**MicroRNAs: role in cardiovascular biology and disease**

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**ABSTRACT**

miRNAs (microRNAs) comprise a novel class of endogenous, small, non-coding RNAs that negatively regulate gene expression via degradation or translational inhibition of their target mRNAs. Recent studies have demonstrated that miRNAs are highly expressed in the cardiovascular system. Although we are currently in the initial stages of understanding how this novel class of gene regulators is involved in cardiovascular biological functions, a growing body of exciting evidence suggests that miRNAs are important regulators of cardiovascular cell differentiation, growth, proliferation and apoptosis. Moreover, miRNAs are key modulators of both cardiovascular development and angiogenesis. Consequently, dysregulation of miRNA function may lead to cardiovascular diseases. Indeed, several recent reports have demonstrated that miRNAs are aberrantly expressed in diseased hearts and vessels. Modulating these aberrantly expressed miRNAs has significant effects on cardiac hypertrophy, vascular neointimal lesion formation and cardiac arrhythmias. Identifying the roles of miRNAs and their target genes and signalling pathways in cardiovascular disease will be critical for future research. miRNAs may represent a new layer of regulators for cardiovascular biology and a novel class of therapeutic targets for cardiovascular diseases.

**INTRODUCTION**

miRNAs (microRNAs) comprise a novel class of endogenous, small, non-coding RNAs that control gene expression by targeting their target mRNAs for degradation and/or translational repression. The first miRNA, lin-4, was identified in 1993 [1,2]. Even though the finding was published in high-profile journals, the lack of homology with other species at that time led lin-4 to be considered as a genetic oddity that was restricted to *Caenorhabditis elegans*. The situation did not change until the second miRNA (let-7) was discovered in 2000, and was found, along with its target lin-41, to be conserved in many species [3–5]. Since then, an increasing number of miRNAs has been identified in mammals. More than 500 miRNAs have been cloned and sequenced in humans, and the estimated number of miRNA genes is as high as 1000 in the human genome [6,7]. As a group, miRNAs regulate approx. 30% of the genes in the human genome [8].

Over the last few years, there has been an explosion in miRNA research because of the important roles of these non-coding RNAs in diverse biological processes. Both basic and clinical studies suggest that miRNAs are important regulators of cell differentiation, growth, proliferation and apoptosis [9–11]. Therefore miRNAs could be pivotal regulators in normal development and physiology, as well as in disease development. An exciting research area is the role of miRNAs in cancer, because cell growth, proliferation and apoptosis are important cellular events in the development of this disease. Indeed, both basic and clinical studies have demonstrated that...
miRNAs are aberrantly expressed in diverse cancers [12–17]. miRNAs are thought to function as both tumour suppressors and oncogenes [18].

miRNAs are also highly expressed in the cardiovascular system [19]; however, their biological roles in the mammalian cardiovascular system have only been elucidated since 2005 [20]. Several studies have demonstrated that miRNAs play important roles not only in cardiovascular development, but also in cardiovascular disease [21–28]. In the present review, research progress regarding miRNAs in the cardiovascular system is summarized and a perspective for this new frontier of cardiovascular research is provided.

**BIOGENESIS OF miRNAs AND THEIR ROLE IN GENE REGULATION**

Mature miRNAs are non-coding single-stranded RNAs of approx. 22 nucleotides and constitute a novel class of gene regulators. As shown in Figure 1, miRNAs are initially transcribed by RNA polymerase II or III (Pol II or Pol III) in the nucleus to form large pri-miRNA transcripts, which are usually several kilobases long, and are capped (MGpppG) and polyadenylated [29,30]. The pri-miRNAs are processed in the nucleus by the RNase III enzyme Drosha, and the dsRNA (double-stranded RNA)-binding protein Pasha (also known as DGCR8), into approx. 70 nucleotide pre-miRNAs, which fold into stem-loop hairpin structures. RanGTP and exportin 5 transport the pre-miRNA into the cytoplasm. Subsequently, another RNase III enzyme Dicer processes the pre-miRNA to generate a transient approx. 18–24 nucleotide duplex. The duplex is loaded into the miRNA-associated miRISC (multiprotein RNA-induced silencing complex), which includes the Argonaute proteins. One strand of the miRNA is preferentially retained in this complex and becomes the mature miRNA; the opposite strand, known as the passenger strand or miRNA*, is eliminated from the complex. In addition to this pathway for miRNA biogenesis, some intronic miRNA precursors are able to bypass Drosha processing to produce miRNAs by Dicer, possibly representing an alternative novel pathway for miRNA biogenesis [31,32].

