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REVIEW TOPIC OF THE WEEK

MicroRNAs in Cardiovascular Disease

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ABSTRACT

Micro-ribonucleic acids (miRNAs) are in the spotlight as post-transcriptional regulators of gene expression. More than 1,000 miRNAs are encoded in the human genome. In this review, we provide an introduction to miRNA biology and research methodology, and highlight advances in cardiovascular research to date. This includes the potential of miRNAs as therapeutic targets in cardiac and vascular disease, and their use as novel biomarkers. Although some miRNA therapies are already undergoing clinical evaluation, we stress the importance of integrating current knowledge of miRNA biology into a systemic context. Discovery studies focus on miRNA effects within one specific organ, whereas the expression of most miRNAs is not restricted to a single tissue. Because most miRNA-based therapies act systemically, this may preclude widespread clinical use. The development of more targeted interventions will bolster well-informed clinical applications, increasing the chances of success and minimizing the risk of setbacks for miRNA-based therapeutics. (J Am Coll Cardiol 2016;68:2577–84) © 2016 by the American College of Cardiology Foundation.

Only 1% of the human genome codes for genes that function in protein synthesis (1). The remaining 99% of deoxyribonucleic acid (DNA) was initially considered to be junk. It is now recognized that the majority of the genome may have biochemical functions, representing regulatory, noncoding ribonucleic acid (RNA). Several subcategories of noncoding RNAs exist, in particular, long noncoding RNAs and small noncoding RNAs. Among the latter, microRNAs (miRNAs/miRs) have thus far attracted most attention since their discovery in *Caenorhabditis elegans* (2). MiRNAs affect the production of proteins by interacting with transcribed messenger RNAs (mRNAs), thus silencing the expression of genes. Here, we aim to provide an overview of miRNA biology for clinicians, discussing their therapeutic and diagnostic potential, as well as their limitations.

BASIC BIOLOGY OF miRNAs

MiRNAs are short (~22 nucleotides), noncoding RNA molecules. They exert their function via the seed region, a sequence of 6 to 8 nucleotides that binds to messenger ribonucleic acid (mRNA), the so-called miRNA targets (3). MiRNA synthesis and silencing have both been extensively reviewed recently (4,5). The key biological concepts are summarized in the **Central Illustration**. Initially, a precursor transcript is produced and then forms double-stranded RNA. Later, the miRNA duplex undergoes unwinding, whereby only a single strand, the so-called guide strand, which is usually the functional unit, is loaded in the RNA-induced silencing complex (RISC). The other strand or passenger strand is often degraded, but may also function as a mature miRNA (6). In the RISC, the miRNA binds to its target mRNA, preventing its translation into a protein. Single miRNAs suppress more than 1 gene, and miRNAs with similar seed regions may suppress a similar, but nonidentical, set of genes, and to differing degrees. Gene suppression is usually partial, rather than total, and a single gene can have binding sites for multiple miRNAs. This organizational complexity, illustrated by a high false-positive rate of target prediction

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ABBREVIATIONS AND ACRONYMS

anti-miRs = inhibitors of miRNAs

CVD = cardiovascular disease

ECM = extracellular matrix

MHC = myosin heavy chain

MI = myocardial infarction

miRNA/miR = micro-ribonucleic acid

mRNA = messenger ribonucleic acid

RISC = ribonucleic acid-induced silencing complex

SMC = smooth muscle cell

algorithms (7), presents challenges in both understanding the functions of miRNAs and manipulating their effects.

MEASUREMENT OF miRNAs

miRNAs are relatively stable, and can be reliably measured in tissues, as well as in biofluids (8). Several techniques have been developed to identify and quantify miRNAs. Benefits and disadvantages of different techniques have been summarized previously elsewhere (8). Here, we briefly discuss the most commonly used methods.

Real-time quantitative polymerase chain reaction has been the cornerstone for miRNA quantification and remains the most reliable technique for quantitative comparison of miRNA expression levels. This technique uses predefined primers to amplify and measure individual miRNAs in a sample. Microarrays use hybridization of miRNAs to specific primers, trading less accurate quantification for higher throughput and lower cost, and measuring hundreds of miRNAs in parallel. Because both of these techniques rely on predefined primer sequences, they are not able to discover previously uncharacterized miRNAs.

