similarly, *miR-21* was shown by some groups to be required for cardiac fibrosis and hypertrophy (12), and by others to be dispensable for this process (13). Some of these conflicting reports likely reflect our poor understanding of the subtleties of miRNA regulation and function or variations in animal models of disease. However, before advancing these and other miRNAs into clinical development, such uncertainties need to be resolved.

One of the most validated miRNAs with respect to its role in cardiovascular disease is *miR-29*, which targets a broad collection of mRNAs encoding collagens and other extracellular matrix proteins involved in fibrosis (14). Members of the *miR-29* family are down-regulated in a variety of fibrotic disorders, which has been proposed to result in excessive extracellular matrix production. Strategies to enhance *miR-29* expression would be expected to suppress fibrosis. Inhibition of *miR-29* has been proposed as a way to enhance extracellular matrix production as a therapeutic intervention for treating aneurysms (15).

Especially fascinating has been the discovery of roles for miRNAs in the control of cardiomyocyte proliferation and heart regeneration (16). Members of the *miR-15* family, for example, are up-regulated in postnatal cardiomyocytes during cell cycle exit, and their inhibition can sustain cardiomyocyte proliferation and confer benefit after myocardial infarction (17). Conversely, *miR-199a* and *miR-590* and members of the *miR-17~92* cluster promote cardiomyocyte proliferation, and their forced expression has been reported to enhance cardiac regeneration (18, 19). Several of these miRNAs also control cancerous cell growth. Thus, strategies to elevate their expression to repair the heart would likely require cardiac-specific delivery or transient regulation. In addition to being mediators of cardiovascular disease, miRNAs have been reported to be capable of reprogramming fibroblasts into cardiomyocytes and to modulate metabolism, suggesting interesting therapeutic possibilities.

CIRCULATING miRNAS

miRNAs have been detected in the blood-stream and in other body fluids, raising interest in their potential use as markers for disease (20, 21). Indeed, distinct expression patterns of extracellular miRNAs have been associated with a variety of cardiovascular disorders, including atherosclerosis, myocardial infarction, heart failure, viral myocarditis, hypertension, and type 2 diabetes. However, whether these miRNAs participate in the disease process or simply serve as markers of disease progression has not been established. Greater patient cohorts will be needed to reach firm conclusions regarding the prognostic power of extracellular miRNAs.

miRNAs can be released into the circulation in membranous structures known as apoptotic bodies during, for example, cell lysis in myocardial infarction. During atherosclerosis, endothelial cells release apoptotic bodies that recruit progenitor cells from the circulation to reduce atherosclerotic plaque formation. Endothelial cell-derived apoptotic bodies containing *miR-126m* have been reported to be transported to adjacent smooth-muscle cells, in which *miR-126m* suppresses atherosclerosis by regulating expression of the chemokine CXCL12 (22). In response to shear stress and atherosclerosis, *miR-145* has also been shown to be transferred from endothelial cells to adjacent smooth-muscle cells within the blood vessel wall and to confer protection against plaque formation (23).

There is also substantial evidence for regulated secretion of miRNAs from intact cells (Fig. 1). The majority of miRNAs within the circulation are associated with AGO2 in nuclease-resistant complexes. miRNAs have also been identified in the circulation within exosomes, which are small membrane vesicles that form within multivesicular bodies and are secreted upon fusion of multivesicular bodies with the plasma membrane. Exosomes contain specific miRNAs rather than the complete spectrum of miRNAs of a cell, indicating specific mechanisms for their recognition, packaging, and secretion. How specific miRNAs are selected for inclusion in exosomes is unknown. miRNAs are also incorporated into high-density lipoprotein and low-density lipoprotein particles through unknown mechanisms. Secreted miRNAs can, in principle, be transferred between distal tissues through the circulation. However, it remains unclear whether or how a miRNA taken up by a cell might achieve a sufficient concentration to inhibit its targets. An intriguing possibility is that extracellular miRNAs may participate in long-range signaling between tissues. In this regard, miRNAs have been reported to act as agonists of Toll-like receptors and to trigger downstream pathway activation in target cells.

TOWARD miRNA THERAPEUTICS

Much of the excitement surrounding the therapeutic potential of miRNAs stems from the ability to inhibit miRNA function with antimiRs (24). These naked oligonucleotides can be delivered by means of subcutaneous or intravenous injection and effectively block the association of miRNAs with their targets. Depending on the tissue, antimiRs can show efficacy at doses acceptable for therapeutic development. Numerous chemical modifications that enhance cellular uptake and stability of antimiRs have been reported. Among these, covalent attachment of cholesterol to the 3' end appears to enhance cellular uptake. A locked

nucleic acid (LNA) modification, in which the 2'-oxygen and the 4' carbon of the ribose moiety of the nucleotide are covalently linked, enhances oligonucleotide binding to miRNAs (25). Because of this high affinity, it has been possible to design "tiny" inhibitors directed specifically against the seed sequence of miRNAs. The relatively limited number of studies to date about the pharmacokinetics and pharmacodynamics of antimiR action suggest that they are taken up from the circulation by endocytosis and accumulate within endosomes or multivesicular bodies, but much remains to be learned about the precise mechanisms of action and cellular handling of antimiRs.

