Non-cardiomyocyte microRNAs in heart failure

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Received 19 August 2011; revised 8 November 2011; accepted 13 December 2011; online publish-ahead-of-print 16 December 2011

Abstract

Multiple structural changes are known to occur in a failing heart. Myocyte hypertrophy, cardiomyocyte apoptosis, interstitial fibrosis, reduced capillary density, and activation of the immune system are all involved in the pathogenesis and progression of heart failure (HF). The molecular mechanisms underlying these changes of the myocardium have been extensively studied, and many pathways involved in these processes have been uncovered. Recently, it has become evident that a novel class of small non-coding RNAs, called miRNAs, also plays a key role in these structural changes of the heart. This review summarizes the current insights on the role of miRNAs outside myocytes in the heart. Specifically, we will discuss miRNA function in fibroblasts, endothelial cells and immune cells in response to myocardial stress as occurs after myocardial infarction and in the pathogenesis of HF.

Keywords

miRNAs • Heart failure • Fibrosis • Angiogenesis • Inflammation

This article is part of the Review Focus on: The Role of microRNA in Cardiovascular Biology and Disease

1. Introduction

Heart failure (HF) is defined as a complex clinical syndrome resulting from any structural or functional disorder that impairs the ability of the ventricles to fill with or eject blood. Underlying pathologies include myocardial infarction (MI), hypertension, valvular heart disease, or inherited mutations in structural or contractile proteins. At the cellular level, HF is associated with cardiomyocyte hypertrophy, cardiomyocyte apoptosis, and changes in the expression of genes regulating energy metabolism, calcium handling, and genes normally expressed in the embryonic heart. Besides these well-described changes in cardiomyocytes, also non-myocytes such as fibroblasts and endothelial cells (ECs), which together account for ~70% of the total number of cardiac cells, are intrinsically involved in the remodelling process that ultimately leads to HF.

MicroRNAs (miRNAs) are ~22 nucleotide long, non-coding RNA sequences that inhibit translation or promote degradation of specific mRNA transcripts by binding to their 3′-untranslated region (3′-UTR). In the human genome, the estimated number of miRNAs is as high as 1000, and their predicted target genes are involved in virtually every biological process. Early evidence that miRNAs are involved in heart disease is derived from gene-expression studies, which revealed that miRNAs are differentially expressed in rodent and human HF. The importance of miRNAs in the heart was first demonstrated by cardiomyocyte-specific deletion of Dicer, an essential component of the miRNA biogenesis pathway. Mice, in which miRNA biogenesis was ablated specifically in cardiomyocytes, died due to HF and exhibited interstitial fibrosis, ventricular dilation with thinned walls, and impaired cardiac function. More recently, several loss-of-function studies in mice (using gene-targeting strategies and oligonucleotide-based inhibitors) firmly established that miRNAs control a variety of cellular processes essential to the heart, including cardiomyocyte hypertrophy and apoptosis, proliferation and activation of fibroblasts, angiogenesis, and possibly also inflammation.

In this review, we summarize the current insights in the function of non-myocyte miRNAs in the heart. Specifically, we will discuss miRNA function in fibroblasts, ECs, and inflammatory cells in the development of HF. We will not discuss miRNA function in smooth muscle cells (SMCs), as there is currently no evidence that links SMC-derived miRNAs to cardiac biology or HF.

2. miRNAs in cardiac fibrosis

Cardiac fibrosis, the excessive accumulation of extracellular matrix (ECM) proteins in the interstitium and perivascular regions of the myocardium, is a hallmark of maladaptive hypertrophy and HF. It is associated with disruption of normal myocardial structures and increased mechanical stiffness, which together contribute to contractile dysfunction of the heart. Fibrosis can also disturb the electrical continuity between cardiomyocytes, leading to conduction slowing and facilitating the occurrence of arrhythmias.

Fibroblasts are responsible for the synthesis of ECM components, both in the healthy heart, as well as in pathological fibrosis. In the
stressed myocardium, fibroblasts differentiate and become active (called myofibroblasts) in response to cytokines and growth factors such as transforming growth factor-β (TGF-β). Activated (myo)fibroblasts proliferate, migrate, and remodel the cardiac interstitium by modulating the secretion of ECM components and matrix metalloproteinases (MMPs), i.e. the enzymes capable of degrading the ECM. Signalling cascades that control ECM synthesis, ECM degradation, and fibroblast proliferation and apoptosis involve SMADs, Rho/Rock, erk-related tyrosine kinase- mitogen activated protein (ERK-MAP), and PI3K/Akt signalling pathways. Thus far, four miRNAs have been implicated in the regulation of cardiac fibrosis: miR-21, miR-29, miR-30, and miR-133. The specific components of the signalling pathways that are targeted by those miRNAs are shown in Figure 1.

