Review

Function and Therapeutic Potential of Noncoding RNAs in Cardiac Fibrosis

Esther E. Creemers, Eva van Rooij

Abstract: Cardiac fibrosis as a result of excessive extracellular matrix deposition leads to stiffening of the heart, which can eventually lead to heart failure. An important event in cardiac fibrosis is the transformation of fibroblasts into myofibroblasts, which secrete large amounts of extracellular matrix proteins. Although the function of protein-coding genes in myofibroblast activation and fibrosis have been a topic of investigation for a long time, it has become clear that noncoding RNAs also play key roles in cardiac fibrosis. This review discusses the involvement of microRNAs and long noncoding RNAs in cardiac fibrosis and summarizes the issues related to translating these findings into real-life therapies. (Circ Res. 2016;118:108-118. DOI: 10.1161/CIRCRESAHA.115.305242.)

Key Words: fibrosis ■ heart disease ■ microRNAs ■ noncoding RNAs ■ therapy

The extracellular matrix (ECM) forms the structural backbone of the heart and provides support for cardiomyocytes and the vasculature. Under normal conditions, the ECM coordinates the proper alignment of cardiomyocytes with neighboring cells to provide efficient mechanical and electrical coupling during contraction. However, the ECM is not merely a passive structure that supports tissue architecture, it also functions as a microenvironment to sequester growth factors and bioactive molecules required for fundamental characteristics of cells, such as proliferation, migration, and differentiation. In the healthy heart, the ECM undergoes a balanced turnover through degradation and synthesis of its constituent proteins, such as collagens, elastin, and basement membranes. However, this balance often becomes disturbed under pathological conditions of the heart, such as injury (eg, myocardial infarction [MI]) or increased loading (eg, hypertension). This misbalance results in excessive deposition of ECM, known as cardiac fibrosis, which can profoundly affect cardiac function and arrhythmias.1,2

An important event in cardiac fibrosis is the transformation of fibroblasts into a more active, smooth muscle–like contractile cell, termed the myofibroblast.3 These myofibroblasts secrete large amounts of ECM and show increased levels of migration.4 The molecular pathways that promote myofibroblast activation and fibrosis are beginning to be elucidated, uncovering a complex signaling network of growth factors, chemokines, and cytokines, such as transforming growth factor β (TGFβ), angiotensin II (AngII), connective tissue growth factor (CTGF), and endothelin-1.5 TGFβ has been identified as a central player of myofibroblast transformation and ECM remodeling (Figure). Not only the canonical TGFβ–Smad pathway appears to be involved, but there is mounting evidence that the noncanonical TGFβ signaling pathway through p38 may play a more central role.3 Compelling evidence also implicates the Ras homolog family member A–myocardin-related transcription factor–serum response factor pathway in the myofibroblast program. Ras homolog family member A signaling can be activated by multiple signals, including TGFβ or mechanical strain, and promotes the nuclear accumulation of myocardin-related transcription factor A through the reorganization of the actin cytoskeleton. In the nucleus, myocardin-related transcription factor activates serum response factor–dependent transcription by binding to CArG elements in the promoters of contractile and smooth muscle cell–specific target genes, such as smooth muscle actin and transgelin-2.6,7 Recently, Molkentin and colleagues added an entirely new signaling module to the regulation of myofibroblast differentiation because they demonstrated an obligate function of the transient receptor potential cation channel C6–calcineurin–nuclear factor of activated T-cells (NFAT) signaling in this process.8,9 Although activated fibroblasts are the main effector cells in the fibrotic heart, inflammatory cells (ie, monocytes, macrophages, lymphocytes, mast cells), vascular cells, and cardiomyocytes also contribute to the fibrotic response by secreting fibrogenic mediators.

Although studies on protein-coding genes have been the focus for many years, it turns out that protein-coding genes represent only 2% of the whole genome, whereas up to 3 quarters of the human genome is transcribed into RNA. These noncoding RNA transcripts can be categorized into small noncoding microRNAs (miRNAs) and long noncoding RNAs (lncRNAs). According to the most recent release of the miRbase...
and NONCODE databases, the human genome encodes for \( \approx 2000 \) different miRNAs and \( \approx 56,000 \) lncRNAs.\(^9,10\) In the context of heart disease, the role of miRNAs has been intensely studied, whereas the role of lncRNAs remains largely unexplored. Thousands of lncRNAs are expressed in the heart, and many of them appear dynamically regulated in the failing heart. Interestingly, the changes in expression appear to be more sensitive in discriminating heart failure of different etiologies than those of either miRNAs or mRNAs.\(^11\) Although this dynamic regulation suggests a profound biological function, to date, clear results on the function of lncRNAs in cardiac fibrosis remain lacking.