RNA sequencing techniques provide "hypothesis-free" identification of RNA species, allowing the discovery of new miRNAs and quantitative analysis of a comprehensive miRNA transcriptome. The use of computational solutions to resolve reads into miRNAs suffers from the risk of reporting putative sequences that do not have real-world correlates (9).

Without added spike-ins and standard curves, all techniques rely on relative rather than absolute quantification, meaning that differences in miRNAs are presented as a "fold change" between paired samples, and not as an absolute unit, requiring information on the context of abundance. Experimental work must show downstream effects of miRNA changes as readout for miRNA function, specifically by comparing the profiles of multiple miRNAs with differential expression of target proteins. Ideally, the miRNA/mRNA duplexes in the RISC are analyzed to prove direct interactions.

THERAPEUTIC MANIPULATION OF miRNAs

The central action of miRNAs is to suppress protein expression through binding and silencing specific target mRNAs, which, in turn, reduces protein synthesis. Therefore, miRNAs offer a tantalizing mechanism for manipulating protein synthesis; in most cases, overexpression of a miRNA will suppress its direct targets, whereas inhibiting an endogenous miRNA will de-repress their expression.

Unmodified RNA strands are degraded upon administration; thus, miRNA therapeutics require either efficient methods of cell type-specific delivery or modifications that enhance stability but preserve miRNA function. For now, clinical studies with miRNA therapeutics mainly use inhibitors of miRNAs (anti-miRs). Anti-miRs are synthetic single strands of RNA, consisting of complementary nucleotides to an endogenous miRNA. Various structural modifications have been designed to increase their half-life in the circulation, bypass degradation in tissues and enhance intracellular delivery (10). Cardiotropic adeno-associated viruses achieve efficient cardiomyocyte-specific miRNA delivery (11). The translational potential of adeno-associated virus-mediated oligonucleotide delivery has been reviewed elsewhere (12). Currently, overexpressing a miRNA is generally considered less safe than inhibiting an endogenous miRNA.

Miravirsen is an anti-miR targeting miR-122 for treatment of hepatitis C (13), which has completed a multicenter phase 2a trial (14) and is currently in a phase 2b trial. The choice of miR-122 as the first therapeutic target highlights the challenges when targeting cardiovascular disease (CVD). First, miR-122 shows exquisite tissue specificity, whereas most miRNAs identified as treatment targets for CVD are ubiquitously expressed, raising concerns for off-target effects. Second, anti-miRs predominantly accumulate in the liver and kidneys, circumventing the need for tissue-specific targeting (13). The latter is further illustrated by the evaluation of anti-miR-21 as a therapy for Alport nephropathy (15). These ongoing clinical trials will provide more insight into the practical use of miRNA therapeutics.

Targeting the heart or vasculature with systemic anti-miRs would require significantly higher dosing, and efficiency may be low. Animal models have shown nephrotoxicity at higher doses of some anti-miRs, although the clinical trial of Miravirsen did not find evidence for renal injury in humans (14). The human immune system has evolved to detect viral RNA. Toll-like receptors recognize both single- and double-stranded RNA (16). High doses of synthetic oligonucleotides may elicit an immune response that could compromise efficacy and safety. Thus, cardiovascular applications will require solutions for local or cell-type-specific delivery, and clinically detectable, reliable readouts to monitor successful target engagement.

miRNAs IN HEART FAILURE

At the cellular level, heart failure is caused by cardiomyocyte dysfunction and fibrosis due to accumulation of extracellular matrix (ECM). These processes remain almost entirely untreated by standard heart failure treatment regimens.

miR-133 is highly abundant in cardiomyocytes, but is reduced in animal models of hypertrophy and in patients with hypertrophic cardiomyopathy (17). In vitro and in vivo studies showed increased hypertrophy upon miR-133 inhibition, and preserved cardiac function upon miR-133 overexpression. Targeting of the beta-1 adrenergic receptor pathway, central to the progression and treatment of heart failure, was implicated as the underlying mechanism (18). In addition to the heart, miR-133 is also present in skeletal muscle, albeit at lower levels than in cardiomyocytes. Here, miR-133 inhibition again increases responsiveness to adrenergic stimuli and promotes differentiation to brown fat tissue (19). miR-133 manipulation also seems to affect the cardiac action potential (20).