### 2.1 miR-21

While miR-21 is expressed in all cell types of the cardiovascular system (cardiomyocytes, vascular SMC, and ECs), its expression is most prominent in cardiac fibroblasts and rather weak in cardiomyocytes. Interestingly, this miRNA is one of the most dynamically regulated miRNAs in response to cardiac stress, as it was found to be 5–10-fold upregulated in mouse models of hypertrophy and in human HF. By in situ hybridization, Thum et al. showed that the increase in miR-21 expression in failing hearts is mainly attributed to fibroblasts. Davis et al. investigated the molecular pathways underlying bone morphogenetic protein (BMP)- and TGF-β-mediated induction of miR-21 in SMCs and showed that the expression of miR-21 is strongly regulated at the post-transcriptional level by SMAD proteins. siRNAs against R-SMADs dramatically decreased the induction of mature miR-21, without affecting the levels of pri-miR-21, suggesting that BMP and TGF-β signalling promote the processing of primary transcripts into mature miR-21. Functional studies revealed that the maturation of miR-21 by SMADs resulted from a functional interaction of SMADs with the Drosha microprocessor complex. Considering the importance of TGF-β-signalling in fibroblasts, it will be highly interesting to know whether this SMAD-dependent maturation mechanism as seen in SMCs, is also responsible for the dramatic upregulation of mature miR-21 in cardiac fibroblasts in the diseased heart.

#### 2.1.1 Inhibition of miR-21 in vivo
Chemically modified antisense oligonucleotides are widely used to study miRNA function. These ‘antimiRs’ harbour the full or partial complementary reverse sequence of the mature miRNA and are able to silence endogenous miRNA levels. The chemistries applied to these antimiRs range from various backbones and sugar modifications such as 2′-O-methyl (2′-OMe), 2′-fluoro (2′-F), locked nucleic acids (LNAs), and phosphothioate linkages between oligonucleotides to cholesterol conjugations. These chemical modifications enhance cellular uptake, increase thermostability of the miRNA/antimiR duplex, and protect against degradation by RNAse. The two

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**Figure 1** Control of cardiac fibrosis by miRNAs and their target genes. Four signalling cascades—SMADs, Rho/Rock, ERK-MAP, and PI3K/Akt (depicted in dark brown)—control ECM turnover in cardiac fibroblasts by influencing processes such as ECM synthesis, ECM degradation, and apoptosis. These signalling cascades are depicted together with the miRNAs and their direct targets that control ECM turnover. Spry1, sprouty homologue 1; PTEN, phosphatase and tensin homologue; CDC42, cell division cycle 42; MMP2, matrix metalloproteinase 2; CTGF, connective tissue growth factor; TGF-β, transforming growth factor-β.
most widely used chemistries for in vivo inhibition of miRNAs in the cardiovascular field are antagonirs and LNA-based antimiRs. Antagonirs are fully complementary to the mature miRNA sequence and are conjugated to cholesterol to promote cellular uptake.\textsuperscript{19} LNA-based antimiRs are either partly or fully complementary to the miRNA and the LNA modification leads to a thermodynamically strong duplex formation with the miRNA. There are some interesting differences between antagonirs and antimiRs. Firstly, antagonirs seem to be one of the few oligonucleotides that truly reduce miRNA levels by degradation of the mature miRNA.\textsuperscript{19} On the other hand, antimiRs seem to inhibit miRNA activity by the formation of a very stable complex with the mature miRNA, visualized by a slower migrating complex on northern blot assays.\textsuperscript{20,21} Secondly, despite effective binding of the miRNA by both antagonirs and anti-miRs, the LNA-modified antimiRs have been shown to require a lower dose.\textsuperscript{18}

The biological role of miR-21 in the heart has recently been uncovered by Thum et al.\textsuperscript{7} by knocking down miR-21 using antagonirs, in a transverse aortic constriction (TAC) model of hypertrophy and HF in mice. They showed that inhibition of miR-21 protected mice against cardiac fibrosis and attenuated hypertrophy and cardiac dysfunction in response to TAC. One of the genes directly targeted by miR-21 in fibroblasts is a negative regulator of the ERK-MAP kinase pathway: Sprouty homology 1 (Spry1). This mechanism was shown to involve FGF secretion and fibroblast survival, which are both important regulators of interstitial fibrosis.\textsuperscript{9} In another study, Roy et al.\textsuperscript{22} revealed that miR-21 induces MMP2 expression in the fibroblasts after ischaemia reperfusion in mice. This was mediated by direct targeting of phosphatase and tension homologue (PTEN), a negative regulator of the phospho-insitol 3-kinase (PI3K)-Akt signalling pathway. Whether this miR-21-mediated upregulation of MMP2 results in enhanced ECM degradation was unfortunately not investigated.\textsuperscript{22} Regulation of fibrosis by miR-21 was supported by three independent groups, studying the function of miR-21 in models of pulmonary and renal fibrosis.\textsuperscript{23–25} Together, these studies indicate that antagonirs against miR-21 may be used as therapy against fibrosis of multiple organs.

However, the role of miR-21 in cardiac fibrosis seems to be more complicated, as was revealed in a recent study by Patrick et al.\textsuperscript{26} They showed that neither genetic deletion of miR-21, nor tiny LNA-dependent oligonucleotides, studying the function of miR-21 in models of multiple organs. Whether this miR-21-mediated upregulation of MMP2 results in enhanced ECM degradation was unfortunately not investigated.\textsuperscript{22} Regulation of fibrosis by miR-21 was supported by three independent groups, studying the function of miR-21 in models of pulmonary and renal fibrosis.\textsuperscript{23–25} Together, these studies indicate that antagonirs against miR-21 may be used as therapy against fibrosis of multiple organs.

