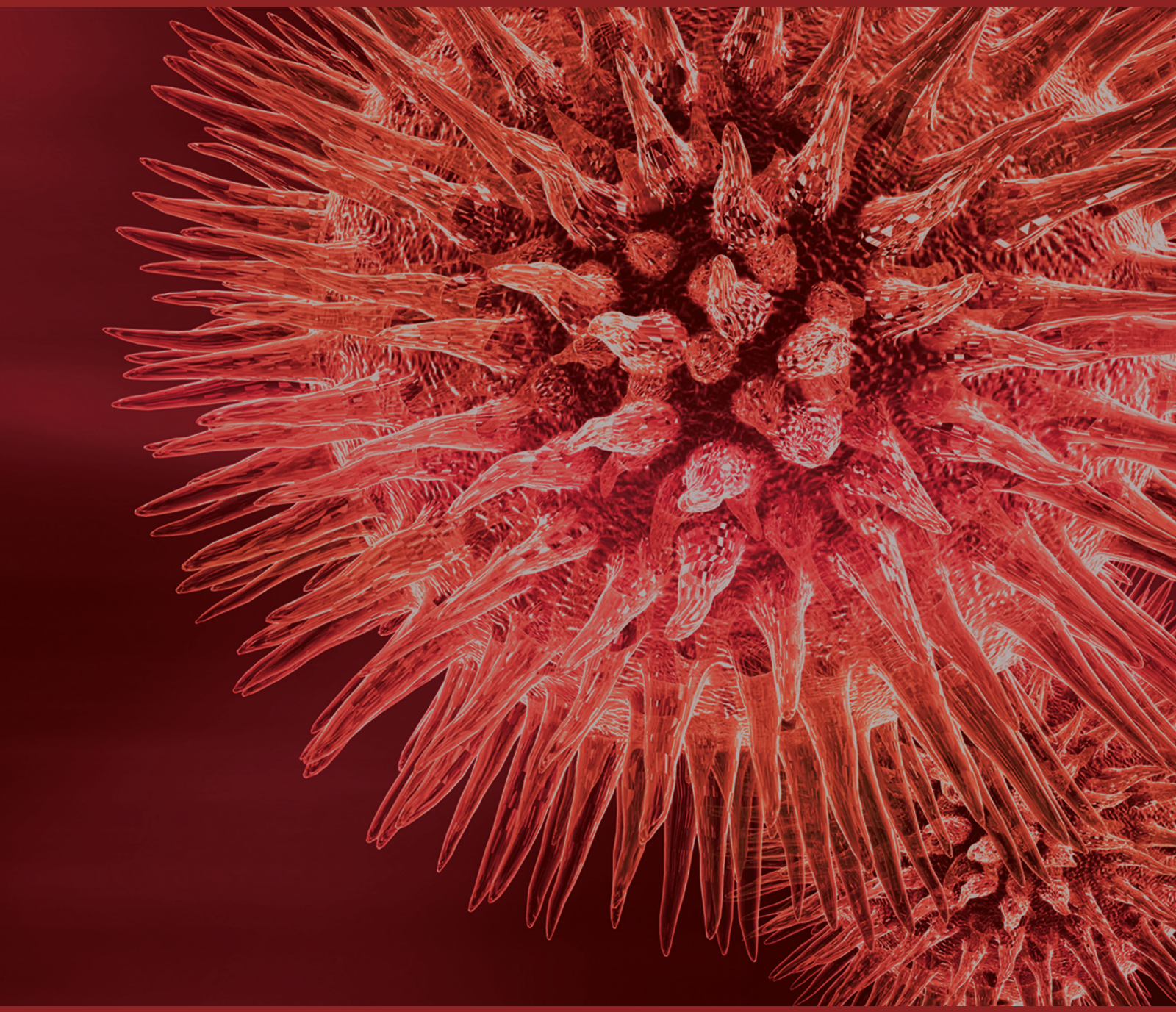
BioMed Research International

MicroRNA and Cardiovascular Disease

Guest Editors: Ling-Qing Yuan, Vinicio A. de Jesus Perez, Xiao-Bo Liao, Magdalena Król, and Chi-Hsiao Yeh



MicroRNA and Cardiovascular Disease

BioMed Research International

MicroRNA and Cardiovascular Disease

Guest Editors: Ling-Qing Yuan, Vinicio A. de Jesus Perez, Xiao-Bo Liao, Magdalena Król, and Chi-Hsiao Yeh

Contents

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 734380, 2 pages
http://dx.doi.org/10.1155/2015/734380

Editorial

MicroRNA and Cardiovascular Disease

Xiao-Bo Liao,[1] Vinicio A. de Jesus Perez,[2] Magdalena Król,[3] Chi-Hsiao Yeh,[4] and Ling-Qing Yuan[5]

[1]*Departments of Cardiothoracic Surgery, Central South University, Changsha 410011, China*
[2]*Division of Pulmonary and Critical Care Medicine, Stanford University Medical Center, Stanford, CA 94305, USA*
[3]*Warsaw University of Life Sciences, 02-787 Warsaw, Poland*
[4]*Chang Gung Memorial Hospital, Keelung 20445, Taiwan*
[5]*Institutes of Metabolism and Endocrinology, Central South University, Changsha 410011, China*

Correspondence should be addressed to Ling-Qing Yuan; allenylq@hotmail.com

Received 31 May 2015; Accepted 31 May 2015

Cardiovascular disease (CVD) is the leading cause of death and a major financial burden in most developed countries around the world, resulting in an estimated death toll of 17 million people in 2011. During the past decade, there have been significant advances in our knowledge of the pathogenesis, diagnosis, and treatment in CVD. Recently, microRNAs (miRNAs) have emerged as one of the most important regulators of gene function and tissue homeostasis that appear to be amenable for development of novel therapies to treat deleterious mutations and restore cellular function in various diseases. Despite the major discoveries made in the past decade, there are still major gaps in our knowledge as to how miRNAs are involved in preserving normal cardiovascular function and how dysregulation in their expression or biological function results in CVD. Available studies strongly support that miRNAs could be targeted in various ways to restore normal cardiovascular function and could find a role in the clinic as therapeutic tools to improve our ability to prevent and effectively treat CVD, likely resulting in improved outcomes and reduced rates of mortality from these diseases. Our current special issue presents a series of original researches and reviews on recent advances in miRNAs regulating the pathophysiology aspects of CVD that will hopefully set the stage for future research initiatives aimed at expanding our understanding of these important mediators of cardiovascular function.

Among the studies included in this special issue, some researchers investigate the miRNA function in different CVD. M. Torrado et al. used the right atrial tachypacing to establish an atrial fibrillation pig model and show that the abnormal expression of miRNAs is associated with modulation of transcription factors in left atrium of this animal model. Their study provides critical insight into how the microRNA-transcription factor regulation network underlies the onset of paroxysmal atrial fibrillation. In another article, A. M. dos Santos et al. report that laminar shear stress (LSS) has a protective effect on human umbilical vein endothelial cells (HUVECs) via concomitant increased expression of miR-126, vascular cell adhesion molecule-1 (VCAM-1), and syndecan-4 (SDC-4). This is supported by in vivo studies using Apo-E KO/CKD mice, a well-established animal model of atherosclerosis and aortic calcification. We see that gain- and loss-of-function studies of miR-126 result in significant changes in the expression of several cytokines by HUVEC that suggest flow might have anti-inflammatory and antiatherosclerosis effect on the endothelium of the systemic circulation. The study by Z.-Y. Xia et al. reveals an interesting interaction between miR-3960/miR-2861 and Runx2 in vascular smooth muscle cells, which regulates osteogenic differentiation of VSMCs. Their studies identify a novel mechanism by which miR-3960/miR-2861 target histone deacetylase 5 or Homeobox A2 that could modify the expression of Runx2 and create a feedback mechanism targeting miR-3960/miR-2861 itself. The authors speculate that this complex regulation feedback loop might play a pivotal role in osteogenic differentiation of VSMCs and contribute to vascular calcification in CVD.

A major focus of studies into miRNA function is the identification of the genes targeted in target cells and tissues. E. Lozano-Velasco et al. report that miRNA might have distinct effects on the same target gene in different cells as shown when miR-125 is overexpressed in HL1 atrial

cardiomyocytes where it increases Mef2d while suppressing this gene in Sol8 cells. Two or more miRNAs could constitute a "microRNA cluster," which can have important roles in controlling physiological and pathological conditions. X. Zhang et al. found miR-17-92, miR-106a-363, and miR-106b-25 clusters were differently expressed between aged and young adult mouse heart and glucose stress could influence their upregulation or downregulation. Using computational algorithms for miRNAs, the authors postulate miR-17-92 cluster and its paralogs might regulate Cdc42-SRF pathway protein components involved in cardiac structure and function. This paper shows that miRNA cluster plays an important role in cardiac morphology and response to environmental stimuli.

There are still several interesting reviews about recent progress on miRNAs and CVD. M. Notari et al. give an excellent review, which discusses the relationship between miRNAs and cardiac development, myocardial regeneration, and cardiovascular disease. They also discuss the potential use of miRNA as a therapeutic option for CVD and the obstacles that must be overcome before these agents reach the clinic. S.-S. Wu et al.'s paper reviews the role of epigenetics in the pathogenesis of arterial calcification. They discuss the relationship between DNA methylation, histone modifications, and microRNAs in regulation of arterial calcification. miR-221 and miR-222 are transcribed from the same miRNA cluster, exhibit high sequence similarity, and share similar target genes. Through discussing the role of miR-221/miR-222 in endothelial cells and VSMCs, D. A. Chistiakov et al. review their role in physiological and atherosclerotic vascular remodeling. W. Zhao et al. discuss the effect of miR-143/145 on VSMCs, endothelial cells, and plasma and suggest that miR-143/145 might be a potential drug target for CVD. X. Fu et al. review some miRNAs involved in the pathogenesis of aortic aneurysm and suggest several miRNAs that might be used as potential diagnostic and prognostic biomarker as well as therapeutic targets for aortic aneurysms. R. Shi et al. review recent studies that stress an essential role of miR-223 as both a regulator and biomarker for platelet reactivity and major cardiovascular events. They suggested miR-223 might be a potential diagnostic tool for recognizing high on-treatment platelet reactivity in clinical practice.

Acknowledgments

We hope that this special issue will help readers become familiarized with recent progress regarding the role of miRNAs in CVD. We want to give special thanks to all the authors who shared their excellent work to be included in our special issue and the reviewers whose input was critical for the selection of the best work. Finally, we want to acknowledge the Editorial Board of Biomed Research International for giving us this opportunity to publish this special issue on microRNA and cardiovascular disease.

