REVIEW

Function and Therapeutic Potential of Non-Coding RNAs in Cardiac Fibrosis

Esther E Creemers¹ and Eva van Rooij²,³

¹Department of Experimental Cardiology, Academic Medical Center, University of Amsterdam, the Netherlands; ²Hubrecht Institute, KNAW, the Netherlands, and; ³Department of Cardiology, University Medical Center Utrecht, the Netherlands.

Running title: Non-coding RNAs in Cardiac Fibrosis

Subject Terms:
Fibrosis
Translational Studies
Gene Expression and Regulation

Address correspondence to:
Dr. Eva van Rooij
Hubrecht Institute
KNAW and University Medical Center Utrecht
Uppsalalaan 8
3584CT Utrecht
The Netherlands
E.vanrooij@hubrecht.eu

DOI: 10.1161/CIRCRESAHA.115.305242
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. While the function of protein-coding genes in myofibroblast activation and fibrosis has been a topic of investigation for a long time, it has become clear that non-coding RNAs also play key roles in cardiac fibrosis. This review discusses the involvement of microRNAs and lncRNAs in cardiac fibrosis and summarizes the issues related to translating these findings into real life therapies.

Keywords:
Heart disease, fibrosis, microRNAs, non-coding RNAs, therapy.

Nonstandard Abbreviations and Acronyms:
ECM extracellular matrix
I/R ischemia / reperfusion
LNA locked nucleic acid
LncRNA long non-coding RNA
MI myocardial infarction
miRNA microRNA
3'UTR 3'untranslated region
TAC transverse aortic constriction

INTRODUCTION

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 in order 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 (e.g. myocardial infarction) or increased loading (e.g. hypertension). This misbalance results in excessive deposition of ECM, known as cardiac fibrosis, which can profoundly affect cardiac function by increasing myocardial stiffness and impairing electrical conduction, both common risk factors for heart failure and arrhythmias1,2.

An important event in cardiac fibrosis is the transformation of fibroblasts into a more active, smooth muscle-like contractile cell, termed the myofibroblast3. These myofibroblasts secrete large amounts of ECM and show increased levels of migration4. 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-15. TGFβ has been identified as a central player of myofibroblast transformation and ECM remodeling (Figure 1). Not only the canonical TGFβ–Smad pathway appears to be involved, but there is mounting evidence that the non-
canonical TGFβ signaling pathway through p38 may play a more central role. Compelling evidence also implicates the RhoA-MRTF-SRF pathway in the myofibroblast program. Ras homolog family member A (RhoA) signaling can be activated by multiple signals, including TGFβ or mechanical strain and promotes the nuclear accumulation of Myocardin-Related Transcription Factor A (MRTF-A) through the reorganization of the actin cytoskeleton. In the nucleus, MRTF-A activates serum response factor (SRF)-dependent transcription by binding to CArG elements in the promoters of contractile and smooth muscle cell-specific target genes, such as smooth muscle actin (SMA) and transgelin-2 (SM22). Recently, Molkentin and colleagues added an entirely new signaling module to the regulation of myofibroblast differentiation, as they demonstrated an obligate function of the transient receptor potential cation channel C6 (TRPC6)-calcineurin-NFAT signaling in this process. While activated fibroblasts are the main effector cells in the fibrotic heart, inflammatory cells (i.e. 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, while up to three quarters of the human genome is transcribed into RNA. These non-coding RNA transcripts can be categorized into small non-coding microRNAs (miRNAs) and long non-coding RNAs (lncRNAs). According to the most recent release of the miRbase and NONCODE databases, the human genome encodes for ~2,000 different miRNAs and ~56,000 lncRNAs. In the context of heart disease, the role of miRNAs has been intensely studied, while 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. While 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 non-coding 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 mRNA are required for its repressive effect on protein expression. miRNAs with identical seed regions are generally grouped into miRNA families, as 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. By regulating the expression of multiple proteins, single miRNAs are able to exert powerful effects on cellular processes such as growth and differentiation. Also in the heart, miRNAs have emerged as crucial regulators of almost every aspect of cardiac biology, including cardiomyocyte hypertrophy and interstitial fibrosis. Some of the key miRNAs involved in the cardiac fibrotic response are outlined below (Figure 1).