MiRNAs constitute a class of small noncoding RNA molecules that inhibit protein expression by imperfect base pairing to complementary sequences located within the 3′ untranslated region (3′ UTR) of target mRNAs. MiRNAs are encoded within the genome as intronic miRNAs, residing within introns of other genes, or as intergenic miRNAs, transcribed under the control of their own promoter. Either way, mature miRNAs originate from longer precursor RNAs, named pri-miRNAs. These pri-miRNAs may be tens of kilobases long and are further processed by enzymes and transporter proteins to small mature miRNAs, containing about 22 nucleotides. In particular, the complementarity between nucleotides 2–8 of the miRNA, called the seed region, and sequences within the 3′ UTR of a target miRNA are required for its repressive effect on protein expression. miRNAs with identical seed regions are generally grouped into miRNA families because these families likely target similar groups of transcripts. Up to 2000 different miRNAs are encoded by the human genome, and each miRNA has numerous high and low affinity targets, averaging roughly 300 conserved targets per miRNA family.\(^10,12\) By regulating the expression of multiple proteins, single miRNAs are able to exert powerful effects on cellular processes, such as growth and differentiation.\(^13\) Also in the heart, miRNAs have emerged as crucial regulators of almost every aspect of cardiac biology,\(^14\) including cardiomyocyte hypertrophy and interstitial fibrosis. Some of the key miRNAs involved in the cardiac fibrotic response are outlined below (Figure).

This review summarizes our current knowledge on miRNA function in cardiac fibrosis and the contribution of exosomal delivery of miRNAs on this process. Additionally, we will comment on the emerging landscape of lncRNA function in regulating gene expression in heart disease, and we finish by discussing the key issues with exploiting these noncoding RNAs as novel therapeutic candidates in the setting of fibrotic heart disease.

### Fibroblast-Enriched miRNAs Involved in Cardiac Fibrosis

MiR-21 is a stress-responsive fibroblast-enriched miRNA, of which its abundance increases during many different conditions of disease, including cardiac failure and remodeling.\(^15\) Its increase augments ERK-MAP (extracellular-signal-regulated kinase/mitogen-activated protein) kinase activity through inhibition of Sprouty homologue 1, thereby regulating fibroblast survival and growth factor secretion. In doing so, miR-21 was...

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**Figure.** A model for the mechanisms of action of microRNAs (miRNAs) in cardiac fibrosis. Control of extracellular matrix (ECM) turnover in cardiac fibroblasts is mediated by at least 4 signaling cascades: TGFβ-SMADs, MAPK-p38, RhoA-actin dynamics-MRTF-A, and TRPC6-calcineurin. These pathways activate the transcription factors SMADs, SRF, and nuclear factor of activated T-cells (NFAT) on promoters of ECM-related genes to control its expression. The miRNAs targeting these genes are depicted in red. MiR-21* and miR-29 can be loaded into exosomes, secreted into the extracellular space, and taken up by other cell types of the heart to influence cardiac remodeling. AngII indicates angiotensin II; MAPK, mitogen-activated protein kinase; MRTF, myocardin-related transcription factor; RhoA, Ras homolog family member A; SRF, serum response factor; TGFβ, transforming growth factor-β; and TRPC6, transient receptor potential cation channel C6. (Illustration Credit: Ben Smith.)
reported to induce cardiac fibrosis and cardiomyocyte hypertrophy, and antagonir-mediated therapeutic inhibition was able to inhibit and even reverse interstitial fibrosis in a pressure overload–induced model of heart failure.\textsuperscript{16} Some debate started after it was reported that miR-21 knockout mice show a comparable cardiac remodeling response as wild-type littermates, including cardiac fibrosis, and that miR-21 inhibition with a locked nucleic acid–modified (LNA-modified) anti-miR also failed to block the remodeling response of the heart to stress.\textsuperscript{17}

Although these results still remain unexplained, a recent report showed an additional role for miR-21 in cardiac fibrosis induced by AngII. In this particular setting, the authors revealed that the increased expression of miR-21 relies on osteopontin. LNA-mediated inhibition of miR-21 under these conditions prevented the development of cardiac fibrosis by increasing its targets PTEN (phosphatase and tensin homolog) and SMAD7.\textsuperscript{18}

Another fibroblast-enriched miRNA that has gained a lot of attention for its clear function in tissue fibrosis is miR-29. MiR-29 has shown strong repressive effects on at least 16 in vivo confirmed ECM genes.\textsuperscript{19} The miR-29 family consists of 3 members, miR-29a, -29b, and -29c, which are downregulated in the injured area of the heart after MI in mice, which correlates with an increase in expression of ECM-related genes required for infarct healing.\textsuperscript{20} Antagomir-mediated inhibition of miR-29 after intravenous injection in mice resulted in de-repression of several fibrosis-related target genes, implying a direct link between miR-29 and the expression of ECM proteins.\textsuperscript{20}

Also for vascular indications, a correlation between miR-29 and ECM was shown. MiR-29 was upregulated in 2 animal models of aortic dilation, as well as in biopsies of human thoracic aneurysms, which correlated to a profound decrease in numerous ECM components.\textsuperscript{21,22} Anti-miR-mediated inhibition of miR-29 blocked aortic dilation after AngII treatment and significantly reduces abdominal aneurysm formation by an increase in collagen expression.\textsuperscript{21-23} Although these data support therapeutic use of anti-miR-29 in various vascular indications, and short-term treatment does not appear to induce liver or kidney fibrosis,\textsuperscript{21} for more chronic treatment regimes, the potential adverse effects of increasing ECM deposition should be taken into account.