The differences in biological effects between the 22-mer cholesterol-modified antagonir-21 of Thum et al.\textsuperscript{9} and the 8-mer LNA-based antimiR-21 of Patrick et al.\textsuperscript{20} probably relate to the differences in chemistry. Although both Thum et al.\textsuperscript{9} and Patrick et al.\textsuperscript{20} convincingly showed efficient miR-21 knockdown by northern blot, qPCR, and target regulation of PDCD4, there may be differences in potency between the inhibitors. Thum et al. subsequently carried out a direct comparison of three different oligonucleotide chemistries directed against miR-21 (22-mer cholesterol-conjugated antagonirs, 22-mer fluoro-modified antimiRs and 8-mer LNA-modified antimiRs), and found that the 8-mer was less efficacious in repressing miR-21 than either one of the 22-mers.\textsuperscript{27} Nevertheless, two groups did show an efficient downregulation of miR-21 using 8-mer oligonucleotides.\textsuperscript{20,28} The cause of this discrepancy is not known but may relate to many factors like (i) the technique to measure knockdown (northern blot and qPCR vs. target regulation), (ii) controls used (missmatched oligonucleotide controls vs. PBS), (iii) subtle differences in 8-mer chemistry between the two studies, (iv) cholesterol-conjugated antagonirs may be more effective than LNA-antimiRs in inhibiting miR-21 function in fibroblasts. Theoretically, it is conceivable that the cholesterol component in cholesterol-conjugated oligonucleotides may have cardioprotective or antifibrotic effects. This is however rather unlikely as the fluoro-modified antimiR-21, which was not linked to cholesterol also showed attenuated cardiac fibrosis.\textsuperscript{27}

In conclusion, the above studies illustrate that the different oligonucleotide chemistries used in different studies may introduce confounding variables. In the future, it will therefore be important to understand the effect of the different oligonucleotide chemistries. Another current limitation in the field is the lack of potential to direct the oligonucleotides to specific cell types. Future studies developing delivery methods to alter miRNA expression in particular cell types within the myocardium will be important to advance the field. Finally, there is also limited insight into the importance of the quantity of agent delivered to target cells and it will be crucial to explore dose–response effects of the different oligonucleotides.

Besides a possible function of miR-21 in fibroblasts, others have revealed anti-apoptotic effects of this miRNA in cardiomyocytes. Responsible target genes include PDCD4, PTEN, and Fas ligand (FasL).\textsuperscript{13,29} This anti-apoptotic effect is confirmed in vivo, in a rat model of ischaemia preconditioning-mediated cardiac protection against ischaemia/reperfusion injury, where inhibition of miR-21 (using cholesterol-conjugated antagonirs) attenuated the protective effect by augmenting apoptosis.\textsuperscript{30} On the contrary, overexpression of miR-21 (using αMHC transgenic mice or adenovirus in rat) protected against cardiac ischaemia injury by a reduction in apoptosis.\textsuperscript{29,30} Interestingly, TAC surgery in these αMHC-miR-21-transgenic mice did not reveal a function in cardiomyocyte hypertrophy,\textsuperscript{31} suggesting that miR-21 is not able to repress cardiomyocyte hypertrophy in a cell-autonomous manner, possibly because its direct target gene Spry1 is not expressed in cardiomyocytes.\textsuperscript{9}

Distinct biological functions of miR-21 in fibroblasts and cardiomyocytes raise the question if long-term silencing of miR-21 would have positive or negative therapeutic effects in HF. On the one hand, miR-21 silencing has been shown to blunt cardiac fibrosis by augmenting expression of its target gene Spry1 in fibroblasts, while on the other hand miR-21 silencing may result in an increase in cardiomyocyte apoptosis. Genetic deletion of miR-21 in mice did not reveal an obvious role for miR-21 in pathological cardiac remodelling after MI and TAC surgeries. Unfortunately, cardiomyocyte apoptosis has not yet been analysed in miR-21 null mice.