Xiao-Bo Liao
Vinicio A. de Jesus Perez
Magdalena Król
Chi-Hsiao Yeh
Ling-Qing Yuan

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 320849, 8 pages
http://dx.doi.org/10.1155/2015/320849

Review Article

The Role of Epigenetics in Arterial Calcification

Shan-Shan Wu,[1,2] Xiao Lin,[2] Ling-Qing Yuan,[2] and Er-Yuan Liao[2]

[1]*Endocrinology Department, Pingxiang People's Hospital, 128 Anyuan Area Square Road, Pingxiang, Jiangxi 337000, China*
[2]*Institute of Metabolism and Endocrinology, The Second Xiangya Hospital, Central South University, 139 Middle Renmin Road, Changsha, Hunan 410011, China*

Correspondence should be addressed to Ling-Qing Yuan; allenylq@hotmail.com and Er-Yuan Liao; eyliao1207@gmail.com

Received 15 August 2014; Accepted 9 December 2014

Academic Editor: Xiao-Bo Liao

Arterial calcification is highly prevalent and correlated with cardiovascular mortality, especially in patients with ESRD or diabetes. The pathogenesis of arterial calcification is multifactorial, with both genetic and environmental factors being implicated. In recent years, several mechanisms contributing to arterial calcification have been proposed. However, these can only explain a small proportion of the variability in arterial calcification, which is a major obstacle for its prevention and management. Epigenetics has emerged as one of the most promising areas that may fill in some of the gaps in our current knowledge of the interaction between the environmental insults with gene regulation in the development of diseases. Epigenetics refers to heritable and acquired changes in gene transcription that occur independently of the DNA sequence. Well-known components of epigenetic regulation include DNA methylation, histone modifications, and microRNAs. Epigenetics research in the regulation of arterial calcification has only recently been elucidated. In this review, we will summarise recent progress in epigenetic pathways involved in arterial calcification and discuss potential therapeutic interventions based on epigenetic mechanisms.

1. Introduction

Arterial calcification (AC), a crucial pathologic component of vascular diseases such as atherosclerosis, coronary artery disease, and peripheral vascular disease, is far more common among patients with end-stage renal disease (ESRD) and diabetes compared with the general population [1–4]. AC not only impairs vasomotor responses, but also influences the stability of atherosclerotic plaques which are prone to rupture, particularly in regions of high background stress, with microcalcifications located in the thin fibrous cap [5–7], eventually leading to myocardial infarction. Thus, AC is a potentially life-threatening condition and understanding the causes of arterial calcification may contribute to the treatment and possibly prevention of this disease. However, there is no available therapy that could reverse arterial calcification at present, even with the recent therapeutic progression, such as bisphosphonates. At present, available therapy just can slow down the progress of arterial calcification. Thus, revealing the pathophysiological mechanism of AC and finding novel therapies that reverse the progress of the vascular remodelling are our target in treating this disease.

AC has been recognised for over a century. Unravelling the mechanism involved has been a topic for many researchers in the past few years. Previously, AC was regarded as a passive, degenerative, end-stage process accompanied by calcium-phosphate mineral precipitation in vessel walls. However, increasing evidence has shown that AC is an active and tightly regulated event that is analogous to mineralisation in bone tissue [8–11]. This is based on the discovery of phenotypic conversion of vascular smooth muscle cells (VSMCs) into osteoblast-like cells, as evidenced by the expression of bone-regulating proteins such as alkaline phosphatase, osteocalcin, and Runx2 (runt-related transcription factor 2)/Cbfa1. Several excellent reviews have been published on AC [12–14], manifesting the vital roles of molecular and genetic factors in this complex disorder. Currently, we are fully aware of the important involvement of epigenetic processes in the regulation of gene expression. Understanding these processes is critical for further insight into the pathogenesis and development of AC.

Since Conrad Waddington first coined the term "epigenetics" back in 1942, research has advanced from genotype to phenotype [15]. Epigenetics refers to heritable alterations

in gene expression without alterations in the genetic code itself [16]; such alterations regulate the dynamics of gene expression and play a crucial role in embryonic development, imprinting, and tissue differentiation [17]. Recent breakthroughs in the field of epigenetics offer us a new perspective on gene regulation, which broaden the conventional cis/trans paradigm of transcriptional processes and transform our conceptualisation of the impact of the environment upon our genes and health [16, 18]. Epigenetics regulation is composed of three main categories: DNA methylation, histone modification/chromatin remodelling, and microRNAs (miRNAs) [19]. However, the group is likely to be expanded in the future [20]. Numerous lines of evidence have implied that epigenetic processes play crucial roles in the development of various diseases (cancers, neurological disorders, autoimmune diseases, and diabetes) [21, 22]. Research investigating the actual role of epigenetics in arterial disorder remains scarce; however, several emerging lines of evidence suggest that epigenetics may be important in the biology of VSMCs and the pathogenesis of arterial calcification. This review will summarise the current knowledge about these subjects.

2. Arterial Calcification

2.1. Mechanisms of Arterial Calcification. The mechanism of AC is complicated. It is not simply composed of precipitation of calcium (Ca) and phosphate (P) but rather is an active and modifiable process in which the VSMCs undergo changes from contractile to secretory phenotype, inducing matrix formation and also attracting local factors that are involved in the mineralisation process. Several different mechanisms for initiating AC have been proposed. First, human and mouse genetic studies have found that blood vessels normally express inhibitors of calcification, indicating that AC is generally inhibited by the physiological function of resident arterial cells. Deficient expression of even one inhibitor of AC is enough to trigger the calcification process [23, 24]. Calcification inhibitors such as matrix GLA protein (MGP) may restrain AC by binding to bone morphogenic proteins (BMP-2) [25]. Likewise, fetuin-A is the most potent circulating inhibitor of extraosseous calcification. Decreased fetuin levels have recently been associated with elevated CVD mortality in haemodialysis patients [26]. Apart from GLA protein or fetuin-A, various other factors have been related to arterial calcification. Among those, BMP-7, osteoprotegerin, osteopontin, and inorganic pyrophosphate, an inhibitor of hydroxyapatite crystal growth, probably counteract detrimental vascular and soft-tissue calcification in CKD [27–29], while BMP-2, RANKL, and leptin have been found to promote arterial calcification [30–32]. Second, the death or "damage" of VSMCs can provide phospholipid-rich membranous debris and apoptotic bodies that may serve as initiation sites for apatite crystallisation [33], particularly in diseases where necrosis and apoptosis are prevalent, such as atherosclerosis [34, 35]. Third, we and others have provided evidence that AC is a process reflecting the transformation of the VSMCs from contractile to secretory phenotype [11, 36–38]. In this process, osteoblast-specific genetic programs are triggered with the presence of osteopontin, BMPs, osteocalcin, and Runx2/Cbfa1 [39], which result in the formation of mineralised matrix, cartilage, and bone, and suggest that osteogenic mechanisms may also play an important role in arterial calcification. The phenotypic modulation of VSMCs can be induced by increased extracellular Ca and P content [40, 41] as well as by various other modulators, such as proinflammatory cytokines (e.g., IL-6, TNF-a) [42], oxidised lipids [43], and microenvironmental and mechanical cues [44]. Finally, accumulating serums Ca, P, and Ca × P are associated with arterial calcification and cardiovascular mortality in patients with ESRD via thermodynamic mechanisms. Elevating Ca or P levels in the culture media leads to enhanced mineralisation and phenotypic changes of VSMCs characterised by a decrease of smooth muscle-specific gene expression and the upregulation of genes associated with bone differentiation [40, 41]. Elevated calcium-induced mineralisation and P-induced phenotypic transition and mineralisation were found to be dependent on the function of a sodium-dependent phosphate cotransporter, Pit-1, based on their ability of being inhibited by phosphonoformic acid [41] and Pit-1-specific small interfering RNA [45]. Briefly, a deficiency of calcification inhibitors, cell death, phenotypic transformation of VSMCs to osteoblastic cells, or/and disturbance in Ca and P metabolism may all initiate and sustain arterial calcification in a concerted manner.

2.2. Pathology and Clinical Consequences of Arterial Calcification. AC is a pathological process that occurs in response to dysregulated or inappropriate environmental stimuli such as advancing age, atherosclerosis, and some metabolic disorders (e.g., CKD, diabetes, and chronic inflammatory disease) and in rare genetic diseases (e.g., Keutel syndrome) [31, 42]. AC develops at two anatomic sites: intima and media layers of the large and medium-sized arterial wall [46]. Intimal calcification occurs in atherosclerotic plaques and progresses in parallel with the plaque evolution [47, 48]. In contrast, medial calcification (also known as Monckeberg's medial sclerosis) could take place independently from atherosclerotic plaques and has been observed in the context of aging, diabetes, and ESRD [49–51]. However, both types of arterial calcification often develop simultaneously in dysmetabolic patients. Intimal calcification is characterised by lipid deposition and macrophage accumulation and has been associated with inflammatory cells and VSMCs. In contrast, in medial calcification, metabolite-induced arterial changes in the absence of lipid deposits or macrophages are viewed as specifically accounting for the upregulation of osteogenic regulatory genes which then induce osteogenic differentiation of mesenchymal cells with following matrix mineralisation, bone, and cartilage formation [42]. Media calcification causes arterial stiffness, linked with high pulse pressure (characterised by increased systolic and decreased diastolic pressure), left ventricular hypertrophy, and reduced coronary perfusion [52]. While intimal calcification mainly results in occlusion of vessels, nevertheless, the consequences of intimal calcification on plaque vulnerability remain less clear, as the determinants of plaque rupture with consecutive

thrombosis are still controversial. Several researches have implied that AC does not elevate plaque vulnerability, which seems more ascribable to a large lipid pool, thin fibrous cap, and intensity of local inflammation [7, 53]. In the end, both count, and they are partly responsible for the morbidity of atherosclerosis, acute coronary events, and even heart failure.

2.3. Epigenetics and Its Roles in Arterial Calcification. Epigenetics was first proposed by Conrad Waddington in a study of "the causal interactions between genes and their products, which bring the phenotype into being" [54]. More recently, epigenetics was redefined as the study of stable changes in gene expression without alterations in the DNA sequence [16]. Such changes are achieved by covalent and noncovalent modifications, which mark the genome and play a role in turning genes on or off [55]. The most well-known epigenetic mechanisms include DNA methylation, histone modification/chromatin remodelling, and miRNAs.