Since the downregulation of miR-29 is observed in a wide variety of diseases associated with tissue fibrosis, many studies have focused on increasing miR-29 levels in an attempt to actually block ECM deposition. Although the therapeutic increase of a miRNA still requires optimization, upregulation of miR-29 levels by either miR-29 mimics or viral delivery has already proven to have therapeutic potential in several fibrotic diseases, like kidney,\textsuperscript{24-26} liver,\textsuperscript{27-29} lung,\textsuperscript{30,31} and systemic sclerosis.\textsuperscript{32}

The miR-15 family consists of 6 evolutionarily conserved miRNAs (miR-15a, miR-15b, miR-16, miR-19b, miR-497, and miR-322), which are abundantly expressed in several cardiac cell types. The expression of multiple miR-15 family members is increased under conditions of cardiac stress or overload.\textsuperscript{15,20,33,34} Initial studies on the role of miR-15 in the heart focused on its function in cardiomyocytes. Gain- and loss-of-function studies in mice validated the miR-15 family as a regulator of cell survival and postnatal cardiomyocyte mitotic arrest by repressing a number of cell cycle genes, including checkpoint kinase 1.\textsuperscript{34,35} Inhibition of the miR-15 family induced cardiomyocyte proliferation, inhibited cardiac remodeling and fibrosis, and improved cardiac function in adult mice subjected to ischemia/reperfusion (I/R) injury.\textsuperscript{34,36}

Tijsen et al recently demonstrated that in fibroblasts, the miR-15 family counteracts the activity of the TGFβ pathway and, thereby, acts as an endogenous inhibitor of ECM remodeling.\textsuperscript{33} The miR-15 family was shown to inhibit the expression of multiple components of the TGFβ pathway, including TGFBR1, SMAD3, SMAD7, p38, and endoglin. Analysis of their 3′UTRs showed ≥1 seed-binding sequences for miR-15, with a maximum of 6 miR-15-binding sites in the 3′UTR of TGFBR1. These 3′UTRs seem to be directly targeted by miR-15 because it was shown in luciferase assays that they responded to knockdown of the miR-15 family. Inhibition of the miR-15 family in vivo, using LNA-based anti-miRs in mice subjected to transverse aortic constrictions (TAC), showed aggravated fibrosis, indicating that the miR-15 family regulates ECM remodeling.\textsuperscript{33} Increased expression of miR-15 family members in the diseased heart could suggest that it is part of an endogenous feedback mechanism to limit TGFβ activity. Why the effects on fibrosis are different for the 2 anti-miR-15 studies remain unclear, but it probably relates to the different types of stress the hearts were exposed to. Although Hullinger et al investigated the effect of miR-15 inhibition on I/R injury, Tijsen et al studied the role of miR-15 in the pressure-overloaded (TAC) heart.\textsuperscript{34} TAC induces a pure hypertrophic response of cardiomyocytes because of an increased afterload on the heart, whereas I/R damages the myocardium by the induction of oxidative stress and cell death. As a consequence, inflammation and angiogenesis are processes that are much more activated in the I/R model compared with the TAC model. Since these biological processes (hypertrophy, inflammation, and angiogenesis) interfere with fibrosis, inhibition of miR-15 may have different outcomes in terms of fibrosis in these different models. Aside from this, in the I/R model, miR-15 inhibition rendered cardiomyocytes resistant to hypoxia-induced cell death, resulting in smaller infarcts. A reduction in scar formation (ie, fibrosis), therefore, simply may have been the result of the smaller infarct size. Finally, another key difference between both studies is the difference in chemistry of the anti-miRs used. In the I/R model, tiny LNA anti-miRs were used that target the complete miR-15 family, whereas in the TAC model, LNA anti-miRs were used that only target miR-15b.

MiR-101 is expressed in cardiac fibroblasts and downregulated in hypertrophic\textsuperscript{15} and postinfarcted hearts.\textsuperscript{37} Forced overexpression of miR-101 suppresses the proliferation of cultured neonatal rat fibroblasts in vitro.\textsuperscript{37} C-fos was identified as an miR-101a target that mediates this proliferative effect through its downstream effector TGFβ. Zhao et al recently identified TGFBR1 as another direct target of miR-101a in cardiac fibroblasts.\textsuperscript{38} Interestingly, adenoviral overexpression of miR-101a revealed a remarkable improvement of cardiac performance after acute MI in rats. This was accompanied by a reduction in interstitial fibrosis and a decrease in apoptotic
cell death in the remote myocardium. These data point to miR-101 as being a potent regulator of fibroblast proliferation via a TGFβ-dependent mechanism.

Nonfibroblast miRNAs Involved in Cardiac Fibrosis

Although numerous miRNAs have been shown to regulate cardiac fibrosis in vivo, for some the mechanism of action may relate to cross talk between different cell types. These miRNAs are expressed in other cardiac cell types than fibroblasts, but have an effect on fibroblast activation by affecting the mechanical environment of the heart, for example, by alterations in cardiomyocyte hypertrophy or by exosomal delivery of miRNAs to fibroblasts.