Non-cardiomyocyte microRNAs in heart failure

575
2.2 miR-29
The miR-29 family is composed of three members, miR-29a, b and c, which differ only by one to two nucleotides. In the heart, the miR-29 family is mainly expressed in fibroblasts and is found to be downregulated in the mouse heart in response to TAC, chronic calcineurin signalling, and in the viable myocardium after MI. In cultured cardiac fibroblasts, miR-29 was downregulated after TGF-β stimulation, suggesting that the decrease in miR-29 in the pathological remodelling of the heart is mediated by TGF-β.
Van Rooij et al. were the first to show that the miR-29 family directly targets a multitude of ECM genes such as col1a1, col3a1, elastin and fibrillin. The reported downregulation of miR-29 in several cardiac pathologies suggests that this loss may actually contribute to the development of cardiac fibrosis, by relieving the repression on ECM gene expression. Knockdown of miR-29 using antagonirs in the healthy mouse heart resulted in increased expression of ECM genes at the mRNA level, but it is currently not known whether this is sufficient to induce excessive fibrosis.
miR-29 is also linked to fibroblast survival. In a screen for miRNAs that are able to modulate p53 activity in NIH3T3 cells, Park et al. found that the miR-29 family induces apoptosis through targeting of CDC42 (a Rho family GTPase) and p85α (the regulatory subunit of PI3K), both of which are known to negatively regulate p53. In the diabetic kidney, miR-29 was shown to directly target the miR-21 target gene, Spry1 and promote activation of Rho kinase. Silencing of miR-29 in this model prevented high glucose-induced apoptosis and fibronectin matrix assembly.
Together these data indicate that miR-29 acts as a regulator of cardiac fibrosis via direct repression of a multitude of ECM genes and possibly also by inducing apoptosis of fibroblasts. Therapeutically, it will be very interesting to test whether overexpression of miR-29 in the heart, using miRNA mimics would be sufficient to prevent or regress pathological fibrosis.

2.3 miR-133 and miR-30
Several groups have reported an anti-hypertrophic effect of miR-133a. In specific, antagonist studies and adenoviral overexpression in mouse TAC studies have been shown to influence left ventricular (LV) weight, wall thickness, cardiomyocyte hypertrophy, and re-expression of the foetal gene programme. Confirmed targets of miR-133 which may be responsible for this anti-hypertrophic effect include calcineurin, NFATc4, SRF, RhoA, CDC42, and Whsc2.
Although miR-133a is exclusively expressed in cardiomyocytes and not in fibroblasts, this miRNA has been shown to regulate cardiac fibrosis. This probably involves a paracrine mechanism, as miR-133 has been shown to control the expression and secretion of the profibrotic growth factors, connective tissue growth factor (CTGF), and TGF-β. In vivo, cardiomyocyte-specific overexpression of miR-133a in mice resulted in decreased levels of apoptosis and fibrosis after TAC, together leading to improved diastolic performance.
Liu et al. generated a knockout mouse for the two miR-133a genes, and showed that complete loss of miR-133a results in late embryonic lethality due to ventricular septum defects, accompanied by an increase in cardiomyocyte proliferation (by direct targeting of CCND2) and a decrease in apoptosis. The 24% of surviving knockout mice showed signs of dilated cardiomyopathy with thinning of the ventricular walls, reduction of cardiac contractility, and interestingly, extensive fibrosis.

In addition to the control of CTGF expression by miR-133, in vitro studies from our laboratory have shown that members of the miR-30 family also bind to the 3′-UTR of CTGF to inhibit its expression. In contrast to miR-133, miR-30 is highly expressed in cardiac fibroblasts, indicating the putative involvement of this miRNA in the control of cardiac fibrosis. Nevertheless, in vivo studies are warranted to confirm whether cardiac fibrosis can be modulated by loss- or gain-of-function of miR-30.
Both miR-133a and members of the miR-30 family are found to be consistently downregulated in rodent and human hypertrophy and HF. The picture emerges that in the healthy heart, high levels of miR-133 and miR-30 are required to balance ECM turnover, by keeping CTGF and TGF-β protein at low levels. During pathological remodelling of the heart, when the expression of miR-133 and miR-30 (but also of miR-29) is decreased, the repression of profibrotic genes is relieved, resulting in enhanced ECM synthesis and fibrosis. Normalization of the expression of these down-regulated miRNAs, by treatment with miRNA mimics may offer an attractive approach to counteract cardiac fibrosis.

3. miRNAs in endothelial cell function
ECs of the heart play a crucial role in regulating and maintaining cardiac function. Besides their apparent role in blood supply and angiogenesis, ECs of the heart release a variety of factors, such as nitric oxide (NO), endothelin, prostaglandins, and angiotensin II, which directly influence cardiac metabolism, growth, and contractile performance of the heart.
Endothelial dysfunction in the heart has been described in diabetes, after MI, ischaemia-reperfusion injury, and in failing hearts. Furthermore, pathological hypertrophy but not physiological hypertrophy as seen in exercise-trained rat hearts, is characterized by a reduction in capillary density. There are various reports which indicate that when growth of the microvasculature does not keep pace with the extent of cardiomyocyte hypertrophy, myocardial hypoxia will cause myocardial dysfunction. This was illustrated in a model of pressure overload-induced hypertrophy, where blockade of VEGF reduced capillary density and accelerated HF. Thus, the balance between cardiac growth and angiogenesis is a critical determinant in the development of HF.

The importance of miRNAs in angiogenesis and EC function was recently revealed in conditional knockout mice for Dicer. In this mouse model, endothelial-specific deletion of Dicer resulted in a reduced angiogenic response to exogenous VEGF, limb ischaemia, and would healing. Specific miRNAs, such as miR-126, the miR-17~92 cluster, and the miR-23~27~24 cluster have subsequently been shown to regulate EC function. The components of the signalling pathways that are targeted by those miRNAs are shown in Figure 2.