MiR-1 is part of the same cluster as miR-133 and shares its abundance, as well as its lower expression in heart failure patients (21). Both increased (22) and reduced (23) expression of miR-1 lead to electrophysiological abnormalities. Interestingly, miR-1 targets insulin-like growth factor-1, which itself represses processing of the pre-miR-1 transcript (21). The insulin-like growth factor-1 pathway is an important contributor to cardiac hypertrophy and arrhythmias, and increasing miR-1 levels seems to improve cardiac function (24).

MiR-208 is also highly enriched in cardiomyocytes, and regulates the balance between the α- and β-myosin heavy chains (MHC). Induction of the β-MHC isotype is a known maladaptive response to cardiac stress and reduces contractility (25). MiR-208 knockout mice, and rats treated systemically with anti-miR-208, had preserved balance between both MHC isotypes in response to experimental cardiac stress, with better cardiac function (26). MiR-208 inhibition has therefore been suggested as a protective treatment in heart failure. However, β-MHC expression is not altered in normal hearts of miR-208 knockout mice, indicating that effects of this miRNA are either dependent on disease context or subject to parallel controls (27). Furthermore, recent deep sequencing data from human hearts suggest that miR-208 expression is relatively low compared with other cardiac miRNAs, such as miR-1 and miR-133 (28). Preclinical trials using miR-208 inhibition in heart failure were announced in 2011, but have not progressed further.

In 1 study, miR-25 expression was repressed in failing human hearts (29), but its expression was increased in another study (30). Where the former study described the targeting of deleterious embryonic gene programs that worsen cardiac function, the latter showed repression of the sarcoplasmic/endoplasmic reticulum calcium adenosine triphosphatase (SERCA), an important contributor to excitation-contraction coupling in cardiomyocytes, and subsequent improvement in cardiac function. Differences in timing and chemical properties of the anti-miR treatment, as well as the study duration, could explain these contradictory results. This highlights the formidable task of determining optimal anti-miR chemistry, given the combinatorial possibilities of modifications that can be introduced, even in small oligonucleotides. Different oligonucleotides targeting the same miRNA may not achieve the same therapeutic benefit.