In vertebrates, miR-1 and miR-133 are generated from a common bicistronic transcript and are both exclusively expressed in muscle cells, including cardiomyocytes for the heart. These miRNAs are fundamental regulators of heart development by balancing cardiomyocyte proliferation through their target genes HAND2, SRF, MEF2A and CCND2.40,41

MiR-1 and miR-133 are downregulated in several models of cardiac hypertrophy and heart failure, where they have been shown to function as inhibitors of cardiomyocyte hypertrophy.42 Therapeutic delivery of miR-1 in the heart, using adeno-associated viruses in a rat model of pressure overload, led to a marked regression of cardiac hypertrophy and myocardial fibrosis. This coincided with improvements in Ca2+ handling, inactivation of the mitogen-activated protein kinase signaling pathways, and inhibition of apoptosis. Fibullin-2, a secreted protein implicated in ECM remodeling, was identified as a direct target of miR-1.43 Ikeda et al demonstrated that miR-1 can repress the calcineurin-NFAT signaling pathway in cardiomyocytes by direct targeting of calmodulin.44

Given the role of calcineurin-NFAT signaling in myofibroblast activation, it will be interesting to test the function of miR-1 in this process. Also miR-133 has been shown to regulate fibrosis. In this regard, Matkovich et al showed that cardiomyocyte-specific transgenic overexpression of miR-133 attenuated pressure overload–induced fibrosis and apoptosis.45 Liu et al generated knockout mice for the 2 miR-133a genes and showed that complete loss of miR-133a resulted in embryonic lethality, which was associated with an increase in cardiomyocyte proliferation and a decrease in apoptosis. The few surviving miR-133a knockout mice showed signs of dilated cardiomyopathy and severe myocardial fibrosis.46 The mechanism may involve cross talk between cardiomyocytes and fibroblasts because in vitro studies revealed that miR-133 directly targets a secreted growth factor, CTGF in cardiomyocytes.47,48 However, in vivo, in the hearts of miR-133 transgenic mice, a downregulation of CTGF was not observed.45 This suggests that in this model, reduced CTGF expression is not responsible for the protection against myocardial fibrosis, and the underlying mechanism remains elusive. So although miR-1 and miR-133 are pivotal regulators of cardiomyocyte hypertrophy and fibrosis, the myocyte specificity of miR-1 and miR-133 indicate the effects on fibrosis to be secondary to a direct effect of the miRNAs in the heart muscle cells.

miR-208a is a cardiomyocyte-specific miRNA involved in the regulation of the myosin heavy chain (MHC) isoform switch towards βMHC during pathological stress conditions of the heart. MiR-208a knockout mice are protected from maladaptive cardiac remodeling, indicating that miR-208a is required for the stress response of the heart.49 Montgomery et al showed that therapeutic inhibition of miR-208a in the setting of heart disease evoked similar effects because systemic delivery of anti-miR-208a in Dahl salt–sensitive rats (which are susceptible to diastolic dysfunction when maintained on a high-salt diet) blocks the pathological induction of βMHC, delays the onset of cardiac dysfunction, blocks hypertrophy, and suppresses the fibrotic response.50 The actions of miR-208a appear to be (at least partially) mediated by its direct target Thrap-1/MED13, a component of the mediator complex.49 Nevertheless, because the miRNA is cardiomyocyte-specific, the effect on fibrosis is again indirect. Previous studies already implied miR-208 to regulate cardiac secretion of factors by the observation that miR-208 has an effect on total body metabolism, which can only be explained by circulating factors.51 It will be interesting to see whether the effect on fibrosis is also mediated by a secretory mechanism.

Antisense strategies to silence miR-34 and miR-199b also led to an attenuated fibrotic response during pathological remodeling of the heart. Multiple miR-34 family members are upregulated in the heart in response to stress. Systemic delivery of anti-miR-34 in mice attenuates pressure overload–induced cardiac remodeling and dysfunction, including fibrosis.52 In vitro studies by Huang et al indicated that miR-34a may function in cardiac fibroblasts by direct targeting of Smad4 and, thus, upregulating TGF-β1 signaling.53 miR-199b is another miRNA which is increasingly expressed in the failing heart and whose inhibition in vivo by antagonims attenuates cardiac hypertrophy and fibrosis. MiR-199b targets the NFAT kinase, Dyrk1a, which represses calcineurin/NFAT signaling in cardiomyocytes.54 Whether the miR-199b effect on fibrosis is indirect or whether miR-199b also stimulates calcineurin/NFAT signaling in fibroblasts will be awaited with great interest.

miR-22 is the most abundantly expressed miRNA in the heart.55 Despite its high expression, this miRNA is dispensable for cardiac development and morphogenesis because the miR-22 knockout mice do not reveal any cardiac abnormalities under basal conditions. However, upon cardiac stress (isoproterenol or TAC), miR-22 knockout mice quickly develop dilated cardiomyopathy accompanied with increased deposition of fibrotic tissue.56,55 In cardiomyocytes, many of the miR-22 target genes appear to be involved in cell metabolism. In cultured cardiac fibroblasts, where miR-22 is also expressed, it directly targets the proteoglycan osteoglycin and controls the migratory activity of fibroblasts.57 Whether this mechanism is active in vivo and whether it contributes to the observed fibrotic response in the miR-22 knockout hearts is currently unknown.