3.1 miR-126
miR-126 is the only miRNA identified thus far that shows EC-specific expression. It is encoded by an intron of the EGF-like-domain multiple 7 (Egfl7) gene, a chemotactant and inhibitor of SMC migration, which is secreted by ECs. miR-126 deficiency in mice and zebrafish revealed that miR-126 acts as a pro-angiogenic factor. In mice, miR-126 deficiency results in late embryonic or neonatal
death in 40% of embryos with severe systemic oedema, multifocal haemorrhages and ruptured blood vessels. This indicates that this miRNA is not absolutely required for differentiation of ECs and embryonic vessel formation, but rather for the maintenance of vascular integrity and postnatal angiogenesis.

Subjection of the surviving miR-126 null mice to MI resulted in increased mortality due to ventricular rupture and defective cardiac neovascularization after MI. The pro-angiogenic action of miR-126 was mediated by enhancing the actions of VEGF and FGF. Direct target genes of miR-126 include negative regulators of these signalling pathways, sprouty-related EVH1 domain containing 1 (Spry1), phophoinositide-3-kinase regulatory subunit 2 (PIK3R2), p21-activated kinase 1 (PAK1), and vascular cell adhesion protein 1 (VCAM-1).

3.2 miR-17~92 cluster

The miR-17~92 cluster encodes seven mature miRNAs—miR-17-5p/3p, miR-18a, miR-19a/b, miR-20a, and miR-92a—which are all transcribed as one common primary transcript. In the heart, the miR-17~92 cluster is expressed in ECs and cardiomyocytes. The exact function of miR-17~92 is cell-type-dependent and in ECs, the cluster is assigned anti-angiogenic properties.

Bonauer et al. showed that forced overexpression of miR-92a in cultured ECs blocks sprout formation in a three-dimensional model of angiogenesis, inhibited vascular network formation in matrigel assays, and reduced EC migration. No effect was found on proliferation or viability of ECs. In mouse models of MI, where neangiogenesis is of crucial importance to restore blood flow to the injured myocardial wall, systemic injections of an antagoni-miR-92a led to enhanced blood vessel growth in the border zone of the infarct. In addition, inhibition of miR-92a after MI suppressed the number of apoptotic cells, reduced the infarct size, and improved cardiac function.

An identified target gene that may regulate the anti-angiogenic function of miR-92a is integrin subunit α5 (ITGA5). Besides the anti-angiogenic properties of miR-92a, subsequent studies from the same laboratory revealed that all other members of this miRNA cluster also exhibit anti-angiogenic properties. miR-17 and miR-20 appear to have the most profound effects in vitro and combined systemic inhibition by antagomir-17/20 promoted neovascularization in vivo.

In contrast to the observed anti-angiogenic properties of the miR-17~92 cluster in ECs, others have reported that the cluster promotes angiogenesis in tumour cells. Overexpression of the entire cluster in myc-induced tumours increased angiogenesis by a paracrine mechanism, involving downregulation of thrombospondin-1 and CTGF, anti-angiogenic factors that are secreted by tumour cells and which turned out to be direct target genes of miR-18 and miR-19. These two miRNAs have also been shown to regulate CTGF and thrombospondin-1 in the ageing heart.

Knockout mice for miR-17~92 with lung hypoplasia, immune defects, and a ventricular septal defect die shortly after birth. Although the precise role of miR-17~92 members in postnatal angiogenesis is not clear, this study does indicate that the cluster is not
required for differentiation of ECs and vessel formation during development. It will be interesting to use this mouse model to conditionally delete miR-17~92 in ECs in models of pathologic cardiac remodeling to study postnatal angiogenesis.

### 3.3 miR-21

Besides the earlier described role of miR-21 in cardiac fibroblasts and myocytes, miR-21 is also expressed in cardiac ECs. Sabaté et al. performed angiogenic assays in Human Umbilical Vein ECs and observed that miR-21 acts as a negative modulator of angiogenesis, by reduced EC proliferation, migration and the ability of these cells to form tubes. The Rho-GTPase RhoB was identified as the target responsible for the anti-angiogenic effects. Interestingly, the previously confirmed target of miR-21, spry1, which has been implicated in ECM deposition by fibroblasts, is also expressed in ECs, where it has been shown to negatively regulate angiogenesis. In human angiogenic progenitor cells, which play a key role in endothelial regeneration and vascular homeostasis, miR-21 was found to control the oxidative stress defence. Overexpression of miR-21 in these progenitor cells resulted in increased oxidative stress and impaired migratory capacity in response to NO synthase inhibitors, whereas miR-21 inhibition blocked these effects. In these angiogenic progenitor cells, miR-21 reduced the expression of superoxide dismutase 2, a key enzyme in the oxidative stress defence. In addition, overexpression of miR-21 led to oxidative stress by ERK/MAP activation via inhibition of its direct target gene Spry2.

In the heart, adenovirus-mediated overexpression of miR-21 in rats decreased infarct size at 24 h after MI and decreased dilatation of the LV at 2 weeks after MI. This protective effect of miR-21 against ischaemia-induced cardiac cell death was attributed to reduced cardiomyocyte apoptosis, due to downregulation of its target gene PDCD4. However, as this adenoviral approach presumably also increased miR-21 expression in ECs of the ischaemic myocardium, it should be taken into consideration that the improved LV remodelling may have resulted, at least partly from enhanced angiogenesis.