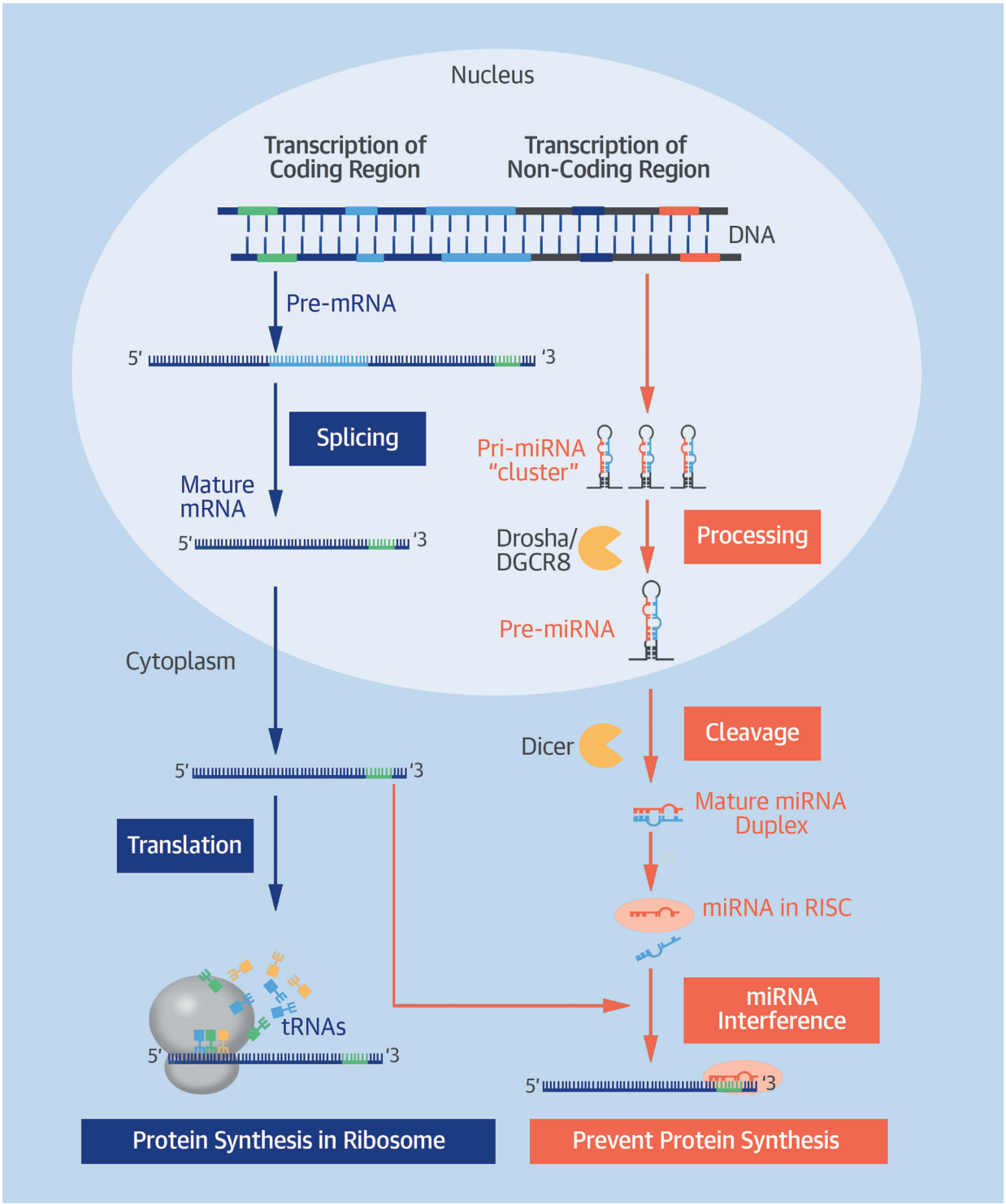
miRNAs IN CARDIAC REGENERATION

MiRNAs have been proposed as an alternative to cell therapy for cardiac regeneration. Studies on neonatal rat cardiomyocyte proliferation highlighted miR-199a and miR-590 as capable of inducing mitosis (31). Injecting these miRNAs into rodent hearts after myocardial infarction (MI) preserved cardiac function. Along similar lines, inhibition of miR-34a improved cardiac function after MI in mice, attenuating cardiomyocyte apoptosis and telomere shortening (32). MiRNA-based therapies for cardiac regeneration and repair still require validation in models with greater translational potential. A miRNA that is pursued currently for therapeutic applications in CVD is miR-92a. Inhibition of miR-92a reduces endothelial inflammation (33) and promotes angiogenesis and functional recovery in ischemic myocardium (34). However, this miRNA is part of a cluster of miRNA genes (miR-17∼92), also known as oncomiR-1 because its members target cell-cycle regulation. This raises concerns about potential side effects of miR-92a therapeutics.

miRNAs IN CARDIAC FIBROSIS

Several miRNAs have been implicated in cardiac fibroblast survival and related signaling pathways. Although some miRNAs directly target genes coding for ECM proteins (35,36), others prevent cardiac fibroblasts from attaining an activated secretory phenotype (37,38). In addition to its role in cardiac hypertrophy, miR-133 is considered antifibrotic

CENTRAL ILLUSTRATION **miRNA Biogenesis and Function**



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through targeting of connective tissue growth factor, a key regulator of the fibrotic process (37), as well as collagen I α-1, a main constituent of the cardiac ECM (39).