The mature miRNA binds to complementary sites in the mRNA target to negatively regulate gene expression in one of two ways that depend on the degree of complementarity between the miRNA and its target, and on other criteria that have yet to be defined. miRNAs that bind to mRNA targets with imperfect complementarity block target gene expression via translational silencing. In contrast, miRNAs that bind to their mRNA targets with perfect complementarity induce target mRNA cleavage [33–36]. However, the above view may not be completely correct, as studies suggest that even imperfect base pairing of miRNA with its target mRNA can lead to a decrease in the abundance of the mRNA [37,38].

**DIFFERENCE BETWEEN miRNAs AND siRNAs (SMALL INTERFERING RNAs) IN MAMMALS**

siRNAs comprise another class of small non-coding RNAs that regulate gene expression via a mechanism similar to that of miRNAs. However, the two are different [39,40], primarily in their origins. siRNAs are produced from long double-stranded (bimolecular) RNAs or long hairpins, often of exogenous origin in mammalian cells [9,41]. In contrast, miRNAs are endogenous, are encoded within the genome and derive from endogenous short hairpin precursors. Therefore miRNAs, rather than siRNAs, appear to have a major role in gene regulation in mammalian systems under normal physiological conditions.

**EXPRESSION OF miRNAs IN THE CARDIOVASCULAR SYSTEM**

Tissue-specific expression is one important characteristic of miRNA expression [19]. Indeed, one miRNA may be highly expressed in one tissue, but may have no or low
expression in other tissues [19]. Identifying an miRNA signature is therefore an essential prerequisite to studying the biological functions of this class of molecules in the cardiovascular system. Using microarray analysis designed to detect the majority of mammalian miRNAs identified thus far, we have recently determined the miRNA signature in both heart and artery samples [21,22]. Overall, out of 180 mature miRNAs arrayed, 140 were found in normal rat carotid arteries, whereas 157 mature miRNAs out of 233 arrayed were found in normal mouse hearts [21,22]. The miRNA signature in the mouse heart has also been demonstrated by three other independent studies [19,23,24]. Our unpublished data also suggest that the miRNA expression profile in rat carotid artery is different from that in the rat heart (Y. Cheng, J. Yang and C. Zhang, unpublished work). For example, the most abundant miRNAs in rat heart are miR-1, let-7, miR-126, miR-30c, miR-133, miR-26a, miR-23 and miR-30c, whereas those in rat artery are miR-145, let-7, miR-125b, miR-125a, miR-23 and miR-143; miR-1 is not abundant in the rat artery. The tissue-specific expression profiles indicate that the physiological functions of miRNAs in each tissue could be unique; identifying these miRNA signatures and clarifying their physiological functions could be important for future studies.

ROLE OF miRNAs IN CARDIOVASCULAR CELL BIOLOGY

In 2005, miR-1 was identified to play a key role in cardiomyocyte differentiation [20]. miR-1 is specifically expressed in cardiac precursor cells, and the miR-1 gene is a direct transcriptional target of muscle differentiation regulators, including SRFs (serum-response factors), MyoD (myogenic differentiation factor D) and Mef2 (myocyte-enhancing factor 2) [20]. Correspondingly, excess miR-1 in the developing heart leads to a decreased pool of proliferating ventricular cardiomyocytes [20]. These results suggest that miR-1 genes modulate the effects of critical cardiac regulatory proteins to control the balance between differentiation and proliferation during cardiogenesis.