### 3.4 miR-23~27~24 cluster

The miRNAs generated by the two miR-23~27~24 clusters are highly expressed in ECs and correspondingly, in highly vascularized tissue. Fiedler et al. recently reported that miR-24 is enriched in ECs and upregulated after cardiac ischaemia. Overexpression of miR-24 induced EC apoptosis, abolished capillary network formation on Matrigel, and inhibited cell sprouting. Blocking of miR-24 in a mouse model of MI limited infarct size by preventing EC apoptosis and enhancement of vascularity, which led to preserved cardiac function and survival. The effects were mediated through direct targeting of the endothelial-enriched transcription factor GATA-2 and the p21-activated kinase PAK4. These findings, which indicate that miR-24 acts as a critical regulator of EC apoptosis and angiogenesis, are in sharp contrast with the study by Qian et al. who reported that miR-24 is highly expressed in cardiomyocytes and fibroblasts, but not detectable in ECs of the heart. In this study, overexpression of miR-24, by local delivery of miR-24 mimics in a mouse model of MI, resulted in the inhibition of cardiomyocyte apoptosis, a reduced infarct size, and an improved LV function. The anti-apoptotic effect on cardiomyocytes was partially mediated by direct repression of the BH3-only domain containing protein Bim, which is known to induce apoptosis. The contrasting conclusions of these two studies, where both overexpression and knockdown of miR-24 in a mouse model of MI-reduced infarct size and preserved LV function, may have resulted from the different time points after MI at which LV remodelling was evaluated and from different chemical modifications or dosing strategies of the oligonucleotides used. However, these studies, together with the different observations of miR-21 inhibition in fibrosis, also point to the gaps in our understanding regarding the precise mechanisms of miRNA modulation by oligonucleotide-based targeting strategies.

The other two members of the cluster, miR-23 and miR-27, were reported to have pro-angiogenic properties, as was illustrated in a model of retinal angiogenesis, where vascularization is inhibited by knockdown of both miRNAs. The angiogenic properties of these miRNAs are mediated by their direct targets sprouty2 and sema6A, which results in a decrease of AKT and ERK1/2 phosphorylation. It is currently unknown whether these miRNAs also have pro-angiogenic properties in the heart.

### 3.5 miR-210

miR-210 is a miRNA shown to be upregulated by hypoxia in all cell types tested to date. Overexpression of miR-210 in ECs increased angiogenesis and VEGF-induced cell migration, while LNA-based antimiR-210 treatment inhibited tube formation and cell migration in response to hypoxia and VEGF. To test the pro-angiogenic potential of miR-210 in the ischaemic heart, Hu et al. tested whether miR-210 overexpression could enhance angiogenesis and rescue cardiac function after MI. Indeed, intramyocardial injections with a minicircle vector carrying miR-210 in a mouse model of MI resulted in smaller infarct sizes, less apoptosis, increased capillary density, and better LV function. Direct miR-210 targets involved in the angiogenic and apoptotic function are Ephrin-A3, Caspase 8-associated protein 2, and Ptp1b. In conclusion, miR-210 may represent a novel therapeutic approach for the treatment of ischaemic heart disease.

### 3.6 Other miRNAs

Other miRNAs that have been implicated in EC function, but which have not been studied yet in heart disease include miR-221/-222, miR-218, and miR-100.

miR-221 and miR-222 are highly expressed in ECs, where they have been shown to reduce proliferation and migration in vitro and in implanted matrigels. Recently, an additional level of control in the Slit-Robo signalling pathway by miR-218 was revealed. The Slit-Robo pathway is known to regulate angiogenesis and to contribute to the stability of the vascular network. Small et al. revealed that the activity of the Robo receptors depends on miR-218, which is encoded by an intron of the Slit2 and Slit3 genes, and which directly targets Robo1 and Robo2. Knockdown of miR-218 resulted in abnormal EC migration and reduced vascularization of the retina. Future studies are warranted to unravel a possible function of this pathway in angiogenesis in the ischaemic heart.

miR-100 modulates proliferation, tube formation, and sprouting activity of ECs. Knockdown of miR-100 in a hind-limb ischaemia model in mice stimulated angiogenesis and resulted in functional improvement of perfusion after femoral artery occlusion. The anti-angiogenic function of miR-100 was mediated by direct repression of mammalian target of rapamycin (mTOR). miR-100 is reported to be upregulated in human HF and it thereby may contribute to the reduced capillary density in HF.
4. miRNAs with a role in inflammation

In patients with congestive HF, a chronic activation of the innate immune system is often observed, as evidenced by increased numbers of T lymphocytes and macrophages in the myocardium and increased proinflammatory cytokine levels (i.e. TNF-α, IL-1β, and IL-6). The levels of these cytokines have been shown to correlate with the severity of symptoms in HF patients. Cytokines are produced and secreted by cells of the immune system and, interestingly, also by cardiomyocytes after reperfusion of ischaemic regions, in haemodynamic overload, or after a systemic inflammatory insult. Activation of a signalling pathway involving Toll-like receptors (TLRs) and the downstream transcription factor nuclear factor-kappaB (NF-κB) has been shown to control the production and secretion of pro-inflammatory cytokines in the heart. These cytokines attract macrophages and natural-killer cells and may exert direct toxic effects resulting in cardiomyocyte apoptosis, disturbances of ECM structures and endothelial dysfunction, which may eventually contribute to the structural and functional deterioration of the failing heart.