MiR-21 has been studied most extensively in the context of fibrosis. This miRNA is increased in heart failure patients and in cardiac fibroblasts of fibrotic mouse hearts (40), and promotes ECM deposition in mouse models of increased afterload (41) and myocardial ischemia (42). In vivo inhibition of miR-21 attenuates the fibrotic response and improves cardiac function in mouse models of heart failure (41). These results were not reproduced in a subsequent study using a different anti-miR, reiterating the importance of optimizing the anti-miR chemistry (43). MiR-21 also serves as an example of a miRNA where both the guide and passenger strands mediate function, with the passenger strand being transferred from fibroblasts to cardiomyocytes, where it exerts a pro-hypertrophic effect (6). This highlights another difficulty in translating findings from preclinical models to patients. If both strands mediate function, then inhibition of just 1 strand by anti-miR treatment may not recapitulate the phenotype observed in knockout mice, where both strands are deleted from the genome.

miRNAs IN NEOINTIMA FORMATION AND ATHEROSCLEROSIS

In addition to its profibrotic role, miR-21 enhances neointimal growth through pro-proliferative and antiapoptotic effects on vascular smooth muscle cells (SMCs) (44). Inhibition of miR-21 reduces in-stent restenosis in animals (45). The development of miRNA-eluting stents (46) could overcome one of the major challenges in miRNA therapies, because local delivery decreases the risk of off-target effects. The same can be argued for miR-29b, which represses ECM production by vascular SMCs (47), whereas inhibition slows abdominal aortic aneurysm progression (48) and promotes favorable plaque remodeling in atherosclerotic mice (49). Delivered locally, miR-29b antagonism could more subtly alter the ECM balance, if stents eluting anti-miR-29b were developed to inhibit aneurysm progression or to stabilize symptomatic atherosclerotic plaques.

Key events in atherosclerosis are endothelial injury and the switch of SMCs from a contractile to a synthetic phenotype. MiR-143 and miR-145 are transcribed together as a cluster and are highly abundant in SMCs, with a marked down-regulation seen in vessels with neointima formation (50,51). Together, these miRNAs regulate vascular SMC differentiation and, consequently, their loss contributes to SMC dedifferentiation and atherosclerosis (52). MiR-126 is highly enriched in endothelial cells (53). It indirectly enhances vascular endothelial growth factor signaling, and therefore has been studied in the context of angiogenesis and endothelial repair. Interestingly, the endothelial effects of this miRNA seem to be mediated by the passenger strand, rather than the guide strand (54). Although miR-126 has been described as endothelial cell-specific (53), this miRNA is expressed in megakaryocytes and may have a role in platelet function as mentioned elsewhere in this paper.

miRNAs IN LIPID METABOLISM

MiR-122 is highly abundant in the liver (55,56). Pharmacological strategies to lower miR-122 levels decreased plasma cholesterol levels (13,55). Unfortunately, initial optimism was dampened by subsequent studies that showed a simultaneous decrease in high-density lipoprotein cholesterol levels (57). Similar findings were obtained for miR-33, an miRNA that regulates cholesterol metabolism. Short-term inhibition was beneficial (58,59), but long-term inhibition in animals fed a high-fat diet had detrimental effects, such as hepatic steatosis (60). More recently, a study in human hepatic cells identified miR-148a as a regulator of the low-density lipoprotein cholesterol receptor (61). Systemic inhibition of miR-148a caused a significant reduction in plasma low-density lipoprotein cholesterol, but also increased high-density

CENTRAL ILLUSTRATION Continued

MicroRNAs (miRNAs) originate from primary transcripts (pri-miRNAs) that are derived from introns (the noncoding regions within a primary mRNA transcript) of protein-coding genes or from intergenic regions within the genome. Primary transcripts are processed in the nucleus to a hairpin-shaped pre-miRNA by the Drosha/DGCR8 complex, transported to the cytoplasm, and then processed to mature miRNA duplexes by the Dicer complex. To exert its function, the mature miRNA is incorporated into an RNA-induced silencing complex (RISC). This complex can then target mRNA through sequence complementarity: the sequence of the incorporated miRNA, with the 6 to 8 nucleotide-long seed sequence on the 5′ end in particular, binds to the targeted mRNA, usually to the untranslated region at the 3′ end. Depending on several factors, including the extent of sequence complementarity, this leads to cleavage or translation repression of the mRNA, preventing a protein from being assembled. mRNA = messenger RNA; RISC = ribonucleic acid-induced silencing complex; tRNA = transfer RNA.