Wang et al recently showed that miR-489 antagonizes cardiac hypertrophy and fibrosis.58 By using αMHC–miR-489 transgenic mice, they show that miR-489 inhibits AngII–induced hypertrophy by direct targeting of myeloid differentiation primary response gene 88 (Myd88), which is known to be involved in cardiomyocyte hypertrophy.59 Interestingly, they also identified IncRNA cardiac hypertrophy–related factor (CHRFL), which may act as an endogenous sponge for miR-489.
The miR-132/212 family has been shown to regulate hypertrophic growth and autophagy of cardiomyocytes. Pharmacological inhibition of miR-132 by antagonir injections rescued cardiac hypertrophy and reduced the development of fibrosis in the murine TAC model. Both miR-132 and miR-212 directly target the anti-hypertrophic transcription factor forkhead-box O3, which suppresses the prohypertrophic calcineurin/NFAT signaling. It is conceivable that the reduction of fibrosis in the antagonir-132-treated TAC animals was secondary to the reduction in cardiomyocyte hypertrophy and apoptosis. Growing evidence indicates that miRNAs can transfer between cells, and miR-132 is no exception because it has recently been shown to be secreted by pericyte progenitor cells. The expression and secretion of miR-132 by transplanted human pericyte progenitor cells in the mouse heart exerts pro-angiogenic, pro-survival, and antifibrotic activity in the MI model. Interestingly, experiments with conditioned medium from these human pericyte progenitor cells on isolated murine fibroblasts confirmed a direct paracrine inhibition on fibroblast growth and on the differentiation into myofibroblasts through a miR-132-mediated mechanism. Besides the uptake of miR-132 by fibroblasts, it can also be taken up in cultured endothelial cells, where miR-132 has been shown to enhance their angiogenic activity. It is not known which cell type (ie, vascular cells, fibroblasts, or others) is most influenced by locally synthesized or exogenously produced miR-132 in vivo. Validated targets of miR-132 include Ras-GTPase activating protein and methyl-CpG-binding protein 2, both expressed in multiple cardiac cell types. Methyl-CpG-binding protein 2 has recently been shown to control an epigenetic pathway that promotes myofibroblast transdifferentiation and fibrosis of the liver.

**Exosomal Transport of miRNAs Influences Cardiac Fibrosis**

miRNAs can influence fibrosis by being expressed within a fibroblast and having a direct effect on ECM-related target genes or by being expressed in a nonfibroblast cell and indirectly influencing fibrosis by changing its host cell whereby somehow triggering the neighboring fibroblast. However, recent data suggest that intercellular delivery of miRNAs via exosomal transfer might also play a role. Exosomes are small (30–100 nm) cell-derived membrane vesicles of endocytic origin that are present in many and perhaps all biological fluids. Although they can contain DNA, mRNAs, miRNAs, and protein, several reports to date ascribe at least part of the functional effects of extracellular vesicles to the active delivery of their content to other cell types and tissues. Exosomes are released into the extracellular environment when multivesicular bodies fuse with the plasma membrane. Because the content of an exosome can vary and likely reflect the cellular origin and physiological condition of a cell, exosomes might regulate a wide variety of local and systemic cellular processes, including cardiac fibrosis.

Ischemic preconditioning has been shown to potentiate cardiac repair by an increase in cardiomyocyte survival and regeneration. Mesenchymal stem cells contribute to this repair process by the potential release of beneficial factors. MiRNA profiling of exosomes from mesenchymal stem cells exposed to ischemic preconditioning showed a significant increase in miR-22 compared with the exosomes from nonconditioned cells. Using a labeled miR-22 mimic, the authors were able to show uptake in cardiomyocytes, indicating the transfer of exosomal content into heart muscle cells. In vivo delivery of exosomes from preconditioned mesenchymal stem cells into infarcted hearts showed a strong reduction in infarct size which appeared to be dependent on miR-22 because parallel inhibition of miR-22 abrogated the protective effect. However, the mechanism by which miR-22 affects cardiac fibrosis remains to be resolved.

Intramyocardial delivery of cardiac progenitor cells (CPCs) has also been shown to be beneficial during MI. Although originally the protective effect was thought to be as a result of cell differentiation and the secretion of growth factors and cytokines, accumulating evidence now points toward extracellular vesicles as being responsible for the therapeutic effects observed. Recently, Gray et al showed a greater cardioprotective effect for exosomes coming from hypoxic CPCs versus exosomes from normoxic CPCs, which for fibroblasts translated into a lesser induction of several fibrosis-related genes. In an attempt to identify the mechanism, the authors determined the exosomal miRNA content of both normoxic and hypoxic CPC-derived exosomes which indeed appeared different based on the cellular condition of the CPCs. Mathematical modeling to determine a relationship between the treatment conditions of the CPCs, the miRNA levels and the putative biological response, resulted in the identification of miRNAs involved in tube formation (as a readout for angiogenesis), CTGF expression (as a readout for fibrosis), or both. The authors showed proof-of-principle by injecting exosomes from hypoxic CPCs into mice, inducing an improvement in function and decline in fibrosis. Although these data are intriguing and confirm the notion that miRNA transfer might contribute to the therapeutic benefits of hypoxic CPCs, follow-up experiments will have to validate a direct biological effect of the transferred miRNAs on angiogenesis or fibrosis after ischemic injury.