Three miRNAs known for their role in inflammation—miR-146, miR-155, and miR-21—are upregulated in mouse models of MI and TAC. This may implicate that these miRNAs are involved in the pathogenesis of HF by influencing the inflammatory response. The components of the signalling pathways that are targeted by these miRNAs are shown in Figure 3.

4.1 miR-146

miR-146 has been shown to be upregulated in inflammatory disorders such as osteoarthritis and rheumatoid arthritis, but also in many cancers. In immune cells, miR-146 is rapidly induced upon lipopolysaccharides (LPS) exposure and this induction is mediated by TLR and NF-κB. Further studies showed that miR-146 exerts an anti-inflammatory function by inhibition of TLR4 and downstream cytokine signalling and interestingly, two downstream mediators of TLR signalling—IRAK1 and TRAF6 were identified as direct target genes of miR-146. This indicates that miR-146 provides a negative feedback loop, in which NF-κB activation induces the expression of miR-146, which in turn represses IRAK1 and TRAF6 protein levels to reduce NF-κB activity.

Besides the abundant expression of miR-146 in immune cells, this miRNA is also highly expressed in the heart and upregulated after TAC and MI. This upregulation seems mediated, at least partly by an influx of inflammatory cells, as evidenced by the increase of miR-146 in the cardiomyocyte-specific Dicer-deficient mice, which show signs of inflammation. miR-146 has also been detected in cardiomyocytes, where it was reported to mediate doxorubicin-induced cardiomyocyte death via its direct target ErbB4. Interventions studies (knockout mice or antimiRs) are warranted to determine the function of this miRNA in the pathogenesis of HF.

4.2 miR-155

miR-155 is abundantly expressed in monocytes, T-cells, and B-cells, where it is induced upon exposure to a variety of inflammatory cytokines. miR-155 is considered a pro-inflammatory miRNA, as was evidenced by the miR-155 knockout mice. These mice are immunodeficient and highly resistant to experimentally induced autoimmune encephalomyelitis. miR-155 targets that have been identified include IKKe, a component of the TLR-pathway that activates NF-κB, and FADD, Ripk1 and PU.1. In the heart, miR-155 is found to be upregulated after MI, in the border zone of the infarct and in the remote myocardium. This induction most probably originates from inflammatory cells that invade the myocardium, since in the absence of cardiomyocyte-derived miRNAs, in the Dicer knockout mice, miR-155 levels still increase in HF. Martin et al. recently identified the angiotensin II type 1 receptor (AT1R) as a miR-155 target gene in vascular SMCs, which may also be of relevance to cardiac biology.

In conclusion, a role for miR-155 in (inflammatory) heart disease has not been reported to date, and therefore, TAC and MI interventions in the miR-155 knockout mice are awaited with great interest.

4.3 miRNA-21

In addition to the expression in fibroblasts, miR-21 is also expressed in immune cells, where it is induced upon LPS exposure. miR-21 protein levels in peripheral blood mononuclear cells, miR-21 regulates its direct target PDCl4, which is also responsible for the anti-apoptotic function of
miR-21 in cardiomyocytes (see section 2.1). However, in peripheral blood mononuclear cells, the miR-21/PDCD4 axis modulates inflammation by negatively regulating TLR4 signalling. In specific, inhibition of miR-21 in these cells increased PDCD4 levels, blunted the IL-10 production, and activated NF-κB and IL-6 production upon LPS stimulation. In the miR-21 knockout mouse generated by Ma et al.,26 it was confirmed that PDCD4 is regulated by miR-21 in embryonic fibroblasts and keratinocytes. The miR-21 knockout mouse of Patrick et al. who studied loss of miR-21 in the heart, however, does not reveal insights in this pathway, as miR-21 target genes were not upregulated. MI and TAC studies in these mice revealed that in the absence of miR-21, LV remodelling was not grossly altered. Nevertheless, subtle changes may have occurred, as cardiac inflammation was not investigated in detail. Thus, in addition to miR-146, miR-21 also acts as an anti-inflammatory miRNA within a negative feedback system: NF-κB is required for miR-21 induction, but by targeting PDCD4, miR-21 inhibits NF-κB and its pro-inflammatory transcriptional targets.