The vascular system is highly regulated by epigenetic mechanisms. Growing evidence has shown that epigenetic markers exert a crucial role in vascular development, endothelial and smooth muscle cell differentiation and function, and allowing a high flexibility in response to sudden physiological or pathological changes. Epigenetic factors also explain how external factors such as diet, environment, and lifestyle may contribute to cardiovascular disease. However, reports on the roles of epigenetics in AC have only emerged in recent years.

2.4. DNA Methylation. DNA methylation, the first mechanism involved in epigenetics, is the most widely studied epigenetic marker [56]. In mammals, DNA methylation occurs mostly within CpG dinucleotides via the addition of a methyl group from SAM to the fifth carbon of a cytosine residue to form 5-methyl-cytosine. The CpG dinucleotides are inclined to cluster in regions called CpG islands, which are identified as a region of more than 200 bases with a CG content of at least 50% [21]. CpG dinucleotides are usually underrepresented in mammalian genomes (~1%). Approximately 70% of human gene promoters reside within CpG islands and are generally unmethylated in normal cells [57]. Some tissue-specific DNA methylation, however, takes place in regions termed CpG island shores as far as 2 kb distant of the promoter region. Furthermore, cancer-specific methylation also occurs at conserved tissue-specific CpG island shores [58]. The basic mechanism of DNA methylation is summarised as follows: enzymes catalyse the addition of methyl groups onto cytosine residues, enzymes modify and remove the methyl group, and then proteins recognise and bind to methyl groups to ultimately influence gene expression. So far, three active DNA methyltransferases (DNMTs), which directly catalyse the addition of methyl groups onto DNA, have been described: DNMT1, DNMT3a, and DNMT3b [21]. DNMT3L, which belongs to the DNMT3 family, lacks the catalytic activity itself, but it is required for the enzymatic activities of DNMT3a and DNMT3b and interacts with them in the nucleus [59, 60]. DNMT2 is a highly conserved protein and possesses tRNA methyltransferase activity [61]. DNMTs are capable of both methylation and demethylation, making the modification reversible [62, 63]. DNA methylation-related proteins, including the MBD (methyl CpG-binding domain) proteins, the UHRF (ubiquitin-like, containing PHD and RING finger domain) proteins, and zinc-finger domain proteins, can bind to 5-mC with a high affinity to inhibit transcription factor binding [64]. DNA methylation represents a hallmark of gene silencing. The mechanism of silencing can be mainly through (1) the promotion of methylated DNA in the recruitment of methyl-CpG-binding domain (MBD) proteins which, in turn, recruit histone-modifying and chromatin-remodelling complexes to methylated sites [65, 66] or (2) a preclusion in the recruitment of DNA binding proteins from their target sites [67].

2.5. DNA Methylation in Arterial Calcification. High phosphate concentration is tightly related to AC in patients with chronic kidney disease. de Oca et al. found that DNMT activity and methylation of the promoter region of the smooth muscle cell-specific protein SM22a increased with high phosphate concentration (3.3 mmol/L), using two *in vitro* models [68]. This was accompanied by a loss of SM22a, a gain of the osteoblast transcription factor Cbfa1, and the increased activity of ALP with subsequent *in vitro* calcification. They also demonstrated that procaine (a demethylating agent) decreased DNMT activity and methylation of the SM22a promoter, which was accompanied by upregulation of SM22a expression and less calcification. In addition, downregulation of SM22a by siRNA or a methyl group donor (S-adenosyl methionine) led to the overexpression of Cbfa1 [68]. This study was the first to link DNA methylation to the loss of smooth muscle lineage marker SM22a in VSMCs incubated with high phosphate and provided epigenetic mechanisms underlying phosphate-induced calcification of VSMCs.

2.6. Histone Modification and Chromatin Remodelling. The next breakthrough came with the identification of histone modifications in the mid-1990s, after which the DNA world developed from one dimension (linear sequence of base pairs) to three dimensions (nuclear topology), with the realisation of the important role of chromatin structure in regulating the genome [69]. The basic unit of chromatin, the nucleosome, is comprised of an octamer of four core histone proteins (H2A, H2B, H3, and H4) which are wrapped around a 147 bp segment of DNA in 1.65 left-handed turns [55]. Histone proteins within the nucleosomal core are predominantly globular except for their N-terminal "tails," which are unstructured and subject to modification [55]. At least eight distinct types of modifications occur on histone tails: acetylation, methylation, phosphorylation, ubiquitination, SUMOylation, ADPribosylation, deimination, and isomerisation. Histone modifications play vital roles in transcriptional regulation, for instance, chromosome condensation [55], DNA replication, alternative splicing [70], and DNA repair [71]. In contrast to methylation, histone modifications are more dynamic and are indirectly correlated

to gene silencing or activation [72]. Furthermore, histone modifications are tightly associated with the context in which they occur and the presence of additional modifications, indicating the existence of a "histone code" [72, 73]. Among the modifications, histone acetylation is the most well-studied. Histone acetylation takes place on lysine (K) residues and refers to the transfer of an acetyl group from acetyl-coenzyme A complexes. In mammals, this reaction is exerted mainly by three histone acetyltransferase (HAT) families, including CBP/p300, GNAT, and MYST [55, 74]. This modification often occurs along with transcriptional activation via stabilising the basic charge of lysine residues and reducing their affinity for DNA and finally preventing the formation of highly condensed chromatin in some cases [74–76]. In contrast, the effect of HAT could be antagonised by the opposing histone deacetylase (HDAC) enzymatic activity which mediates the removal of acetyl groups from lysine residues. At least four classes of HDACs have been identified: class I (HDAC1–3, HDAC8), class II (HDAC4–7, HDAC 9-10), class III sirtuins (SIRT1–7), and class IV (HDAC11) [74]. These HDACs are Zn^{2+}-dependent, except class III HDACs, which are NAD-dependent. This enzymatic activity has been related to cell-cycle progression, gene silencing, differentiation, and DNA damage-induced response [76]. Since HATs and HDACs are two large enzymes families of antagonistic actions, the balance between them accounts for a pivotal regulatory mechanism for gene expression, developmental processes, and disease progression. In addition to histone modifications, chromatin can be remodelled by ATP-dependent chromatin remodelling complexes [77], which make use of ATP hydrolysis to alter the histone-DNA interaction so that nucleosomal DNA becomes much more accessible to interacting proteins [78]. These variations in chromatin structure result in changes in transcription in numerous sorts of biological processes and provide enormous modifications of functional responsiveness.

2.7. Histone Modification in Arterial Calcification. The switch of VSMCs from a contractile to synthetic phenotype is controlled by a series of transcription factors, particularly serum response factor (SRF) and its main cofactor, myocardin [79]. Almost all SMC-specific protein genes and genes that are important for SMC phenotypic switching contain the CArG box DNA sequence within their promoter [80]. Binding of SRF to the CArG box sequences activates VSMCs-specific contractile genes. Recent data indicate that the SRF binding in SMCs is modulated at the level of chromatin structure. Experiments *in vivo* and *in vitro* have shown that the binding of SRF to CArG boxes of SMC-specific genes such as SM22 is highly dependent on histone modifications [81, 82]. These include acetylation of lysine 9 in histone H3, acetylation of histone H4, and dimethylation of lysine residues 4 and 79 in histone H3. Moreover, deacetylation of histone H4 was accompanied by a loss of SRF binding to CArG box DNA during attenuation of SMC differentiation in response to vascular injury [82]. SRF-mediated transcriptional activation could be enhanced by its coactivator myocardin via interaction with the MADS box of SRF. Myocardin can recruit p300, a HAT that is associated with the transcriptional activation domain of myocardin and increases the transcriptional activity of specific genes [83]. In contrast, class II HDACs suppress smooth muscle gene activation by interacting with a domain of myocardin different from the p300-binding domain.

2.8. miRNAs. A newly discovered epigenetic mark involves miRNAs, a novel class of small, noncoding RNAs that are not translated into proteins [84]. miRNAs are initially transcribed in the nucleus by RNA polymerase II or III, forming long precursor transcripts [85]. These precursor RNA molecules subsequently undergo a specific cleavage driven by the Drosha and Dicer enzymes in the nucleus and cytoplasm, respectively. The resulting mature miRNA is single-stranded and composed of 18–22 nucleotides. miRNA is loaded into a miRNA-induced silencing complex (RISC) and directed to the target mRNA by pairing with sequences in the 3′UTR of target mRNA, leading to either degradation of mRNA or translational suppression. Studying miRNAs and their effects on translation is one of the most novel and active areas of epigenetic research. Approximately 1500 different miRNAs have been identified in humans so far, and the number will probably increase in the future [86]. The potential impact of miRNA-mediated biological regulation is estimated to be considerable. Numerous miRNAs have been found to be critical modulators of vascular pathologies, such as atherosclerosis, arterial remodelling, angiogenesis, and apoptosis [87].

2.9. miRNAs in Arterial Calcification. The research into miRNAs is rapidly growing and recent studies have disclosed a significant role of miRNAs in vascular biology and disease [87]. To date, an array of miRNAs have been also shown to be associated with AC (Table 1). Recent study demonstrated miR-125b targets transcription factor SP7 [88] or Ets1 [89] in regulating the osteogenic transdifferentiation of VSMCs; moreover, miR-125b was found to be inhibited during atherosclerotic plaque formation and was downregulated in calcified vessels [88], providing the first report concentrating on the effect of miRNAs on arterial calcification. Du et al. revealed a novel regulatory role of the miR-29/ADAMTS-7/COMP axis during arterial calcification *in vitro* and *in vivo* [90]. MiR-29a/b level was repressed in high-phosphate-induced calcifying VSMCs or blood vessels with chronic kidney disease. Additionally, Balderman et al. disclosed that the downregulation of miR-30b and miR-30c by BMP-2 increased Runx2 expression and facilitated VSMCs calcification [91]. Our group showed that miR-133a and miR-204 modulated VSMCs calcification by targeting Runx2 [92, 93]. We found that miR-133a was also significantly decreased during osteogenic differentiation of VSMCs treated with β-glycerophosphate. Overexpression of miR-133a inhibited VSMCs transdifferentiation into osteoblast-like cells, as proven by a decrease in ALP activity, OC secretion, Runx2 expression, and mineralised nodule formation. Conversely, the knockdown of miR-133a with a miR-133a inhibitor promoted osteogenic differentiation of VSMCs by increasing ALP activity, OC secretion, and Runx2