Although it is known that remote ischemic conditioning protects the heart from ischemic damage, more recently, it was shown that it can also attenuate cardiac remodeling in response to MI. Repeated rounds of transient limb ischemia resulted in a decrease in functional decline and less fibrosis 4 weeks after MI in rats. MiRNA analysis of the exosomal content of the animals exposed to remote ischemic conditioning before MI indicated a significant elevation in miR-29a compared with the untreated rats. Although this would fit with the antifibrotic function of miR-29, further investigation is required to show that the antifibrotic effect of remote ischemic conditioning is because of exosomal delivery of miR-29a to the heart. The link between exosomal transfer of miR-29 and ECM remodeling was also shown in the setting of exercise. Chaturvedi et al recently showed that the level of miR-29 is elevated in exosomes in response to exercise, which corresponds to a lowering in the expression of the miR-29 target MMP9. Because MMP9 regulates matrix remodeling, this could imply that the increased exosomal transfer of miR-29 regulates the beneficial effects on cardiac remodeling observed in response to exercise via an MMP9-mediated mechanism.
Although data indicate that exosomal miRNAs might have an effect on cardiac fibrosis, cardiac fibroblast themselves also appear to secrete exosomes that can influence cardiac remodeling. Although the biological relevance of the exosomes and their cargo is likely determined by the state of their donor, as well as the recipient cell, exosomes derived from a diseased heart seem to transmit a negative signal, as shown by the observation of Bang et al that cardiac fibroblasts promote cardiomyocyte hypertrophy by the exosomal transfer of miR-21*.

Analysis of the miRNA content of exosomes coming from cardiac fibroblasts showed 50 miRNAs to be present above detection cutoff with a high propensity for star (*) miRNAs (the strand of the miRNA duplex that is detected at a lower level). miR-21* was shown to be enriched in fibroblast-derived exosomes and delivered to cardiomyocytes, where it induced cardiomyocyte hypertrophy. AntagomiR-mediated inhibition of miR-21* showed a reduction in AngII-induced cardiac remodeling.

Although it remains unclear why these effects on fibrosis and hypertrophy were not observed after genetic overexpression or deletion of miR-21* (because both miRNA strands are overexpressed or deleted in the miR-21 mouse models), it is very interesting to see that fibroblasts can communicate with cardiomyocytes through exosomal transfer of miRNAs.

### IncRNAs as New Players in Cardiac Biology

It has become increasingly clear that transcription of the genome is far more extensive and complex than previously appreciated. Present estimates are that protein-coding genes make up only 1%–2% of the genome, whereas ≤3 quarters of the human genome is copied into RNA. A large portion of these RNA species is classified as IncRNAs, a heterogeneous group of noncoding transcripts longer than 200 nucleotides that do not encode for proteins. These IncRNAs are generally expressed lower than protein-coding genes and poorly conserved among species. This low evolutionary conservation and low expression has challenged the functional importance of IncRNAs, and it was initially suggested that IncRNAs are the result of transcriptional noise. Contrarily, the finding that their promoters are often highly conserved and their expression is

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### Table. miRNAs Implicated in Myocardial Fibrosis, Based on In Vivo Intervention Studies in Mice

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<td>miR-199b</td>
<td>Antagomir-199b</td>
<td>TAC</td>
<td>Reduced fibrosis</td>
<td>Dyrk1A</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>miR-22 KO</td>
<td>TAC</td>
<td>More fibrosis</td>
<td>Ogn</td>
<td>56–58</td>
</tr>
<tr>
<td>miR-489</td>
<td>CMHC-miR-489</td>
<td>AngIi</td>
<td>Reduced fibrosis</td>
<td>Myd88</td>
<td>59</td>
</tr>
</tbody>
</table>

AAV9 indicates adeno-associated viruses; AngII, angiotensin II; CTGF, connective tissue growth factor; Fbln2, fibulin-2; FoxO3, forkhead-box O3; MeCP2, methyl-CpG-binding protein 2; MHC, myosin heavy chain; MI, myocardial infarction; PTEN, phosphatase and tensin homolog; RasGAP, Ras-GTPase activating protein; SPRY, Sprouty homologue 1; TAC, transverse aortic constriction; and TGFβ, transforming growth factor-β.
generally more tissue-specific than protein-coding genes does suggest a functional relevance for at least some of them.\textsuperscript{74} In fact, functional studies have revealed that lncRNAs participate in a variety of biological processes, including transcriptional regulation by recruiting chromatin regulatory proteins to specific genomic locations, genomic imprinting, organization of protein complexes, and shaping distinct nuclear structures.\textsuperscript{75,76}

Recent RNA sequencing studies in mouse and human hearts revealed that lncRNAs are dynamically regulated during disease.\textsuperscript{11,77-79} In human hearts, a total number of 18,480 lncRNAs were detected, of which \( \approx 600 \) were differentially expressed in failing hearts with either ischemic or non-ischemic origin. Interestingly, the expression signature of lncRNAs, but not miRNAs or mRNAs, could distinguish heart failure of different etiologies. Although many lncRNAs have been identified in the heart, to date, only a small number of lncRNAs have been functionally characterized.