5. Conclusion and perspective

The myocardium comprises a complex tissue structure, in which different cell types are interconnected within a complex ECM network. To understand how miRNAs may play a role in the pathogenesis of HF, it is important to realize that cardiac remodelling is caused by collective changes in individual cell types such as myocytes, fibroblasts, ECs, SMCs, and immune cells. As summarized in this review, growing evidence indicates that miRNAs contribute to pathological remodelling of the heart by regulating the expression of target genes that are involved in fibrosis, EC function, angiogenesis, and inflammation. While some miRNAs have very specific functions in one cell type (e.g. miR-126 in ECs), other miRNAs are more ubiquitously expressed and regulate gene expression in multiple cell types. An example of the latter is miR-21, which has been described to act pro-fibrotic in fibroblasts, act as an inhibitor of angiogenesis in ECs, and exerts anti-inflammatory functions in immune cells (Figure 4).

5.1 miRNAs in cell-to-cell communication

Besides cell autonomous effects of miRNAs, they are also involved in communication between cells, either by influencing the secretion of cytokines and growth factors or, as recent evidence suggests, by exerting a direct signalling function. An example of the first mode of communication represents the miR-133a-mediated regulation of CTGF and TGF-β in cardiomyocytes, which after secretion regulate fibroblast function. A first indication that miRNAs are physically involved in cell-to-cell communication stems from the discovery that extracellular miRNAs circulate in the bloodstream in a highly stable form. Although the precise cellular release mechanism of miRNAs remains largely unknown, the first studies reveal that these circulating miRNAs may be delivered to recipient cells, where they can regulate translation of target genes. In an elegant study by Zernecke et al.,84 it was recently shown that miR-126 may be involved in cell-to-cell communication. In the context of atherosclerosis, they reported high levels of miR-126 in the circulation, where this miRNA was carried by apoptotic bodies, derived from dying ECs. Interestingly, when ApoE null mice (atherosclerotic mouse model)

![Figure 4](https://academic.oup.com/cardiovascres/article-abstract/93/4/573/436535)

**Figure 4** miR-21 regulates gene expression in multiple cell types in the heart. miR-21 acts profibrotic in fibroblasts, anti-apoptotic in cardiomyocytes, anti-angiogenic in ECs, and anti-inflammatory in immune cells. Direct targets of miR-21 responsible for these effects are depicted in dark brown in the respective cell types. Spry1, sprouty homologue 1; PTEN, phosphatase and tensin homologue; MMP2, matrix metalloproteinase 2; FasL, fas ligand; PDCD4, programmed cell death 4; RhoB, ras homologue family member B.
were injected with apoptotic bodies from wild-type mice, they were protected against athero-progression, as evidenced by reduced infiltration of macrophages and an increased number of SMCs in the carotid arteries. In contrast, when the same number of apoptotic bodies was injected from miR-126-deficient mice, the protective effect on atherosclerosis was lost. In conclusion, this is the first study to show that miRNAs, present in the circulation can be taken up by distant ECs where they can exert specific cellular functions. Besides delivery of miRNAs to ECs, in vitro studies revealed that miRNAs can also be efficiently transferred to mouse embryonic fibroblasts and H9C2 cardiomyocytes. The presence of endogenous miRNAs in the bloodstream has also indicated that circulating miRNAs may serve as biomarkers for cardiovascular disease. Our laboratory has recently demonstrated that miR-423-Sp is enriched in the plasma of HF patients. It will be interesting to investigate if these miRNAs have a signalling function in HF.

5.2 Therapeutic potential of miRNAs

Several animal studies have demonstrated that pharmacological modulation of miRNAs by antisense oligonucleotides is able to influence myocardial remodelling, and as a consequence, miRNAs are now emerging as therapeutic targets in the treatment of HF. That miRNA manipulation is becoming a feasible therapeutic approach is illustrated by silencing of miR-122 in non-human primates. Here, miR-122 knockdown resulted in a long-lasting lowering of plasma cholesterol levels and reduced serum and liver RNA levels of the Hepatitis C virus in chronically infected chimpanzees. The miR-122 inhibitor used in these studies is the first miRNA-targeted drug that has entered a human clinical trial.

In the cardiovascular field, miRNA inhibitors are mainly based on cholesterol-linked oligonucleotides (antisense) or LNA-modified oligonucleotides (antimiRs). The half-life of these miRNA inhibitors and delivery to different cell types importantly depends on the chemical properties. This might also be the reason why different oligonucleotide chemistries used to silence the same miRNA resulted in different cardiac phenotypes (see section 2.1). This underscores the gap in our understanding of the precise mechanism of action of these miRNA inhibitors in vivo, and indicates that more research is needed before heart patients can possibly be treated with miRNA-based therapeutics. This research should be aimed to advance insights into the different effects elicited by the different chemistries used, into the dose-dependent effects of these oligonucleotides and to developing cell-type-specific delivery methods to alter miRNA expression in particular cell types within the myocardium. Perhaps, technical advances will eventually allow us to modulate cardiac miRNAs in a cell type-specific manner with minimal off-target effects. This may be clinically useful to improve the various aspects of the remodelling process that independently contributes to the progression of HF.

Conflict of interest: Dr Pinto is a co-founder of and holds less than 5% equity in ACS Biomarker BV, a company commercializing cardiovascular biomarkers.

Funding

This work was supported by the Dutch Heart Foundation grants NHS2007-B077 and NHS2007-B167.

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