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 non-coding 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\textsuperscript{15}. Its increase augments ERK-MAP kinase activity through inhibition of Sprouty homologue 1 (Spry1), thereby regulating fibroblast survival and growth factor secretion. In doing so miR-21 was 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 wildtype littermates, including cardiac fibrosis, and that miR-21 inhibition with an locked nucleic acid-modified (LNA-modified) antimiR also failed to block the remodeling response of the heart to stress\textsuperscript{17}.

While 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 and SMAD\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 \textit{in vivo} confirmed extracellular matrix genes\textsuperscript{19}. The miR-29 family consists of three members, miR-29a, -29b and -29c, which are downregulated in the injured area of the heart after myocardial infarction (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}. AntimiR-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, 22, 23}. While these data support therapeutic use of antimiR-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. While 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 six evolutionarily conserved miRNAs (miR-15a, miR-15b, miR-16, miR-195, miR-497, 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, 26, 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 (Chek1)\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}. DOI: 10.1161/CIRCRESAHA.115.305242
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. 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 one or more seed binding sequences for miR-15, with a maximum of six miR-15 binding sites in the 3’UTR of TGFBR1. These 3’UTRs seem to be directly targeted by miR-15, as 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 antimiRs in mice subjected to transverse aortic constrictions (TAC) showed aggravated fibrosis indicating that the miR-15 family regulates ECM remodeling. 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 two antimiR-15 studies remains unclear, but it probably relates to the different types of stress the hearts were exposed to. Whereas 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 (Tijsen, 2014 #26). TAC induces a pure hypertrophic response of cardiomyocytes due to an increased afterload on the heart, while 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 to the TAC model. Since these biological processes (hypertrophy, inflammation, 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 (i.e. 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 antimiRs used. In the I/R model, tiny LNA antimiRs were used that target the complete miR-15 family, while in the TAC model, LNA antimiRs were used that only target miR-15b.

MiR-101 is expressed in cardiac fibroblasts and down-regulated in hypertrophic and post-infarcted hearts. Forced overexpression of miR-101 suppresses the proliferation of cultured neonatal rat fibroblasts in vitro. C-fos was identified as a 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. 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.

Non-fibroblast miRNAs involved in cardiac fibrosis.

While 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. MiR-1 and miR-133 are down-regulated in several models of cardiac hypertrophy and heart failure, where they have been shown to function as inhibitors of cardiomyocyte hypertrophy. Therapeutic delivery of miR-1 in the heart, using adeno-associated viruses (AAV9) in a rat model of pressure overload, led to a marked regression of cardiac hypertrophy and...
myocardial fibrosis. This coincided with improvements in Ca\(^{2+}\) handling, inactivation of the mitogen-activated protein kinase (MAPK) signaling pathways and inhibition of apoptosis. Fibulin-2 (Fbln2), a secreted protein implicated in ECM remodeling was identified as a direct target of miR-142. Ikeda et al. demonstrated that miR-1 can repress the calcineurin-NFAT signaling pathway in cardiomyocytes by direct targeting of calmodulin mRNA. 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. Liu et al. generated knockout mice for the two 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. The mechanism may involve cross-talk between cardiomyocytes and fibroblasts, as in vitro studies revealed that miR-133 directly targets a secreted growth factor, connective tissue growth factor (CTGF) in cardiomyocytes. However, in vivo, in the hearts of miR-133 transgenic mice, a downregulation of CTGF was not observed. 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 while 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 MHC 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. Montgomery et al. showed that therapeutic inhibition of miR-208a in the setting of heart disease evoked similar effects, as systemic delivery of antimiR-208a in Dahl salt-sensitive rats (which are susceptible to diastolic dysfunction when maintained on a high-salt diet) blocks the pathological induction of MHC, delays the onset of cardiac dysfunction, blocks hypertrophy, and suppresses the fibrotic response. The actions of miR-208a appear to be (at least partially) mediated by its direct target Thr-1/MED13, a component of the mediator complex. Nevertheless, since 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. 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 antimiR-34 in mice attenuates pressure overload-induced cardiac remodeling and dysfunction including fibrosis. 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. miR-199b is another miRNA which is increasingly expressed in the failing heart, and whose inhibition in vivo by antagomirs attenuates cardiac hypertrophy and fibrosis. MiR-199b targets the NFAT kinase, Dyrk1a, which represses calcineurin/NFAT signaling in cardiomyocytes. 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. Despite its high expression, this miRNA is dispensable for cardiac development and morphogenesis, as 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. In cardiac myocytes, 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\textsuperscript{58}. Whether this mechanism is active \textit{in vivo} and whether it contributes to the observed fibrotic response in the miR-22 knockout hearts is currently unknown.