Table 1: miRNAs involved in the regulation of arterial calcification.

miRNAs	Observation	Ref.
miR-125b	Target SP7, inhibiting osteogenic transdifferentiation of VSMCs	[88, 89]
miR-133a	Target Runx2, inhibiting the osteogenic differentiation of VSMCs	[92]
miR-135a*/762/714/712*	Targets NCX1, PMCA1, and NCKX4, promoting VSMCs calcification	[94]
miR-204	Target Runx2, inhibiting VSMCs calcification	[93]
miR-221/222	Targets Enpp1 and Pit-1, contributing to arterial calcification	[95]
miR-223	Targets Mef2c and RhoB, inducing VSMCs migration and calcification	[96]
miR-29a/b	Target ADAMTS-7, inhibiting VSMCs calcification	[90]
miR-30b/30c	Target Runx2, inhibiting VSMCs calcification	[91]

expression [92]. Moreover, we demonstrated that *in vitro* miR-204 was decreased in mouse aortic VSMCs during β-glycerophosphate-induced calcification, whereas Runx2 protein levels were elevated. Overexpression of miR-204 by transfection of miR-204 mimics suppressed Runx2 protein levels and attenuated β-glycerophosphate-induced osteoblastic differentiation of VSMCs, whereas miR-204 inhibition by transfection of miR-204 inhibitors significantly increased Runx2 protein levels and promoted osteoblastic differentiation of VSMCs, suggesting the role of miR-204 as an endogenous attenuator of Runx2 in VSMCs calcification. *In vivo* overexpression of miR-204 by injection of miR-204 agomirs in mice alleviated vitamin D3-induced medial artery calcification [93]. Contrary to the negative role of the above miRNAs, it has been reported that some miRNAs promote AC. Gui et al. identified that the increased expression of miR-135a*, miR-762, miR-714, and miR-712* in VSMCs may be associated with VSMCs calcification by disrupting the potential Ca^{2+} efflux target proteins NCX1, PMCA1, and NCKX4 [94]. Mackenzie et al. proved that miR-221 and miR-222 act concomitantly to influence the trans-differentiation of murine VSMCs and contribute to the pathological process of arterial calcification *in vitro*, which may probably occur via the calcification regulators Enpp1 and Pit-1 [95]. Rangrez et al. also found that overexpressing miR-223 in VSMCs targeted Mef2c and RhoB and tended to increase VSMCs migration and calcification [96]. Taken together, all of these studies revealed the vital roles of miRNAs in osteogenic transdifferentiation and calcification of VSMCs (Table 1).

It is reported that epigenetic marks vary during aging, including a global decrease in the sufficiency of 5-methylcytosines and some histone modifications [97]. Since AC is an age-related disease, it could be speculated that those age-related changes in epigenetic marks are involved in the pathophysiology of the calcification. However, this remains to be demonstrated.

3. Conclusions and Perspectives

As reviewed above, epigenetic modifications such as DNA methylation, histone modifications, and miRNAs offer a new perspective in the control of gene expression, with significant applications to AC. Apart from deepening our understanding of disease mechanisms, epigenetics may be targeted for future therapies and genetic interventions. For instance, the demethylating agents 5-aza-20-deoxycytidine and procaine have been utilized in several experimental studies [98, 99]. Some HATs and HDACs inhibitors are applied in the treatment of cancer, cardiovascular disease, and neurological disorders [100, 101]. Nevertheless, their potential application is limited attributed to their nonspecific activation of genes and other genomic elements not only in diseased cells but also in normal cells. Thus, agents that are capable of regulating the epigenetic control of genes specifically in a given pathway would be much more useful. In accordance with this, therapies based on miRNAs or small interfering RNAs might be more specific and promising [102, 103]. However, studies of drugs focused on epigenetics in AC that act at the risk-factor level are still scarce. Additional studies are undoubtedly required to further elucidate how epigenetic phenomena impact the development of AC exactly and for the design of alternative treatment strategies, aimed at interfering in these epigenetic processes for the management of a variety of cardiovascular diseases related to AC.

Conflict of Interests

The authors state no conflict of interests and have received no payment in preparation of this paper.

Authors' Contribution

Shan-Shan Wu and Xiao Lin contributed equally to this work.

Acknowledgments

This work was supported by funding from The National Natural Science Foundation of China (Grant nos. 81270962 and 81370973), the National Basic Research Program of China (973 Program) (2014CB942903), the Fundamental Research Funds for the Central Universities of Central South University (Grant no. 2013zzts326), and the Program for New Century Excellent Talents in University (NCET-13-0601).

References

[1] W. G. Goodman, J. Goldin, B. D. Kuizon et al., "Coronary-artery calcification in young adults with end-stage renal disease who are undergoing dialysis," *The New England Journal of Medicine*, vol. 342, no. 20, pp. 1478–1483, 2000.

[2] F. Verbeke, W. van Biesen, E. Honkanen et al., "Prognostic value of aortic stiffness and calcification for cardiovascular events and mortality in dialysis patients: outcome of the Calcification Outcome in Renal Disease (CORD) study," *Clinical Journal of the American Society of Nephrology*, vol. 6, no. 1, pp. 153–159, 2011.

[3] A. N. Qasim, S. S. Martin, N. N. Mehta et al., "Lipoprotein(a) is strongly associated with coronary artery calcification in type-2 diabetic women," *International Journal of Cardiology*, vol. 150, no. 1, pp. 17–21, 2011.

[4] I. E. Schauer, J. K. Snell-Bergeon, B. C. Bergman et al., "Insulin resistance, defective insulin-mediated fatty acid suppression, and coronary artery calcification in subjects with and without type 1 diabetes the CACTI study," *Diabetes*, vol. 60, no. 1, pp. 306–314, 2011.

[5] R. Virmani, A. P. Burke, A. Farb, and F. D. Kolodgie, "Pathology of the vulnerable plaque," *Journal of the American College of Cardiology*, vol. 47, no. 8, pp. C13–C18, 2006.

[6] Y. Vengrenyuk, L. Cardoso, and S. Weinbaum, "Micro-CT based analysis of a new paradigm for vulnerable plaque rupture: cellular microcalcifications in fibrous caps," *Molecular and Cellular Biomechanics*, vol. 5, no. 1, pp. 37–47, 2008.

[7] T. Hoshino, L. A. Chow, J. J. Hsu et al., "Mechanical stress analysis of a rigid inclusion in distensible material: a model of atherosclerotic calcification and plaque vulnerability," *The American Journal of Physiology—Heart and Circulatory Physiology*, vol. 297, no. 2, pp. H802–H810, 2009.

[8] M. J. Duer, T. Friščić, D. Proudfoot et al., "Mineral surface in calcified plaque is like that of bone further evidence for regulated mineralization," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 28, no. 11, pp. 2030–2034, 2008.

[9] Y. Du, Y. Wang, L. Wang et al., "Cartilage oligomeric matrix protein inhibits vascular smooth muscle calcification by interacting with bone morphogenetic protein-2," *Circulation Research*, vol. 108, no. 8, pp. 917–928, 2011.

[10] L.-Q. Yuan, J.-H. Zhu, H.-W. Wang et al., "RANKL is a downstream mediator for insulin-induced osteoblastic differentiation of vascular smooth muscle cells," *PLoS ONE*, vol. 6, no. 12, Article ID e29037, 2011.

[11] Q.-H. Liang, Y. Jiang, X. Zhu et al., "Ghrelin attenuates the osteoblastic differentiation of vascular smooth muscle cells through the erk pathway," *PLoS ONE*, vol. 7, no. 4, Article ID e33126, 2012.

[12] T. M. Doherty, L. A. Fitzpatrick, D. Inoue et al., "Molecular, endocrine, and genetic mechanisms of arterial calcification," *Endocrine Reviews*, vol. 25, no. 4, pp. 629–672, 2004.

[13] F. Rutsch, Y. Nitschke, and R. Terkeltaub, "Genetics in arterial calcification: pieces of a puzzle and cogs in a wheel," *Circulation Research*, vol. 109, no. 5, pp. 578–592, 2011.

[14] R. Wallin, N. Wajih, G. Todd Greenwood, and D. C. Sane, "Arterial calcification: a review of mechanisms, animal models, and the prospects for therapy," *Medicinal Research Reviews*, vol. 21, no. 4, pp. 274–301, 2001.

[15] C. H. Waddington, "The epigenotype," *International Journal of Epidemiology*, vol. 41, no. 1, pp. 10–13, 2012.

[16] A. D. Goldberg, C. D. Allis, and E. Bernstein, "Epigenetics: a landscape takes shape," *Cell*, vol. 128, no. 4, pp. 635–638, 2007.

[17] M. Esteller, "Epigenetics in cancer," *The New England Journal of Medicine*, vol. 358, pp. 1148–1159, 2008.

[18] A. P. Feinberg, "Epigenetics at the epicenter of modern medicine," *JAMA*, vol. 299, no. 11, pp. 1345–1350, 2008.

[19] M. Esteller, "Cancer epigenetics for the 21st century: what's next?" *Genes and Cancer*, vol. 2, no. 6, pp. 604–606, 2011.

[20] M. Münzel, D. Globisch, and T. Carell, "5-hydroxymethylcytosine, the sixth base of the genome," *Angewandte Chemie International Edition*, vol. 50, no. 29, pp. 6460–6468, 2011.

[21] A. Portela and M. Esteller, "Epigenetic modifications and human disease," *Nature Biotechnology*, vol. 28, no. 10, pp. 1057–1068, 2010.

[22] L. M. Villeneuve and R. Natarajan, "The role of epigenetics in the pathology of diabetic complications," *The American Journal of Physiology—Renal Physiology*, vol. 299, no. 1, pp. F14–F25, 2010.

[23] G. Weissen-Plenz, Y. Nitschke, and F. Rutsch, "Mechanisms of arterial calcification: spotlight on the inhibitors," *Advances in Clinical Chemistry*, vol. 46, pp. 263–293, 2008.

[24] F. Rutsch and R. Terkeltaub, "Deficiencies of physiologic calcification inhibitors and low-grade inflammation in arterial calcification: lessons for cartilage calcification," *Joint Bone Spine*, vol. 72, no. 2, pp. 110–118, 2005.

[25] A. Sweatt, D. C. Sane, S. M. Hutson, and R. Wallin, "Matrix Gla protein (MGP) and bone morphogenetic protein-2 in aortic calcified lesions of aging rats," *Journal of Thrombosis and Haemostasis*, vol. 1, no. 1, pp. 178–185, 2003.