The role of miRNAs in cardiac myocyte growth has been documented in three recent studies [22–24]. Overexpression of miR-23a, miR-23b, miR-24, miR-195 or miR-214 via adenovirus-mediated gene transfer induced hypertrophic growth of cultured cardiomyocytes, whereas overexpression of miR-150 or miR-181b caused a decrease in cardiomyocyte cell size [23]. We have shown recently that miRNAs are aberrantly expressed in cultured neonatal hypertrophic cardiomyocytes that are stimulated by AngII (angiotensin II) or PE (phenylephrine) [22]. Modulating an aberrantly up-regulated miRNA, miR-21, via antisense-mediated depletion (knockdown), has a significant negative effect on cardio-myocyte hypertrophy in vitro [22]. In contrast, overexpression of an aberrantly down-regulated miRNA, miR-1, via adenovirus-mediated gene transfer, is sufficient to prevent hypertrophic growth of cardiac myocytes that are stimulated by either serum or ET-1 (endothelin-1) [24]. The cellular effects of miRNAs in the heart have been confirmed further both in vitro and in vivo [25–28].

In our recent study of the potential roles of miRNAs in VSMC (vascular smooth muscle cell) proliferation and apoptosis [21], we found that depletion of miR-21, an miRNA that is up-regulated in proliferative VSMCs, results in decreased cell proliferation and increased cell apoptosis in a dose-dependent manner in cultured rat aortic VSMCs. This suggests that miR-21 has a pro-proliferative and anti-apoptotic effect on VSMCs. In addition, our unpublished data also demonstrate that miR-21 has a pro-migratory effect on cultured VSMCs (Y. Cheng, J. Yang and C. Zhang, unpublished work); the mechanisms responsible for these effects are unclear, but our preliminary study suggests that PTEN (phosphatase and tensin homology deleted on chromosome 10) and Bcl-2 might be involved [21]. The effect of miR-221 and miR-222 on vascular endothelial cell migration was initially determined by tube formation and wound healing assays [42]. The results suggest that the influence of miR-221 and miR-222 on endothelial cell migration occurs, at least in part, through their target c-Kit. The effects of some other miRNAs, such as let-7, on human endothelial cell migration were also demonstrated in two recent studies [43,44]. Obviously, the effects of other highly expressed miRNAs in endothelial cells on cell migration, proliferation and apoptosis need to be determined in future studies.

ROLE OF miRNAs IN CARDIOVASCULAR DEVELOPMENT

The role of miRNAs in cardiac development has been well described in several studies [20,45–47]. miR-1 is specifically expressed in cardiac and skeletal muscle of embryonic mice, and this expression is controlled by several key heart transcription factors such as SRF and Mef2 [20]. Overexpression of miR-1 results in thin-walled ventricles, heart failure and developmental arrest at embryonic day 13.5, due to a significant decrease in the number of cycling myocardial cells. In addition, overexpression of miR-1 decreased the level of Hand2 protein without changing its mRNA level, suggesting that Hand2 is a target of miR-1 during heart development. The essential role of miRNAs in cardiovascular development is demonstrated indirectly in Dicer-deficient mice that lose miRNAs: both heart and vessel development are seriously impaired [48]. Furthermore, targeted deletion of the muscle-specific miRNA miR-1-2 also implicates miRNAs as key players in cardiovascular development [47]. Consistent with the
above animal studies, miRNAs also play an important role in *Drosophila* heart development [45,46].