Wang \textit{et al.} recently showed that miR-489 antagonizes cardiac hypertrophy and fibrosis\textsuperscript{59}. 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\textsuperscript{60}. Interestingly, they also identified LncRNA CHRF, 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\textsuperscript{61}. 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 (FoxO3), which suppresses the prohypertrophic calcineurin/NFAT signaling\textsuperscript{61, 62}. 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, as it has recently been shown to be secreted by pericyte progenitor cells\textsuperscript{63}. The expression and secretion of miR-132 by transplanted human pericyte progenitor cells in the mouse heart exerts proangiogenic, 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\textsuperscript{63}. It is not known which cell type (i.e. vascular cells, fibroblasts or others) is most influenced by locally synthesized or exogenously produced miR-132 \textit{in vivo}. Validated targets of miR-132 include Ras-GTPase activating protein (RasGAP) and methyl-CpG-binding protein 2 (MeCP2), both expressed in multiple cardiac cell types. MeCP2 has recently been shown to control an epigenetic pathway that promotes myofibroblast transdifferentiation and fibrosis of the liver\textsuperscript{64}.

Exosomal transport of microRNAs 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 non-fibroblast cell and indirectly influencing fibrosis by changing its host 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 (30-100nm) cell-derived membrane vesicles of endocytic origin that are present in many and perhaps all biological fluids. While 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\textsuperscript{65}. Since the content of an exosome can vary and likely reflects 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 (MSCs) contribute to this repair process by the potential release of beneficial factors\textsuperscript{66}. MiRNA profiling of exosomes from MSCs exposed to ischemic preconditioning showed a significant increase in miR-22 compared to the exosomes from non-conditioned 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. \textit{In vivo} delivery of exosomes from pre-conditioned MSCs into infarcted hearts showed a strong reduction in infarct size.
which appeared to be dependent on miR-22, as 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. While originally the protective effect was thought to be due to cell differentiation and the secretion of growth factors and cytokines, accumulating evidence now points towards 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 in 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. While 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.

While it is known that remote ischemic conditioning (RIC) 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 RIC before MI indicated a significant elevation in miR-29a compared to the untreated rats. While this would fit with the anti-fibrotic function of miR-29, further investigation is required to show that the anti-fibrotic effect of RIC is due to 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. Since 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. While 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 appear 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 cut-off 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.

While it remains unclear why these effects on fibrosis and hypertrophy were not observed after genetic overexpression or deletion of miR-21* (since 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.
LncRNAs 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, while up to three quarters of the human genome is copied into RNA. A large portion of these RNA species is classified as lncRNAs, a heterogeneous group of non-coding transcripts longer than 200 nucleotides that do not encode for proteins. These lncRNAs are generally lower expressed than protein coding genes and poorly conserved among species. This low evolutionary conservation and low expression has challenged the functional importance of lncRNAs, and it was initially suggested that lncRNAs are the result of transcriptional “noise”\(^72\). Contrarily, the finding that their promoters are often highly conserved\(^73\) and their expression is generally more tissue-specific than protein-coding genes does suggest a functional relevance for at least some of them\(^74\). 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\(^75, 76\).

Recent RNA sequencing studies in mouse and human hearts revealed that lncRNAs are dynamically regulated during disease\(^11, 77-79\). In human hearts a total number of 18,480 lncRNAs were detected, of which ~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 has been functionally characterized.

Two LncRNAs, myosin heavy chain-associated RNA transcript (Mhrt) and Cardiac hypertrophy related factor (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\(^80\). This lncRNA is robustly downregulated in various types of human failing hearts and in the mouse heart after pressure overload. Restoring Mhrt expression to pre-stress 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 (Brg1) by binding to its helicase domain and preventing Brg1 from recognizing its genomic DNA targets. Brg1 has previously been shown to be reactivated in cardiac stress, where it forms a complex with histone deacetylases (HDAC) and members of the poly(ADP-ribose) polymerase family (PARP) to control pathological gene expression\(^81\). Thus, the reactivation of Brg1 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 Tnnt2-Mhrt transgenic mice. Since Mhrt was overexpressed in 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\(^59\). Also CHRF is an evolutionary 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\(^59\).