[26] M. Ketteler, P. Bongartz, R. Westenfeld et al., "Association of low fetuin-A (AHSG) concentrations in serum with cardiovascular mortality in patients on dialysis: a cross-sectional study," *The Lancet*, vol. 361, no. 9360, pp. 827–833, 2003.

[27] S. M. Moe and N. X. Chen, "Mechanisms of vascular calcification in chronic kidney disease," *Journal of the American Society of Nephrology*, vol. 19, no. 2, pp. 213–216, 2008.

[28] T. Wada, M. D. McKee, S. Steitz, and C. M. Giachelli, "Calcification of vascular smooth muscle cell cultures: inhibition by osteopontin," *Circulation Research*, vol. 84, no. 2, pp. 166–178, 1999.

[29] P. A. Price, H. H. June, J. R. Buckley, and M. K. Williamson, "Osteoprotegerin inhibits artery calcification induced by warfarin and by vitamin D," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 21, no. 10, pp. 1610–1616, 2001.

[30] K. A. Hruska, S. Mathew, and G. Saab, "Bone morphogenetic proteins in vascular calcification," *Circulation Research*, vol. 97, no. 2, pp. 105–114, 2005.

[31] P. Collin-Osdoby, "Regulation of vascular calcification by osteoclast regulatory factors RANKL and osteoprotegerin," *Circulation Research*, vol. 95, no. 11, pp. 1046–1057, 2004.

[32] G.-Y. Liu, Q.-H. Liang, R.-R. Cui et al., "Leptin promotes the osteoblastic differentiation of vascular smooth muscle cells from female mice by increasing RANKL expression," *Endocrinology*, vol. 155, no. 2, pp. 558–567, 2014.

[33] L. L. Demer and Y. Tintut, "Vascular calcification: pathobiology of a multifaceted disease," *Circulation*, vol. 117, no. 22, pp. 2938–2948, 2008.

[34] A. Tanimura, D. H. McGregor, and H. C. Anderson, "Matrix vesicles in atherosclerotic calcification," *Proceedings of the Society for Experimental Biology and Medicine*, vol. 172, no. 2, pp. 173–177, 1983.

[35] D. Proudfoot, J. N. Skepper, L. Hegyi, M. R. Bennett, C. M. Shanahan, and P. L. Weissberg, "Apoptosis regulates human vascular calcification in vitro: evidence for initiation of vascular calcification by apoptotic bodies," *Circulation Research*, vol. 87, no. 11, pp. 1055–1062, 2000.

[36] P.-F. Shan, Y. Lu, R.-R. Cui, Y. Jiang, L.-Q. Yuan, and E.-Y. Liao, "Apelin attenuates the osteoblastic differentiation of vascular smooth muscle cells," *PLoS ONE*, vol. 6, no. 3, Article ID e17938, 2011.

[37] X.-B. Liao, X.-M. Zhou, J.-M. Li et al., "Taurine inhibits osteoblastic differentiation of vascular smooth muscle cells via

the ERK pathway," *Amino Acids*, vol. 34, no. 4, pp. 525–530, 2008.

[38] M. Schoppet, R. C. Shroff, L. C. Hofbauer, and C. M. Shanahan, "Exploring the biology of vascular calcification in chronic kidney disease: what's circulating?" *Kidney International*, vol. 73, no. 4, pp. 384–390, 2008.

[39] L. L. Demer, Y. Tintut, and F. Parhami, "Novel mechanisms in accelerated vascular calcification in renal disease patients," *Current Opinion in Nephrology and Hypertension*, vol. 11, no. 4, pp. 437–443, 2002.

[40] H. Yang, G. Curinga, and C. M. Giachelli, "Elevated extracellular calcium levels induce smooth muscle cell matrix mineralization in vitro," *Kidney International*, vol. 66, no. 6, pp. 2293–2299, 2004.

[41] S. Jono, M. D. McKee, C. E. Murry et al., "Phosphate regulation of vascular smooth muscle cell calcification," *Circulation Research*, vol. 87, no. 7, pp. e10–e17, 2000.

[42] M. Abedin, Y. Tintut, and L. L. Demer, "Vascular calcification: Mechanisms and clinical ramifications," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 24, no. 7, pp. 1161–1170, 2004.

[43] J. D. Miller, Y. Chu, R. M. Brooks, W. E. Richenbacher, R. Peña-Silva, and D. D. Heistad, "Dysregulation of antioxidant mechanisms contributes to increased oxidative stress in calcific aortic valvular stenosis in humans," *Journal of the American College of Cardiology*, vol. 52, no. 10, pp. 843–850, 2008.

[44] C. Y. Y. Yip, J.-H. Chen, R. Zhao, and C. A. Simmons, "Calcification by valve interstitial cells is regulated by the stiffness of the extracellular matrix," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 29, no. 6, pp. 936–942, 2009.

[45] X. Li, H.-Y. Yang, and C. M. Giachelli, "Role of the sodium-dependent phosphate cotransporter, Pit-1, in vascular smooth muscle cell calcification," *Circulation Research*, vol. 98, no. 7, pp. 905–912, 2006.

[46] K. Amann, "Media calcification and intima calcification are distinct entities in chronic kidney disease," *Clinical Journal of the American Society of Nephrology*, vol. 3, no. 6, pp. 1599–1605, 2008.

[47] J. L. Hunt, R. Fairman, M. E. Mitchell et al., "Bone formation in carotid plaques: a clinicopathological study," *Stroke*, vol. 33, no. 5, pp. 1214–1219, 2002.

[48] A. P. Burke, A. Taylor, A. Farb, G. T. Malcom, and R. Virmani, "Coronary calcification: insights from sudden coronary death victims," *Zeitschrift fur Kardiologie*, vol. 89, supplement, no. 2, pp. S49–S53, 2000.

[49] A. Farzaneh-Far, D. Proudfoot, C. Shanahan, and P. L. Weissberg, "Vascular and valvar calcification: recent advances," *Heart*, vol. 85, no. 1, pp. 13–17, 2001.

[50] M. E. Edmonds, N. Morrison, J. W. Laws, and P. J. Watkins, "Medial arterial calcification and diabetic neuropathy," *British Medical Journal*, vol. 284, no. 6320, pp. 928–930, 1982.

[51] J. G. Mönckeberg, "Über die reine Mediaverkalkung der Extremitätenarterien und ihr Verhalten zur Arteriosklerose," *Virchows Archiv für pathologische Anatomie und Physiologie und für klinische Medizin*, vol. 171, no. 1, pp. 141–167, 1903.

[52] M.-C. Wang, W.-C. Tsai, J.-Y. Chen, and J.-J. Huang, "Stepwise increase in arterial stiffness corresponding with the stages of chronic kidney disease," *The American Journal of Kidney Diseases*, vol. 45, no. 3, pp. 494–501, 2005.

[53] T. C. Lin, Y. Tintut, A. Lyman, W. MacK, L. L. Demer, and T. K. Hsiai, "Mechanical response of a calcified plaque model to fluid shear force," *Annals of Biomedical Engineering*, vol. 34, no. 10, pp. 1535–1541, 2006.

[54] L. van Speybroeck, "From epigenesis to epigenetics: the case of C. H. Waddington," *Annals of the New York Academy of Sciences*, vol. 981, pp. 61–81, 2002.

[55] T. Kouzarides, "Chromatin modifications and their function," *Cell*, vol. 128, no. 4, pp. 693–705, 2007.

[56] R. Holliday and J. E. Pugh, "DNA modification mechanisms and gene activity during development. Developmental clocks may depend on the enzymic modification of specific bases in repeated DNA sequences," *Science*, vol. 187, no. 4173, pp. 226–232, 1975.

[57] S. Saxonov, P. Berg, and D. L. Brutlag, "A genome-wide analysis of CpG dinucleotides in the human genome distinguishes two distinct classes of promoters," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 5, pp. 1412–1417, 2006.

[58] R. A. Irizarry, C. Ladd-Acosta, B. Wen et al., "The human colon cancer methylome shows similar hypo- and hypermethylation at conserved tissue-specific CpG island shores," *Nature Genetics*, vol. 41, no. 2, pp. 178–186, 2009.

[59] Z.-X. Chen, J. R. Mann, C.-L. Hsieh, A. D. Riggs, and F. Chédin, "Physical and functional interactions between the human DNMT3L protein and members of the de novo methyltransferase family," *Journal of Cellular Biochemistry*, vol. 95, no. 5, pp. 902–917, 2005.

[60] C. Holz-Schietinger and N. O. Reich, "The inherent processivity of the human de *Novo* methyltransferase 3A (DNMT3A) is enhanced by DNMT3L," *The Journal of Biological Chemistry*, vol. 285, no. 38, pp. 29091–29100, 2010.

[61] M. G. Goll, F. Kirpekar, K. A. Maggert et al., "Methylation of $tRNA^{Asp}$ by the DNA methyltransferase homolog Dnmt2," *Science*, vol. 311, no. 5759, pp. 395–398, 2006.

[62] S. Kangaspeska, B. Stride, R. Métivier et al., "Transient cyclical methylation of promoter DNA," *Nature*, vol. 452, no. 7183, pp. 112–115, 2008.

[63] R. Métivier, R. Gallais, C. Tiffoche et al., "Cyclical DNA methylation of a transcriptionally active promoter," *Nature*, vol. 452, pp. 45–50, 2008.

[64] L. D. Moore, T. Le, and G. Fan, "DNA methylation and its basic function," *Neuropsychopharmacology*, vol. 38, no. 1, pp. 23–38, 2013.

[65] L. Lopez-Serra and M. Esteller, "Proteins that bind methylated DNA and human cancer: reading the wrong words," *British Journal of Cancer*, vol. 98, no. 12, pp. 1881–1885, 2008.

[66] M. Esteller, "Epigenetic gene silencing in cancer: the DNA hypermethylome," *Human Molecular Genetics*, vol. 16, no. 1, pp. R50–R59, 2007.

[67] A. Kuroda, T. A. Rauch, I. Todorov et al., "Insulin gene expression is regulated by DNA methylation," *PLoS ONE*, vol. 4, no. 9, Article ID e6953, 2009.

[68] A. M. de Oca, J. A. Madueño, J. M. Martinez-Moreno et al., "High-phosphate-induced calcification is related to *SM22α* promoter methylation in vascular smooth muscle cells," *Journal of Bone and Mineral Research*, vol. 25, no. 9, pp. 1996–2005, 2010.