Two lncRNAs, MHC–associated RNA transcript (Mhrt) and CHRF (AK048451), have thus far been implicated in cardiac hypertrophy. Recently, Han et al identified an evolutionarily conserved cluster of antisense lncRNA transcripts from the MHC locus, which they named Mhrt.\textsuperscript{80} This lncRNA is robustly downregulated in various types of human failing hearts and in the mouse heart after pressure overload. Restoring Mhrt expression to prestress levels was found to protect the heart from hypertrophy and failure in the murine TAC model and identified Mhrt as a cardioprotective lncRNA. Mechanistically, Mhrt antagonizes the function of the chromatin-remodeling factor BRM/SWI2-related gene 1 by binding to its helicase domain and preventing BRM/SWI2-related gene 1 from recognizing its genomic DNA targets. BRM/SWI2-related gene 1 has previously been shown to be reactivated in cardiac stress, where it forms a complex with histone deacetylases and members of the poly(ADP-ribose) polymerase family to control pathological gene expression.\textsuperscript{81} Thus, the reactivation of BRM/SWI2-related gene 1 during cardiac stress seems to be mediated by the reduced expression of Mhrt. With regard to cardiac fibrosis, it was found that the development of fibrosis was virtually absent 6 weeks after TAC in \textit{Tnnt2-Mhrt} transgenic mice. Because Mhrt was overexpressed specifically in cardiomyocytes, this effect on fibrosis seems indirect.

CHRF was identified as an lncRNA that is upregulated in the mouse heart after AngII treatment or TAC surgeries.\textsuperscript{82} Also CHRF is an evolutionarily conserved lncRNA and is significantly upregulated in human heart failure samples. CHRF was found to induce cardiomyocyte hypertrophy and apoptosis in vitro by acting as a decoy for miR-489. Given the observation that transgenic overexpression of miR-489 antagonized cardiac fibrosis after AngII treatment, it will be interesting to explore the role CHRF in cardiac fibrosis.\textsuperscript{83}

Besides the function of lncRNAs in cardiomyocyte hypertrophy, there is also evidence showing that lncRNAs regulate cardiomyocyte metabolism,\textsuperscript{84} differentiation, and proliferation of cardiomyocytes.\textsuperscript{85} In endothelial cells and smooth muscle cells, several lncRNAs (ie, MALAT1 and SENCR) have been shown to control migration and differentiation.\textsuperscript{84,85} In fibroblasts, TERRA lncRNAs (eg, telomeric repeat-containing RNA) are implicated in regulating the structure and processing of deprotected telomeres.\textsuperscript{86,87} Deprotected telomeres elicit a DNA damage response leading to chromosome instability, which is in turn associated with cellular senescence. Abdelmohsen et al compared lncRNA expression in early-passage, young WI-38 fibroblasts with late-passage, old WI-38 fibroblasts by RNA sequencing and identified numerous senescence-associated lncRNAs.\textsuperscript{88} Among these lncRNAs, 3 transcripts were shown to be able to modulate the onset of senescence or cell viability in fibroblast cultures. It will be interesting to test these lncRNAs in a cardiac setting and investigate whether manipulation of these lncRNAs have the potential to reduce (age-dependent) cardiac fibrosis.

The noncoding repressor of NFAT is an lncRNA that was identified in a screen for noncoding RNAs able to modulate the activity of the transcription factor NFAT. Knockdown of noncoding repressor of NFAT in human embryonic kidney \textsuperscript{16} 293 cells or mouse 3T3 fibroblasts resulted in increased NFAT activity by regulating its subcellular localization.\textsuperscript{89} Given the requirement of the calcineurin/NFAT signaling pathways in the conversion of cardiac fibroblasts to myofibroblasts,\textsuperscript{2} insights into a possible function of noncoding repressor of NFAT in the cardiac fibroblasts may help us to better understand fibrogenic processes in the heart.

Finally, it has been reported that competing endogenous RNAs regulate the distribution of miRNA molecules on their targets and, thereby, impose an additional level of post-transcriptional regulation. In particular, a muscle-specific lncRNA, lnc-MD1, sponges miR-133 to regulate the expression of MAML1 (mastermind-like 1) and MEF2C (myogenic enhancer factor 2C), transcription factors that activate muscle-specific gene expression. It was found that HuR, which is under the repressive control of miR-133, is derepressed because of the splicing activity of linc-MD1 on miR-133. This study, therefore, uncovered a feedforward positive loop involving muscle transcription factors, RNA-binding proteins, miRNAs, and an lncRNA that controls the early phases of myogenesis. Interestingly, the levels of lnc-MD1 are strongly reduced in muscle cells of patients with Duchenne Muscular Dystrophy.