Besides the function of lncRNAs in cardiomyocyte hypertrophy, there is also evidence showing that lncRNAs regulate cardiomyocyte metabolism\(^82\), differentiation and proliferation of cardiomyocytes\(^83\). In endothelial cells and smooth muscle cells, several lncRNAs (i.e. MALAT1 and SENCR) have been shown to control migration and differentiation\(^84, 85\). In fibroblasts, TERRA LncRNAs (e.g. telomeric repeat-containing RNA) are implicated in modulating the structure and processing of deprotected
telomeres. 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. Among these lncRNAs three 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 non-coding repressor of NFAT (NRON) is an lncRNA that was identified in a screen for non-coding RNAs able to modulate the activity of the transcription factor NFAT. Knockdown of NRON in human embryonic kidney 293 cells or mouse 3T3 fibroblasts resulted in increased NFAT activity by regulating its subcellular localization. Given the requirement of the calcineurin/NFAT signalling pathways in the conversion of cardiac fibroblasts to myofibroblasts, insights into a possible function of NRON in the cardiac fibroblasts may help us to better understand fibrogenic processes in the heart.

Finally, it has been reported that competing endogenous RNAs (ceRNAs) 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 and MEF2C, transcription factors that activate muscle-specific gene expression. It was found that HuR, which is under the repressive control of miR-133, is derepressed due to the sponging activity of lnc-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 non-coding RNAs.

The recent interrogation into the function of non-coding 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 antifibrotic 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. In this regard, the involvement of non-coding 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 show 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. 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, since antimiRs 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 antimiR will also inhibit the miRNA in other tissues or cell types where the antimiR 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. While currently no miRNA-based therapies for fibrotic indications have entered the clinic, Regulus therapeutics is actively exploring the use of antimiR-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 antimiR-21 in a mouse model of Alport nephropathy improved survival and improved histological endpoints. These results demonstrate that inhibition of miR-21 represents a potential therapeutic strategy for chronic kidney diseases including Alport nephropathy.
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 down-regulated in disease. While the development of miRNA mimic therapies has been lagging behind on the antimiR applications, the first reports describing the therapeutic use of mimics are now appearing. MiRagen therapeutics is pursuing miR-29 for cutaneous and pulmonary fibrosis. While miR-29 was originally discovered as a regulator of cardiac fibrosis, 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.

While 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. Delivery approaches that can increase targeting to the heart could help in using these miRNA-based therapies for cardiac fibrosis.

The biological relevance of lncRNAs has triggered interest in the therapeutic applications of lncRNA-modifying drugs. Compared to miRNAs, these non-coding RNAs appear to have a more restricted expression pattern, which would increase the specificity of the lncRNA-targeting therapies. Both antisense oligonucleotides (ASO) and small interfering RNAs (siRNA) can be used to target lncRNAs, by binding to and cleaving the target RNA. An extra hurdle for lncRNA-therapeutics is that lncRNAs, 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 lncRNAs appear more complex than miRNAs. While miRNAs mainly regulate gene expression by targeting mRNAs, lncRNAs 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 a lncRNA to assess the effect of therapeutic regulation will likely be even more difficult than to do so for a miRNA.

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 lncRNA modulation. Feasibility of lncRNA targeting was indicated by Gutschner et al. showing that subcutaneous injection of an ASO against MALAT1, albeit at 50 mg/kg, five times a week for five weeks, gave a therapeutic benefit on preventing lung cancer metastasis.

Even though there are currently no active programs pursuing non-coding RNAs for cardiac fibrosis, the lack of good treatment options and the potency of these non-coding RNAs, warrants a serious pursuit of targeting these non-coding 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.
SOURCES OF FUNDING
EEC was supported by grants from the Netherlands Organization for Scientific Research (NWO) [grant numbers 825.13.007 and 836.12.002] and the Netherlands Cardiovascular Research Initiative [grant number CVON 2011-11]. EvR was supported by grants from the European Research Council (ERC) [grant number 615708], Trans-Atlantic Network of Excellence grant from the Leducq Foundation and the Netherlands Cardiovascular Research Initiative [grant number CVON 2014-27]

DISCLOSURES
EvR is co-founder of miRagen Therapeutics, Inc.