[69] B. M. Turner, "Histone acetylation as an epigenetic determinant of long-term transcriptional competence," *Cellular and Molecular Life Sciences*, vol. 54, no. 1, pp. 21–31, 1998.

[70] R. F. Luco, Q. Pan, K. Tominaga, B. J. Blencowe, O. M. Pereira-Smith, and T. Misteli, "Regulation of alternative splicing by histone modifications," *Science*, vol. 327, no. 5968, pp. 996–1000, 2010.

[71] D. Huertas, R. Sendra, and P. Muñoz, "Chromatin dynamics coupled to DNA repair," *Epigenetics*, vol. 4, no. 1, pp. 31–42, 2009.

[72] M. S. Cosgrove and C. Wolberger, "How does the histone code work?" *Biochemistry and Cell Biology*, vol. 83, no. 4, pp. 468–476, 2005.

[73] T. Jenuwein and C. D. Allis, "Translating the histone code," *Science*, vol. 293, no. 5532, pp. 1074–1080, 2001.

[74] G. G. Wang, C. D. Allis, and P. Chi, "Chromatin remodeling and cancer, part I: covalent histone modifications," *Trends in Molecular Medicine*, vol. 13, no. 9, pp. 363–372, 2007.

[75] A. Kimura, K. Matsubara, and M. Horikoshi, "A decade of histone acetylation: marking eukaryotic chromosomes with specific codes," *Journal of Biochemistry*, vol. 138, no. 6, pp. 647–662, 2005.

[76] S. Thiagalingam, K.-H. Cheng, H. J. Lee, N. Mineva, A. Thiagalingam, and J. F. Ponte, "Histone deacetylases: unique players in shaping the epigenetic histone code," *Annals of the New York Academy of Sciences*, vol. 983, pp. 84–100, 2003.

[77] A. Lusser and J. T. Kadonaga, "Chromatin remodeling by ATP-dependent molecular machines," *BioEssays*, vol. 25, no. 12, pp. 1192–1200, 2003.

[78] P. B. Becker and W. Hörz, "ATP-dependent nucleosome remodeling," *Annual Review of Biochemistry*, vol. 71, pp. 247–273, 2002.

[79] J. M. Miano, X. Long, and K. Fujiwara, "Serum response factor: master regulator of the actin cytoskeleton and contractile apparatus," *The American Journal of Physiology—Cell Physiology*, vol. 292, no. 1, pp. C70–C81, 2007.

[80] J. M. Miano, "Serum response factor: toggling between disparate programs of gene expression," *Journal of Molecular and Cellular Cardiology*, vol. 35, no. 6, pp. 577–593, 2003.

[81] P. Qiu and L. Li, "Histone acetylation and recruitment of serum responsive factor and CREB-binding protein onto SM22 promoter during SM22 gene expression," *Circulation Research*, vol. 90, no. 8, pp. 858–865, 2002.

[82] O. G. McDonald, B. R. Wamhoff, M. H. Hoofnagle, and G. K. Owens, "Control of SRF binding to CArG box chromatin regulates smooth muscle gene expression in vivo," *The Journal of Clinical Investigation*, vol. 116, no. 1, pp. 36–48, 2006.

[83] D. Cao, Z. Wang, C.-L. Zhang et al., "Modulation of smooth muscle gene expression by association of histone acetyltransferases and deacetylases with myocardin," *Molecular and Cellular Biology*, vol. 25, no. 1, pp. 364–376, 2005.

[84] V. Ambros, "The functions of animal microRNAs," *Nature*, vol. 431, no. 7006, pp. 350–355, 2004.

[85] M. Faller and F. Guo, "MicroRNA biogenesis: there's more than one way to skin a cat," *Biochimica et Biophysica Acta: Gene Regulatory Mechanisms*, vol. 1779, no. 11, pp. 663–667, 2008.

[86] I. Bentwich, A. Avniel, Y. Karov et al., "Identification of hundreds of conserved and nonconserved human microRNAs," *Nature Genetics*, vol. 37, no. 7, pp. 766–770, 2005.

[87] M. S. Jamaluddin, S. M. Weakley, L. Zhang et al., "MiRNAs: roles and clinical applications in vascular disease," *Expert Review of Molecular Diagnostics*, vol. 11, no. 1, pp. 79–89, 2011.

[88] C. Goettsch, M. Rauner, N. Pacyna, U. Hempel, S. R. Bornstein, and L. C. Hofbauer, "MiR-125b regulates calcification of vascular smooth muscle cells," *The American Journal of Pathology*, vol. 179, no. 4, pp. 1594–1600, 2011.

[89] P. Wen, H. Cao, L. Fang et al., "miR-125b/Ets1 axis regulates transdifferentiation and calcification of vascular smooth muscle cells in a high-phosphate environment," *Experimental Cell Research*, vol. 322, no. 2, pp. 302–312, 2014.

[90] Y. Du, C. Gao, Z. Liu et al., "Upregulation of a disintegrin and metalloproteinase with thrombospondin motifs-7 by miR-29 repression mediates vascular smooth muscle calcification," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 32, no. 11, pp. 2580–2588, 2012.

[91] J. A. F. Balderman, H.-Y. Lee, C. E. Mahoney et al., "Bone morphogenetic protein-2 decreases microRNA-30b and microRNA-30c to promote vascular smooth muscle cell calcification," *Journal of the American Heart Association*, vol. 1, no. 6, Article ID e003905, 2012.

[92] X.-B. Liao, Z.-Y. Zhang, K. Yuan et al., "MiR-133a modulates osteogenic differentiation of vascular smooth muscle cells," *Endocrinology*, vol. 154, no. 9, pp. 3344–3352, 2013.

[93] R.-R. Cui, S.-J. Li, L.-J. Liu et al., "MicroRNA-204 regulates vascular smooth muscle cell calcification in vitro and in vivo," *Cardiovascular Research*, vol. 96, no. 2, pp. 320–329, 2012.

[94] T. Gui, G. Zhou, Y. Sun et al., "MicroRNAs that target Ca^{2+} transporters are involved in vascular smooth muscle cell calcification," *Laboratory Investigation*, vol. 92, no. 9, pp. 1250–1259, 2012.

[95] N. C. W. Mackenzie, K. A. Staines, D. Zhu, P. Genever, and V. E. MacRae, "miRNA-221 and miRNA-222 synergistically function to promote vascular calcification," *Cell Biochemistry and Function*, vol. 32, no. 2, pp. 209–216, 2014.

[96] A. Y. Rangrez, E. M'Baya-Moutoula, V. Metzinger-Le Meuth et al., "Inorganic phosphate accelerates the migration of vascular smooth muscle cells: evidence for the involvement of miR-223," *PLoS ONE*, vol. 7, no. 10, Article ID e47807, 2012.

[97] M. F. Fraga and M. Esteller, "Epigenetics and aging: the targets and the marks," *Trends in Genetics*, vol. 23, no. 8, pp. 413–418, 2007.

[98] A. J. Patterson, M. Chen, Q. Xue, D. Xiao, and L. Zhang, "Chronic prenatal hypoxia induces epigenetic programming of PKCε gene repression in rat hearts," *Circulation Research*, vol. 107, no. 3, pp. 365–373, 2010.

[99] Y. Saito, G. Liang, G. Egger et al., "Specific activation of microRNA-127 with downregulation of the proto-oncogene BCL6 by chromatin-modifying drugs in human cancer cells," *Cancer Cell*, vol. 9, no. 6, pp. 435–443, 2006.

[100] V. Santini, A. Gozzini, and G. Ferrari, "Histone deacetylase inhibitors: molecular and biological activity as a premise to clinical application," *Current Drug Metabolism*, vol. 8, no. 4, pp. 383–394, 2007.

[101] A. Granger, I. Abdullah, F. Huebner et al., "Histone deacetylase inhibition reduces myocardial ischemia-reperfusion injury in mice," *FASEB Journal*, vol. 22, no. 10, pp. 3549–3560, 2008.

[102] E. Fabbri, E. Brognara, M. Borgatti et al., "miRNA therapeutics: delivery and biological activity of peptide nucleic acids targeting miRNAs," *Epigenomics*, vol. 3, no. 6, pp. 733–745, 2011.

[103] J. Krützfeldt, N. Rajewsky, R. Braich et al., "Silencing of microRNAs in vivo with "antagomirs"," *Nature*, vol. 438, no. 7068, pp. 685–689, 2005.

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 354517, 18 pages
http://dx.doi.org/10.1155/2015/354517

Review Article

Human miR-221/222 in Physiological and Atherosclerotic Vascular Remodeling

Dmitry A. Chistiakov,[1,2] Igor A. Sobenin,[3,4] Alexander N. Orekhov,[3,5] and Yuri V. Bobryshev[3,6,7]

[1] *Department of Medical Nanobiotechnology, Pirogov Russian State Medical University, Moscow 117997, Russia*
[2] *The Mount Sinai Community Clinical Oncology Program, Mount Sinai Comprehensive Cancer Center, Mount Sinai Medical Center, Miami Beach, FL 33140, USA*
[3] *Laboratory of Angiopathology, Institute of General Pathology and Pathophysiology, Russian Academy of Sciences, Moscow 125315, Russia*
[4] *Laboratory of Medical Genetics, Russian Cardiology Research and Production Complex, Moscow 121552, Russia*
[5] *Institute for Atherosclerosis Research, Skolkovo Innovative Center, Moscow 121609, Russia*
[6] *Faculty of Medicine and St Vincent's Centre for Applied Medical Research, University of New South Wales, Sydney, NSW 2052, Australia*
[7] *School of Medicine, University of Western Sydney, Campbelltown, NSW 2560, Australia*

Correspondence should be addressed to Yuri V. Bobryshev; y.bobryshev@unsw.edu.au

Received 6 June 2014; Accepted 29 October 2014

Academic Editor: Ling-Qing Yuan

A cluster of miR-221/222 is a key player in vascular biology through exhibiting its effects on vascular smooth muscle cells (VSMCs) and endothelial cells (ECs). These miRNAs contribute to vascular remodeling, an adaptive process involving phenotypic and behavioral changes in vascular cells in response to vascular injury. In proliferative vascular diseases such as atherosclerosis, pathological vascular remodeling plays a prominent role. The miR-221/222 cluster controls development and differentiation of ECs but inhibits their proangiogenic activation, proliferation, and migration. miR-221/222 are primarily implicated in maintaining endothelial integrity and supporting quiescent EC phenotype. Vascular expression of miR-221/222 is upregulated in initial atherogenic stages causing inhibition of angiogenic recruitment of ECs and increasing endothelial dysfunction and EC apoptosis. In contrast, these miRNAs stimulate VSMCs and switching from the VSMC "contractile" phenotype to the "synthetic" phenotype associated with induction of proliferation and motility. In atherosclerotic vessels, miR-221/222 drive neointima formation. Both miRNAs contribute to atherogenic calcification of VSMCs. In advanced plaques, chronic inflammation downregulates miR-221/222 expression in ECs that in turn could activate intralesion neoangiogenesis. In addition, both miRNAs could contribute to cardiovascular pathology through their effects on fat and glucose metabolism in nonvascular tissues such as adipose tissue, liver, and skeletal muscles.