lipoprotein cholesterol levels. Long-term side effects of miR-122 and miR-33 inhibition, combined with the advent of novel therapeutic options for dyslipidemia, may limit the clinical use of miRNAs to modulate lipid metabolism.

miRNAs AS BIOMARKERS

MiRNAs are present, stable, and detectable in the circulation (62), both in plasma or serum, where they are either bound to protein complexes or contained in microvesicles or lipoproteins. Microvesicle- and lipoprotein-borne miRNAs have been suggested to affect protein expression when delivered to cells (63–66). For example, miR-223 is relatively abundant in plasma, and may transduce an endocrine signal between blood cells and vascular cells (67). Because absolute levels of circulating miRNAs are low, it remains to be proven whether miRNA transfer is sufficient to achieve effective target repression in recipient cells.

Several cardiac miRNAs are detectable in blood early after MI, potentially reducing time to diagnosis (68). However, head-to-head comparisons with established biomarkers, such as high-sensitivity troponins, found that detection of miRNAs did not improve on the accuracy or usefulness of current methods (69). Furthermore, current miRNA detection techniques are time consuming and do not allow for the rapid diagnosis required in patients with MI. In the context of hypertrophic cardiomyopathy, higher levels of miR-29a correlate with both hypertrophy and fibrosis (70), but its clinical benefits beside current diagnostic tools remain unclear.

New biomarker searches should focus on unmet clinical needs, rather than areas where well-performing, established markers already exist. For example, current risk prediction models for MI could improve. Three studies, albeit with differing methodologies, have detected differentially expressed miRNAs in patients who went on to suffer acute MI (71–73). Karakas et al. (71) found a surprisingly strong correlation of single miRNAs with the risk of cardiovascular death, although this was in a highly selected population, and was not compared with traditional risk models. No single miRNA conferred a clinically significant change in risk of acute MI in either the study by Bye et al. (72) or by Zampetaki et al. (73), but the combined usefulness of an miRNA panel improved the predictive power of traditional Framingham risk models. The miRNAs selected by Zampetaki et al. (73) also predicted mortality in a cohort of patients with symptomatic coronary artery disease (74). Mechanistic links have been reported between 2 of these miRNAs and platelet function: miR-126 (75) and miR-223 (76). Circulating miRNAs can be derived from a range of cell types, but platelets contribute substantially (75,76). Levels of circulating miRNAs are affected by the administration of antiplatelet therapy (77), and correlate with existing platelet reactivity assays in patients post-MI (75). Platelet miRNAs have been linked to hyper-reactive platelets (78).

CONCLUSIONS

MiRNAs can regulate protein expression, and so are the subject of intense interest in understanding and treating CVD. Treatment strategies currently focus on systemic anti-miR delivery, which raises concerns for off-target effects, including platelet activation (79). Future efforts should be aimed at evaluating cell-type-specific strategies or local delivery. For the cardiovascular system, enhanced targeting could be achieved through the use of adeno-associated virus vectors for cell-type-specific miRNA delivery or nanoparticle-bound anti-miRs or miRNA mimics (80).

Preclinical research has focused mainly on identifying mechanisms within a single tissue or cell type. However, caution needs to be exercised to avoid moving toward clinical evaluation too quickly. MiRNA regulation of protein expression is highly dependent on context and cell type, and their ubiquitous expression makes side effects of miRNA therapies unpredictable. Targeting individual miRNAs therefore requires meticulous evaluation of systemic effects. A careful approach in advancing miRNA therapies may slow progression toward clinical application, but may spare miRNA therapeutics a setback similar to gene therapy. The great potential of miRNAs justifies the exercise of apprehension before large-scale clinical studies for CVD.

Circulating miRNAs are expressed differentially across disease phenotypes and are implicated as novel biomarkers. Their platelet origin could make circulating miRNAs particularly relevant in the context of CVD. Platelet reactivity may confer cardiovascular risk, but there is no single accepted biomarker. More mechanistic studies and validation in larger cohorts are required to establish the clinical utility of miRNA biomarkers.

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