In contrast to classical pharmacological agents, the onset of action of antimiRs appears to be delayed, often taking several days. The protracted onset of activity likely reflects the time required to rebalance the proteome of a target cell as a consequence of the modest changes in myriad miRNA targets. Conversely, the actions of antimiRs are remarkably long-lived, owing to their high stability and accumulation within intracellular depots from which they may be slowly released. Whereas the sustained activity of antimiRs allows for effective treatment of chronic diseases with periodic dosing, the long-term consequences of antimiR accumulation in different tissues and the inability to rapidly reverse the activity or eliminate the presence of a toxic antimiR raise obvious concerns. An additional challenge with respect to the development of miRNA-based drugs is the inability to correlate target engagement with mechanism and therapeutic efficacy. Because of their many targets and the summation of relatively small repressive effects that contribute to the therapeutic actions of miRNAs, it is difficult or impossible to directly ascribe the activity of an antimiR to a specific target.

AntimiRs accumulate predominantly in the liver and kidney, necessitating substantially higher doses to achieve efficacy in the cardiovascular system. This raises challenges with respect to achieving sufficient intracellular concentrations of oligonucleotides in cardiovascular tissues so as to evoke a therapeutic effect without causing liver and renal toxicity. Another major challenge in the systemic delivery of antimiRs directed against miRNAs that are widely expressed is the avoidance of adverse effects in nondiseased tissues. Because miRNAs act on numerous targets, an individual miRNA may have a beneficial activity in one tissue and an adverse activity in another. In this regard, methods for local delivery that reduce the doses of antimiRs needed for therapeutic efficacy and diminish possibilities for off-target effects are needed. Localized delivery of antimiRs or miRNA mimics for treating vascular disorders of the retina is a potentially promising clinical approach (26). The use of oligonucleotide-coated stents to modulate miRNA function in the blood vessel wall also warrants consideration.

The first miRNA-based therapeutic to reach the clinic is an LNA-modified antimiR directed against the liver-specific miRNA *miR-122*, which is required for replication of the hepatitis C virus (HCV). *miR-122* stabilizes the viral RNA by binding two adjacent sequences near the 5' end of the HCV genome and recruiting a RISC-like complex containing Ago2. Patients receiving five weekly subcutaneous injections of antimiR-122 in a phase 2a trial showed dramatic dose-dependent reduction of HCV RNA levels, including suppression to undetectable levels in several patients (27). No dose-limiting toxicities or desensitizing mutations in the HCV genome were observed. Intriguingly, as observed in studies of antimiRs in mice, the onset of efficacy was delayed by several weeks after initial dosing,

and inhibition was sustained after termination of drug treatment. Whereas these findings provide promising proof-of-concept for the systemic delivery of antimiRs, it should be noted that the liver is a much more accessible target tissue than is the heart. Moreover, the mechanism of action of *miR-122* in HCV replication is clearly distinct from the canonical mechanism of miRNA repression of mRNA translation, leaving open questions as to whether similar efficacy of inhibition will be observed for other miRNAs.

The majority of miRNAs are likely to play beneficial rather than pathogenic roles. Thus, strategies for elevating expression of miRNAs with salutary roles in disease will be important. However, methods for miRNA mimicry have yet to be optimized. miRNA mimics are double-stranded synthetic oligonucleotides that are processed into single-stranded miRNAs when introduced into cells. In contrast to antimiRs, which can be chemically modified to enhance stability and binding, miRNA mimics do not tolerate extensive modification. Recent advances in lipid formulations for enhancing uptake of small interfering RNAs may offer opportunities in this regard. The first miRNA mimic to be tested in humans has been delivered intravenously in a liposome formulation for liver cancer (28). Adenoviral delivery methods, particularly with adenoviruses such as AAV9, which show tropism to the heart, also warrant consideration for enhancing expression of beneficial miRNAs in disease settings. A concern with miRNA overexpression is the likelihood for off-target effects due to excessive repression of target mRNAs and the promiscuous inhibition of false targets.

PERSPECTIVE

miRNAs have become a source of great excitement as regulators of cardiovascular and other diseases because they provide new insights into disease mechanisms and can be therapeutically targeted. However, much remains to be learned about the precise mechanisms of miRNA action within complex disease pathways and whether such modest regulators can, indeed, be modulated in settings of chronic disease and in a tissue-specific manner. There has been a tendency to oversimplify the mechanistic basis of miRNA functions in the context of single downstream targets, but this is clearly not their primary mode of action. Thus, we need a deeper understanding as well as systems biology approaches to fully explain miRNA activity under conditions of homeostasis and disease. Despite these uncertainties and given the pace of this field, it seems likely that some of the many miRNAs implicated in cardiovascular disease will emerge as viable biomarkers and drug targets as well as links to previously unrecognized mechanisms of disease.

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Fig. 1. miRNA regulation and processing

miRNAs associate with the AGO protein in the RISC complex and bind to the 3'UTRs of target mRNAs to block protein expression. Stress signals such as ischemia and cytokine signaling regulate miRNA expression and their repressive activity. miRNAs are packaged into microvesicles and exosomes and may be secreted into the bloodstream, where they are transported to adjacent cells or distal tissues. miRNAs are also found in apoptotic bodies and lipoproteins. AntimiRs targeted against miRNAs are taken up by cells and sequestered in intracellular vesicles from which they are gradually released, enabling them to associate with their miRNA targets.

Fig. 2. miRNAs in cardiovascular disease

Shown are pathological processes in the cardiovascular system and the miRNAs that control these processes. miRNAs regulate cardiomyocyte proliferation, hypertrophy, and apoptosis. Other miRNAs act on fibroblasts and inflammatory cells to control fibrosis and inflammation, respectively. Blood vessel growth and stability, and angiogenesis and smooth-muscle cell proliferation, are also regulated by miRNAs acting on endothelial cells and smooth-muscle cells, respectively.