1. Introduction

In the body, the vascular system fulfills a variety of vital functions. Blood vessels transport nutrients and oxygen to every cell and take off wastes and carbon dioxide. The vasculature is involved in maintaining body temperature, pH, and mineral homeostasis. Blood and lymph vessels transfer immune cells essential for host defense against various pathogens. The vascular network is composed of differently sized vessels like the micro-, small, medium, and large vessels.

The vascular tissues include several types of cells including endothelial cells (ECs), vascular smooth muscle cells (VSMCs), pericytes, fibroblasts, resident macrophages, resident mesenchymal stem cells (MSCs) and progenitors, and

connective tissues. The classical three-layer structure of the vascular wall involves the intima, media, and adventitia flanked with the elastic laminae [1].

The vessel wall is adopted for various functional requirements that could be changed depending on each segment of the circulatory system. For example, aorta and large arteries are enriched with elastic fibers in order to support sufficient flow and pressure for delivery of blood components to the extreme peripheral tissues [2]. The circulatory network is a dynamic system that constitutively develops and matures to be better adapted to the rapid changes in microenvironmental conditions. Physiological processes associated with structural modifications in the vascular wall and related to vascular developmental changes during embryogenesis and adaptive responses such as neovascularization are termed vascular remodeling [3, 4].

In embryogenesis, vascular remodeling is an essential mechanism that supports the development and formation of the mature vascular network. In humans, vascular remodeling starts at day 21 when the immature heart begins to beat pushing blood through the early vasculature [5]. Indeed, biomechanical and hemodynamic forces and characteristics such as shear stress, cylinder stress, pressure, velocity, and flow become applicable to the developing vessels and induce signaling cascades that contribute to angiogenesis, vessel sprouting, vascular branching, hierarchy, maturation, and arterial-vein identity [6]. These signaling pathways are activated in ECs and VSMCs through the mechanism of mechanotransduction associated with upregulation and downregulation of certain genes involved in vasculogenesis, cell differentiation, proliferation, migration, differentiation, adhesion, and cell-matrix interactions [7].

To date, mouse is a leading model system for studying the physical and molecular regulation of vascular development and embryogenic vascular remodeling. In cultured mouse ECs, mechanical shear stress was shown to activate a signal transduction complex composed of three receptors such as vascular endothelial growth factor receptor 2 (VEGFR2)/fetal liver kinase 1 (FLK1), vascular endothelial cell cadherin (VE-cadherin), and platelet endothelial cell adhesion molecule 1 (PECAM1). As a result, cells realigned parallel to the direction of flow become mechanosensitive [8]. Direct transmission of mechanical force occurs through PECAM1 to VE-cadherin that acts as an adaptor protein activating Flk1 and catalyzing the activation of the phosphatidylinositol-3-OH kinase (PI3K) signaling cascade [9]. This leads to the induction of a panel of transcription factors including Krüppel-like factors- (KLF-) 4 and 6 and T cell acute lymphocytic leukemia 1 (Tal1) essential for both hematopoiesis and vasculogenesis [10, 11].

Vascular remodeling is a crucial mechanism of the routine endothelial replacement and repair of damaged vessel wall in order to maintain vascular integrity and function and prevent thrombosis [12]. This process involves many cell types including resident and nonresident stem and nonstem cells. In arterial injury, bone mesenchymal stem cells were shown to have a capacity to differentiate to neo-ECs and contribute to reendothelization [13]. The reendothelization is controlled by bone-marrow derived transcription factor KLF-10 [14]. Medial VSMCs and adventitial fibroblasts undergo phenotypic changes associated with induction of proliferation, migration, and differentiation and enhanced production of extracellular matrix proteins and adhesion molecules and release of reactive oxygen species, chemokines, cytokines, growth factors, and matrix metalloproteinases (MMPs) that, collectively, affect medial VSMC contractility and growth directly and that stimulate recruitment and retention of circulating inflammatory and progenitor cells to the vascular wall [15]. Pericytes characterized with significant phenotypic plasticity and ability to transdifferentiate to several vascular cell types such as VSMCs, fibroblasts, and macrophages also contribute to vascular repair [16].

However, vascular remodeling can occur as a maladaptive response against vessel injury. In that case, the remodeling could be associated with various vascular abnormalities such as endothelial dysfunction, hypertrophy, or fibrosis and be a part of a pathophysiological mechanism in the development of hypertension, thrombosis, restenosis, cardiomyopathy, atherosclerosis, and other cardiovascular disease.

Atherosclerosis is characterized with marked structural alterations in the arterial wall induced by the subendothelial accumulation of modified lipoproteins followed by the chronic inflammatory response [17, 18]. Vascular remodeling results from a close interplay of changes in the vascular tone and structure. An important concept for arterial remodeling (so-called Glagov's phenomenon) considers that arteries remodel to maintain constant flow despite increases in atherosclerotic lesion mass [19]. Indeed, atherosclerotic vascular remodeling is an adaptive response of the circulatory system to the growth of atherosclerotic plaques [3].

Atherogenic vascular remodeling involves various cell types, with a prominent role of ECs and VSMCs. Inflammatory monocytes and macrophages recruited in the atherosclerotic plaque contribute to the atherosclerosis-associated changes in the vascular wall via stimulation of ECs and VSMCs [20]. Briefly, inflammatory stimuli and changes in hemodynamic characteristics of blood flow in atherosclerotic vessels such as elevated blood pressure and increased shear stress led to the proinflammatory activation of arterial endothelium and advanced apoptosis of ECs, with subsequent denudation of the arterial wall and increased risk of thrombosis [21, 22]. In contrast, VSMCs lack their quiescence and "contractile" phenotype and acquire capacity to proliferate and migrate. This leads to the neointimal formation associated with intimal thickening and ectopic calcification of atherosclerotic arteries [23]. Vascular remodeling is accompanied with upregulation or downregulation of various molecular subsets including microRNAs (miRNAs).

Within the last decade, a research focused on evaluation of a role of miRNAs in physiological and pathological structural changes in vessels becoming more and more intensive [24]. This especially concerns studying involvement of tissue-specific (e.g., vascular or heart) miRNAs that normally control embryonic angiogenesis, cardiogenesis, and/or vascular repair. Human miRNA- (miR-) 221 and its paralogue miR-222 show notable activities in the vascular network by influencing angiogenic properties of ECs [25] and phenotypic changes in

VSMCs [26]. miR-221/222 have been found to regulate essential physiological vascular processes such as angiogenesis [27], neointimal hyperplasia [28], vessel wound healing [29], and vascular aging [30]. Furthermore, this miRNA is involved in a variety of vascular-related pathological mechanisms including tumor angiogenesis [31], atherosclerotic inflammation and vascular remodeling [32], fibrosis [33], vascular calcification [34], cardiac hypertrophy [35], angiotensin II dependent hypertension [36], and diabetic hyperglycemia-induced endothelial dysfunction [37]. In this review, we provide the information about the contribution of miR-221/222 to vascular remodeling in normal and atherosclerotic vessels.

2. Biogenesis and mRNA-Silencing Function of miR-221/222

In human DNA, the miR-221/222 gene cluster is located on chromosome Xp11.3 [38]. The miR-221 and miR-222 genes are separated by a distance of 726 bp (Figure 1). Nucleotide sequences of both genes share high similarity to each other. In fact, the genes are paralogues arisen from the duplication of the ancestral gene. They are transcribed as a single long noncoding RNA precursor with RNA polymerase II [39]. The promoter region contains two canonical TATA boxes located on 550 and 190 base pairs (bp) upstream of pre-miR-222. Three poly(A) signals are located downstream of pre-miR-221. The expression of the miR-221/222 gene cluster is positively regulated by angiotensin II [36] and downregulated by a repressive complex formed by estrogen receptor α and two nuclear receptor corepressors NCOR1 and NCOR2 [38].

In the nucleus, the common pri-miR-221/222 transcript then is spliced and split by the "microprocessor" complex Drosha/DiGeorge syndrome critical region gene 8 (DGSR8) [40] with formation of the individual pre-miR-221 and pre-miR-222 precursors that both have the length of 110 nucleotides (nt). Pre-miRNAs are transferred to the cytoplasm by the nuclear transporter exportin-5, a Ran-GTP RNA-binding protein [41], where Dicer cleaves the miRNA precursors to mature miRNA duplexes. Each miRNA duplex is stabilized with the heterotrimeric complex Argonaute-2 (Ago2)/TAR RNA-binding protein (TRBP)/protein kinase R-activating protein (PACT) [42] that is a core of the RNA-induced silencing complex (RISC). Double stranded miRNA is generally a transient imperfect duplex molecule consisting of a passenger strand and a mature miRNA strand (also referred to as guide strand) [43]. The duplex was then cleaved by Dicer with the formation of a functional single stranded mature miRNA bound to the RISC complex (miRISC). During miRISC complex formation, several additional proteins are recruited including GW182 (glycine-tryptophan protein of 182 kDa), MTDH (metadherin), and SND1 (staphylococcal nuclease domain-containing protein 1). GW182 contains two repression domains, which trigger translational repression with mild effect on mRNA decay, and multiple glycine/tryptophan repeats essential for formation and maintaining stability of the multiprotein RISC complex [44]. SND1 is a coactivator of several eukaryotic transcription factors and a component of ribonucleoprotein complexes such as spliceosome and RISC complex that increases their functional efficiency and kinetics [45]. In the RISC complex, MTDH acts as a scaffold protein possessing RNA-binding properties [46]. Generally, only one strand is incorporated into the miRISC selected on the basis of its thermodynamic instability [47]. Indeed, maturation of the pre-miR-221 results in the formation of either mature 23nt-long miR-221-5p or miR-221-3p. For miR-222, generating two mature 21nt-long miRNAs (miR-222-5p and miR-222-3p) is possible (Figure 1).