**Therapeutic Perspective for Noncoding RNAs**

The recent interrogation into the function of noncoding RNAs in cardiac fibrosis has not only advanced our understanding of the pathogenesis of this condition, but also provided novel targets for therapeutic intervention. Despite intense research efforts, success in developing antibiotic therapies has been limited. At present, there are no therapeutic strategies to treat cardiac fibrosis directly, except maybe inhibitors of the renin–angiotensin–aldosterone system.\textsuperscript{90} In this regard, the involvement of noncoding RNAs in ECM remodeling during heart disease poses an interesting opportunity to explore novel approaches to influence the onset and development of cardiac fibrosis.

Preclinical studies have shown that all cardiac cell types, including cardiac fibroblasts, can be targeted by miRNA inhibitors and that inhibition of miRNAs can have profound effects on cardiovascular function.\textsuperscript{91} The conservation of miRNAs, their known sequence and the fact that they are small, make them relatively easy and attractive to target in vivo.
However, because anti-miRs are designed knowing that they will affect all genes that are regulated by the miRNA, the drug will likely also regulate unrelated genes and possibly produce unexpected (potentially undesired) changes in gene expression. Another off-target effect of miRNA therapeutics can be caused by the broad tissue distribution pattern of most miRNAs. Systemic administration of an anti-miR will also inhibit the miRNA in other tissues or cell types where the anti-miR is delivered, which could lead to unwanted side effects of the therapy. Nonetheless, the potency of some miRNAs creates enough enthusiasm to pursue them in a clinical setting. Although currently no miRNA-based therapies for fibrotic indications have entered the clinic, Regulus therapeutics is actively exploring the use of anti-miR-21 in an inherited form of kidney disease caused by mutations in the type IV collagen genes, named Alport syndrome. Earlier this year, Gomez et al reported that subcutaneous delivery of anti-miR-21 in a mouse model of Alport nephropathy improved survival and improved histological end points. These results demonstrate that inhibition of miR-21 represents a potential therapeutic strategy for chronic kidney diseases, including Alport nephropathy.95

MiRNA mimics can be used to increase the level of beneficial miRNAs in settings of disease. These are often double-stranded, conjugated oligos that can be used to replace miRNAs that are lost or downregulated in disease.96 Although the development of miRNA mimic therapies has been lagging behind on the anti-miR applications, the first reports describing the therapeutic use of mimics are now appearing. MiRagen therapeutics is pursuing miR-29 for cutaneous and pulmonary fibrosis. Although miR-29 was originally discovered as a regulator of cardiac fibrosis,20 a more recent study by miRagen and collaborators showed that intravenous injection of synthetic RNA duplexes can increase miR-29 levels in vivo for several days. Moreover, therapeutic delivery of these miR-29 mimics in a mouse model of pulmonary fibrosis was able to decrease collagen expression and block and even reverse pulmonary fibrosis.31

Although both miR-21 and miR-29 were initially studied for their involvement in cardiac fibrosis, the fact that they are currently being pursued for indications in other tissues likely reflects the targeting efficiency of the heart. Systemic delivery of miRNA therapeutics does lead to cardiac targeting, but with a lower efficiency than other tissues, like liver, kidney, and lung.33,34 Delivery approaches that can increase targeting to the heart could help in using these miRNA-based therapies for cardiac fibrosis.

The biological relevance of IncRNAs has triggered interest in the therapeutic applications of IncRNA-modifying drugs. Compared with miRNAs, these noncoding RNAs appear to have a more restricted expression pattern, which would increase the specificity of the IncRNA-targeting therapies.94 Both antisense oligonucleotides and small interfering RNAs can be used to target IncRNAs by binding to and cleaving the target RNA.95 An extra hurdle for IncRNA therapeutics is that IncRNAs, unlike miRNAs, often reside in the nucleus and may therefore be less accessible to target. The lack of sequence conservation makes it difficult to use preclinical animal models to assess the effect and outcome in patients. Also functionally, IncRNAs seem more complex than miRNAs. Although miRNAs mainly regulate gene expression by targeting miRNAs, IncRNAs influence gene expression by a broad range of actions like chromatin remodeling, genomic imprinting, miRNA regulation, and organization of protein complexes. Pinpointing an exact biological function of an IncRNA to assess the effect of therapeutic regulation will likely be even more difficult than to do so for an miRNA.95

However, despite the difficulties in translating these findings to the clinic, it is to be expected that in due time, many reports will appear showing the therapeutic effects of IncRNA modulation. Feasibility of IncRNA targeting was indicated by Gutschner et al showing that subcutaneous injection of an antisense oligonucleotide against MALAT1, albeit at 50 mg/kg, 5 times a week for 5 weeks, gave a therapeutic benefit on preventing lung cancer metastasis.96

Even though there are currently no active programs pursuing noncoding RNAs for cardiac fibrosis, the lack of good treatment options and the potency of these noncoding RNAs warrants a serious pursuit of targeting these noncoding RNAs therapeutically. If we are able to overcome some of the issues related to regulating these RNAs, the potential benefit for patients suffering from cardiac fibrosis could be tremendous.

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E. van Rooij is co-founder of miRagen Therapeutics, Inc. The other authors report no conflicts.

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