## ROLE OF miRNAs IN ANGIOGENESIS

Angiogenesis is an important physiological and pathological process, both in normal development and in diseases such as ischaemic cardiovascular diseases and cancers [49,50]. The first evidence that miRNAs may play important roles in mammalian angiogenesis was obtained from Dicer-deficient mice [48], where blood vessel formation and maintenance in Dicer-deficient embryos and yolk sacs were severely compromised. This impairment is consistent with the concurrent altered expression of several crucial genes involved in embryonic angiogenesis such as *vegf*, *flt1*, *kdr* and *tie1* in these mice [48]. An essential role of miRNAs in tumour angiogenesis was established in a recent study [51], which showed that a miR-17-92 cluster was significantly up-regulated in c-Myc-expressing colonocytes. Furthermore, miR-17-92 knockdown with antisense 2′-O-methyl oligonucleotides inhibited tumour angiogenesis, whereas retroviral-mediated overexpression of miR-17-92 enhanced tumour angiogenesis. These findings establish a role for miRNAs in tumour angiogenesis.

HUVECs (human umbilical vein endothelial cells) are a valuable in vitro model of angiogenesis because of their ability to form capillary-like structures in response to appropriate stimuli [52]. The miRNA signature of HUVECs was recently determined, and 15 highly expressed miRNAs were found to have angiogenic growth factor receptors as putative targets [42]. In particular, miR-221 and miR-222 affect c-Kit expression and, as a consequence, the angiogenic properties of its ligand SCF (stem cell factor) [42]. An interaction between miR-222 and c-Kit is probably part of a complex circuit that controls the ability of endothelial cells to form new capillaries. The knockdown of Dicer in endothelial cells also alters the expression of several key regulators of endothelial biology and angiogenesis, such as TEK/Tie-2, KDR/VEGFR2 (vascular endothelial growth factor receptor 2), Tie-1, eNOS (endothelial nitric oxide synthase) and IL-8 (interleukin-8). These changes may explain the impairment of angiogenic properties in Dicer-knockout endothelial cells [43]. More recently, miR-27b and let-7f were found to modulate angiogenesis (Figure 2); the let-7f-mediated effect was shown to occur by targeting of the angiogenesis inhibitor thrombospondin-1 [44].

It is well known that the homeobox genes GAX and HOXA5 (homoeotic A5) inhibit angiogenesis in vascular endothelial cells [53,54]. Recently, one study demonstrated that GAX and HOXA5 are targets for miR-130a [55]. Forced expression of miR-130a inhibits GAX and HOXA5 expression through their specific 3′-UTR (untranslated region) sequence [55]. The results indicate that miR-130a is a regulator of the angiogenic phenotype of vascular endothelial cells, largely through its ability to modulate the expression of GAX and HOXA5.

## ROLE OF miRNAs IN CARDIAC HYPERTROPHY AND HEART FAILURE

Cardiac hypertrophy is a common pathological response to a number of cardiovascular diseases, such as hypertension, ischaemic heart disease, valvular diseases and endocrine disorders. Cardiac hypertrophy often leads to heart failure in humans and is a major determinant of mortality and morbidity in cardiovascular diseases. miRNAs are important regulators for the differentiation and growth of cardiac cells, and it is therefore reasonable to hypothesize that miRNAs play important roles in cardiac hypertrophy and heart failure. In 2005, a Japanese research group originally determined the expression profile of miRNAs in the kidney and heart of salt-sensitive hypertensive rats [56]. No difference was found...
in miRNA expression between hypertrophic hearts and control hearts, but this unexpected finding could be a result of the low sensitivity of the method used for that study.