REFERENCES

DOI: 10.1161/CIRCRESAHA.115.305242 12


92. van Rooij E, Kauppinen S. Development of microrna therapeutics is coming of age. *EMBO molecular medicine*. 2014

DOI: 10.1161/CIRCRESAHA.115.305242
**FIGURE LEGEND**

**Figure 1. A model for the mechanisms of action of miRNAs in cardiac fibrosis.** Control of ECM turnover in cardiac fibroblasts is mediated by at least four signaling cascades: TGFβ-SMADs, MAPK-p38, RhoA-actin dynamics-MRTFA, TRPC6-Calcineurin. These pathways activate the transcription factors SMADs, SRF and 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. (Illustration Credit: Ben Smith).
Table 1. miRNAs implicated in myocardial fibrosis, based on *in vivo* intervention studies in mice

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Intervention</th>
<th>fibrosis model</th>
<th>Effect on fibrosis</th>
<th>Known targets</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-21</td>
<td>antagomir-21</td>
<td>-TAC</td>
<td>-reduced fibrosis</td>
<td>SPRY1</td>
<td>16</td>
</tr>
<tr>
<td>miR-21 KO</td>
<td>-TAC</td>
<td>-no change</td>
<td>PTEN</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>Tiny antimiR-21</td>
<td>-TAC</td>
<td>-no change</td>
<td>SMAD7</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>antimiR-21</td>
<td>-AngII</td>
<td>-reduced fibrosis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-29</td>
<td>antagomir-29</td>
<td>-normal heart</td>
<td>-Increased ECM expression</td>
<td>Collagens Elastin Fibrillin-1</td>
<td>19, 20</td>
</tr>
<tr>
<td>miR-15</td>
<td>Tiny antimiR-15</td>
<td>-MI</td>
<td>-reduced fibrosis</td>
<td>TGFBRI</td>
<td>33, 34</td>
</tr>
<tr>
<td>antimiR-15b</td>
<td>-TAC</td>
<td>-more fibrosis</td>
<td>SMAD3 SMAD7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-101</td>
<td>adenovirus miR-101a</td>
<td>-MI</td>
<td>-reduced fibrosis</td>
<td>C-fos</td>
<td>37, 38</td>
</tr>
<tr>
<td>miR-132</td>
<td>antagomir-132</td>
<td>-TAC</td>
<td>-reduced fibrosis</td>
<td>FoxO3 RasGAP MeCP2</td>
<td>61, 62</td>
</tr>
<tr>
<td>miR-1</td>
<td>AAV9 miR-1</td>
<td>-TAC</td>
<td>-reduced fibrosis</td>
<td>Fbln2 CaM</td>
<td>42, 44</td>
</tr>
<tr>
<td>miR-133</td>
<td>αMHC-miR-133a Tg</td>
<td>-TAC</td>
<td>-reduced fibrosis</td>
<td>CTGF</td>
<td>45-47</td>
</tr>
<tr>
<td>miR-133a double KO</td>
<td>-spontaneous</td>
<td>-reduced fibrosis</td>
<td>severe cardiac fibrosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-208a</td>
<td>miR-208 KO</td>
<td>-TAC</td>
<td>-reduced fibrosis</td>
<td>MED13</td>
<td>49, 50</td>
</tr>
<tr>
<td>antimiR-208a</td>
<td>-hypertension</td>
<td>-reduced fibrosis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-34</td>
<td>antimiR-34</td>
<td>-TAC</td>
<td>-reduced fibrosis</td>
<td>SMAD4</td>
<td>52, 53</td>
</tr>
<tr>
<td>miR-199b</td>
<td>antagomir-199b</td>
<td>-TAC</td>
<td>-reduced fibrosis</td>
<td>Dyrk1A</td>
<td>54</td>
</tr>
<tr>
<td>miR-22</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>αMHC-miR-489</td>
<td>-AngII</td>
<td>-reduced fibrosis</td>
<td>Myd88</td>
<td>59</td>
</tr>
</tbody>
</table>
Function and Therapeutic Potential of Non-Coding RNAs in Cardiac Fibrosis
Esther E Creemers and Eva van Rooij

Circ Res. published online November 4, 2015;
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2015 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://circres.ahajournals.org/content/early/2015/11/04/CIRCRESAHA.115.305242

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in
Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial
Office. Once the online version of the published article for which permission is being requested is located, click
Request Permissions in the middle column of the Web page under Services. Further information about this process is
available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/