The problem has been solved with the development of more sensitive methods for detecting miRNAs, such as microarray analysis, qRT-PCR (quantitative real-time PCR) and improved Northern blot analysis. Almost simultaneously, three independent groups reported striking results on the miRNA expression signature in mouse hearts that were made hypertrophic by either aortic binding or the expression of activated calcineurin [22–24], showing that miRNAs are aberrantly expressed in hypertrophic hearts. The results were confirmed by in vitro studies of cardiac myocytes with hypertrophy [22–26]. Furthermore, the overexpression of some miRNAs that are up-regulated in hypertrophic hearts induces cardiac myocyte hypertrophy, whereas the overexpression of some miRNAs that are down-regulated in hypertrophic hearts prevents cardiac myocyte hypertrophy. On the other hand, inhibition of miR-21, an miRNA that is up-regulated in hypertrophic animal and human hearts, inhibits hypertrophic hearts in vitro [22]. The role of miR-21 was confirmed further by another group [28]. However, a recent study points to a different role of miR-21 in cardiac hypertrophy [25]. In vivo, the overexpression of miR-195, an miRNA that is up-regulated in hypertrophic hearts, was sufficient to drive cardiac hypertrophy [23] (Figure 3A), whereas a gene mutation or ‘decoy’ approach has confirmed the role of miR-208 (Figure 3B) and miR-133 in cardiomyocyte hypertrophy [26,27,57]. Taken together, these findings demonstrate that multiple miRNAs are involved in cardiac hypertrophy and that modulating one aberrantly expressed miRNA is sufficient to modulate hypertrophy. However, the molecular mechanisms responsible for individual miRNA-mediated effects on cardiac hypertrophy are unclear.

More recently, the roles of miRNAs in human cardiac hypertrophy and heart failure have been elucidated in several clinical studies [23,24,28,29,58,59]. Northern blot analysis of the hypertrophy-regulated miRNAs in idiopathic end-stage failing human hearts shows that the expression of miR-24, miR-125b, miR-195, miR-199a and miR-214 is significantly increased compared with control hearts [23]. A total of 43 out of 87 miRNAs detected are aberrantly expressed in hearts with ischemic cardiomyopathy, dilated cardiomyopathy or aortic stenosis [58], indicating that miRNAs are indeed involved in the pathophysiology of human cardiac hypertrophy and heart failure.

**ROLE OF miRNAs IN NEOINTIMAL LESION FORMATION**

Neointimal lesion formation is a common pathological lesion found in diverse cardiovascular diseases, such as atherosclerosis, coronary heart diseases, postangioplasty restenosis and transplantation arteriopathy [60]. Using microarray analysis and a well-established neointimal formation model, we determined the miRNA expression profile in the vascular wall with neointimal lesion formation [21]. Compared with normal uninjured arteries, microarray analysis demonstrated that aberrant miRNA expression was a characteristic of vascular walls after angioplasty. At 7 days after balloon injury, 113 artery miRNAs were differentially expressed: 60 were up-regulated, and 53 were down-regulated. At 14 days after injury, 110 artery miRNAs were differentially expressed (63 up-regulated and 47 down-regulated), whereas 102 miRNAs were differentially expressed (55 up-regulated and 47 down-regulated) at 28 days after angioplasty. Those miRNAs that were highly expressed in the rat carotid artery, i.e. up-regulated more than 1-fold or down-regulated by 50% after angioplasty, were verified
further by qRT-PCR and/or Northern blot analysis [21]. It should be noted that the aberrant expression is not limited to the vascular wall with acute neointimal lesion formation. Our unpublished data suggest that miRNAs are also abnormally expressed in ApoE (apolipoprotein E)-knockout mouse arteries with chronic atherosclerotic neointimal lesion formation (Y. Cheng, J. Yang and C. Zhang, unpublished work). Modulating an aberrantly overexpressed miRNA, miR-21, via antisense-mediated knockdown, has a significantly negative effect on neointimal lesion formation in rat artery after angioplasty (Figure 4). These results indicate that miRNAs are important regulators in the development of proliferative vascular diseases.

ROLE OF miRNAs IN CARDIAC ARRHYTHMIAS

Cardiac arrhythmias in the setting of ischaemic heart disease remain a serious health problem because of their sudden and unpredictable nature, and their potentially fatal consequences. In a rat model of myocardial infarction and in human hearts with coronary heart disease, the muscle-specific miRNA miR-1 was significantly upregulated in ischaemic heart tissue [61]. To determine further the role of miR-1 in arrhythmogenesis, both gain-of-function and loss-of-function approaches have been applied to enhance or inhibit miR-1 expression in the infarcted myocardium. The results show that the injection of mature miR-1 exacerbates arrhythmogenesis, whereas elimination of miR-1 by an antisense inhibitor suppresses arrhythmias. This indicates that miR-1 has pro-arrhythmic as well as arrhythmogenic effects [61]. Silencing the genes for the ion channels GJA1 and KCNJ2 verified that these proteins are important players in the miR-1-mediated arrhythmogenic effect [61].

miR-133 expression is up-regulated in the diabetic rabbit heart, and ERG (ether-a-go-go-related gene), a long-QT syndrome gene encoding a key K⁺ channel (I\(_{\text{Kr}}\)) in cardiac cells, was confirmed to be a target for miR-133 [62]. Delivery of exogenous miR-133 into the rabbit myocytes and cell lines resulted in post-transcriptional repression of ERG, down-regulating the ERG protein level without altering its transcript level and causing a substantial depression of I\(_{\text{Kr}}\), an effect that was abrogated by the miR-133 antisense inhibitor [62]. Thus depression of I\(_{\text{Kr}}\) via repression of ERG by miR-133 may contribute to repolarization slowing and, thereby, QT prolongation and the associated arrhythmias in diabetic hearts.

In cardiac cells, KCNQ1 assembles with KCNE1 and forms a channel complex constituting the slow delayed rectifier current I\(_{\text{KS}}\). Expression of KCNQ1 and KCNE1 is regionally heterogeneous and changes with the pathological states of the heart; however, the molecular mechanisms responsible for these changes are unclear. Recently, one study has characterized KCNQ1 and KCNE1 as targets of the muscle-specific miRNAs miR-133 and miR-1 respectively [63]. The heterogeneous
expression of miR-1 and miR-133 offers an explanation for the well-recognized regional differences in expression of KCNQ1 and KCNE1 and the disparity between the levels of their mRNA and protein in each region [63].

HCN2 and HCN4 are two important cardiac pacemaker channel proteins that control rhythmic activity of the heart. A recent study has demonstrated that HCN2 mRNA is a target of miR-1 and miR-133, and HCN4 mRNA is a target of miR-1 [64]. Xiao et al. [64] developed two new therapeutic approaches to explore the possibility of using the principles of miRNA action in a gene-specific manner, namely the gene-specific miRNA-mimic and miRNA-masking antisense approaches. They demonstrated that gene-specific miRNA mimics, which are 22 nucleotide RNAs designed to target the 3′-UTRs of HCN2 and HCN4, were efficient in abrogating the expression and function of HCN2 and HCN4, respectively. Meanwhile, the miRNA-masking antisense, based on the target sites of miR-1 and miR-133 in the 3′-UTRs of HCN2 and HCN4, markedly enhanced HCN2/HCN4 expression and function. Thus these two therapeutic approaches, based on the principles of miRNA action, could provide novel gene therapy strategies for cardiac arrhythmias [64].

**FUTURE DIRECTIONS**

Investigating the role of miRNAs in cardiovascular disease is a new frontier for cardiovascular research. In fact, we are just beginning to understand how this novel class of gene regulators is involved in cardiovascular biology. Given the vast number of miRNAs and the profound effects of the few that have been identified so far on the cardiovascular system, it is certain that many new and unanticipated roles of miRNAs in the control of cardiovascular function are waiting to be discovered. Further information regarding the functions of these aberrantly expressed miRNAs in cardiovascular disease is needed. More importantly, identifying their gene targets and signalling pathways responsible for their cardiovascular effects is critical for future studies. The combination of computational analysis, bioinformatics and cardiovascular cell biology approaches, as well as loss-of-function and gain-of-function approaches, will be needed for the identification of miRNA target genes [65]. As miRNA inhibitors may have some non-specific effects and miRNA knockouts may result in lethality for animals, conditional knockout mice targeting specific miRNAs in specific tissue types and cells will provide a greater insight into their biological roles in the cardiovascular system. Human studies will move research on the function of miRNA in cardiovascular diseases to the next level. The ultimate goal, of course, is the development of new therapeutic and diagnostic strategies for cardiovascular diseases.

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