Following assembly of the miRISC complexes, there is increasing evidence that miRNAs exert their posttranscriptional regulatory functions in the context of processing bodies (P-bodies). P-bodies are distinct compartments (foci) in the cytoplasm involved in RNA degradation and turnover [48]. Furthermore, P-bodies were recognized to serve as the functional site of miRNA-mediated gene silencing since duplex miRNA-containing RISC complexes were found in these compartments [49]. The miRISC complex binds target mRNA through Watson-Crick base pairing between the guide strand and the $3'$ untranslated region (UTR) of the target [50]. The precision of target recognition heavily depends on base pairing between the seeds (nt 2–8 at the $5'$ end) of the miRNA guide [51].

Extensive base pairing between the miRNA guide and mRNA target induces the degradation of mRNA target through Ago2-mediated cleavage and deadenylation that is dependent on the activity of the complex comprised by Pop2 (pyrin domain containing 2), Ccr4 (mRNA deadenylase), and Not1 (CCR-NOT transcription complex subunit 1) [52]. Deadenylated mRNA is then degraded with exoribonuclease Xrn1p and decapped with mRNA-decapping (Dcp) enzymes [53]. In the cytoplasm, mRNAs actively translated by polyribosomes are targeted by miRISC complexes to suppress translation [54]. These mRNAs could then be delivered to P-bodies for degradation or storage in order to preserve a pool of transcripts whose translation is rate-limited for cell growth [55].

3. miR-221/222 Function in Physiological Vascular Remodeling

3.1. Antiangiogenic Activity of the miR-221/222 Cluster in Vascular ECs. Inside the vascular tissue, ECs are responsible for critical functions [56, 57]. They provide inner lining to the blood vessels and the heart, secrete a variety of bioactive molecules to affect the local environment of the cells, and transfer the molecules from the blood to the interstitial fluid. During the developmental stages and in diseases, ECs tightly cooperate with the nearby muscles in mediating angiogenesis and neovascularization. Overall, ECs contribute to the maintenance and growth of normal tissues [56, 57].

It was shown that human blood cord-derived CD34+ HPCs, endothelial progenitor cells (EPCs) [58], and human umbilical vein endothelial cells (HUVECs) [59] as well as quiescent ECs [60] highly express miR-221/222 thereby

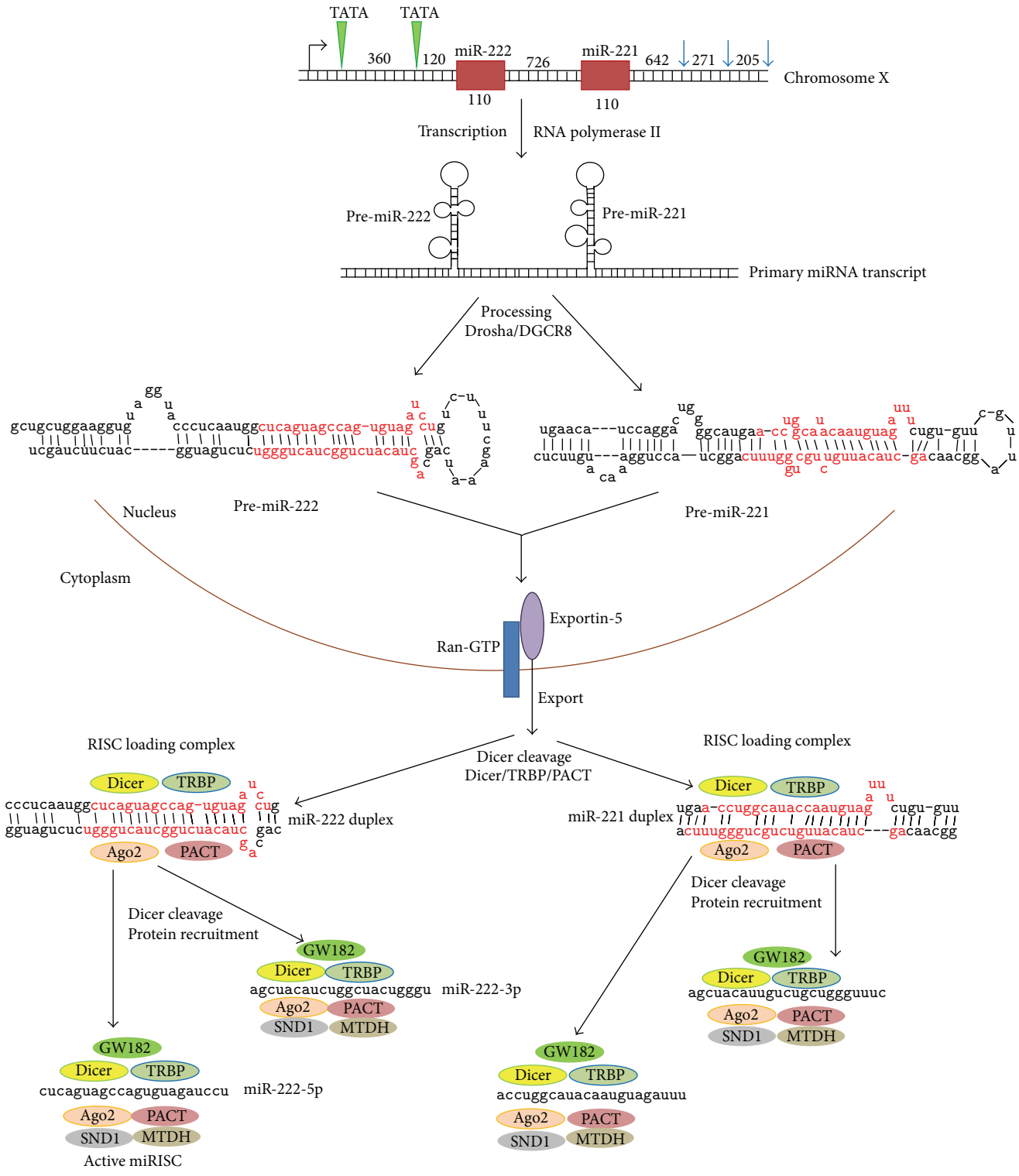


Figure 1: Biogenesis of human miR-221/222. miRNA-encoding genes are shown in red boxes. In the common promoter, TATA boxes are marked by green triangles. Poly(A) signals are displayed with blue arrows. Numbers designate the distance between the regulatory transcription elements (in bp) and the length of each miRNA gene and intergenic spacer. The sequence of a mature miRNA duplex is presented in red color. Ago2: Argonaute-2; DGCR8: DiGeorge syndrome critical region gene 8; GW182: glycine-tryptophan protein of 182 kDa; MTDH: metadherin; PACT: protein kinase R-activating protein; Ran-GTP: GTP-binding nuclear protein Ran; SND1: staphylococcal nuclease domain-containing protein 1; TRBP: TAR RNA-binding protein.

suggesting a critical role of this miRNA cluster in regulating development and function of the vascular endothelium. However, in vascular ECs, the role of miR-221/222 greatly varies depending on the developmental stages and microenvironment.

ECs were shown to express both SCF and its receptor c-kit [60–63]. Indeed c-kit-dependent angiogenic properties of ECs are targeted by miR-221/222 [25]. miR-221/222 were shown to be transcribed in c-kit-positive HUVECs suggesting a coordinated transcriptional regulation. miR221/222 downregulates c-kit on the posttranslational level since reduction in c-kit protein levels but not in mRNA levels was observed in HUVECs transfected with miR221/222 [25].

In embryonic ECs, miR-221 was shown to drive angiogenesis and especially proliferation and migration of tip ECs in sprouting neovessels [64]. In miR-221-deficient zebrafish embryos, proliferation of ECs is blocked [65]. miR-221 supports embryonic angiogenesis through targeting two key regulatory molecules, CDKN1B and PI3KR1. CDKN1B is a cyclin-dependent kinase (CDK) inhibitor $p27^{Kip1}$ that negatively regulates cell growth and prevents proliferation by inhibiting cyclin-dependent kinases [66]. PI3KR1 is the p85-regulatory subunit α of the phosphoinositide-3-kinase (PI3K) complex, an essential component of PI3K signaling that is crucial for vascular development [67]. PI3KR1 inhibits the PI3K p110 catalytic subunits but is also necessary for activity of the membrane-bound enzyme and stimulation by a receptor tyrosine kinase [68]. miR221 is likely to alter an appropriate balance in regulatory and catalytic subunits of PI3K that may indeed affect the receptor tyrosine kinase-mediated activation of PI3K and local PI3K activity in separate subcellular compartments such as growing filopodia of tip embryonic ECs.

miR-221 directs proliferation of embryonic ECs through vascular endothelial growth factor C (VEGF-C)/Fms-related tyrosine kinase 4 (Flt4) signaling [64] that is modulated by PI3K3R1 that stimulates interaction of PI3K with VEGF-C-activated Flt4 (VEGFR3), a receptor for VEGF-C and VEGF-D [69]. Activation of notch signaling in stalk ECs was shown to inhibit both Flt4 and miR-221 that leads to increase in CDKN1B levels and reducing proliferation [64]. Specification of hemogenic ECs from the primordial endothelium is controlled by c-kit, notch signaling, and $p27^{Kip1}$-dependent cell cycle control [66]. miR-221 is involved in crosstalk with all signaling components essential phenotypic definition of hemogenic ECs and therefore plays a key role at early differentiation stages of the vascular endothelial development.

In EPCs, miR-221 was found to suppress serine/threonine-protein kinase PAK1 [70] (Figure 2). PAK1 belongs to the family of $p21^{Waf1/Cip1}$-activated kinases and serves as a target for small GTPases Cdc42 and Rac [71]. EPCs are known to play an essential role in vascular repair and maintenance of vascular homeostasis through reendothelialization and neovascularization. EPCs transfected with miR-221/miR-222 loose capacity to do wound healing and tube formation [63].

PAK1 is involved in cytoskeleton reorganization and activation of EPC motility and proliferation is response to vascular injury [72, 73]. PAK1 was found to activate c-Raf/MEK/ERK signaling by phosphorylation of MEK on serine 298 [74] and RAF on serine 338 [75]. Therefore, miR-221-mediated PAK1 targeting attenuates proliferation of EPCs and impairs their function through downregulating c-Raf/MEK/ERK pathway thereby displaying antiangiogenic properties and contributing to maintenance of quiescent phenotype [70]. Interestingly, biologically active molecular components of garlic extract such as diallyl disulfide and diallyl trisulfide inhibit miR-221 expression and restore angiogenic properties of EPCs via derepressing c-kit and activating signaling protein kinases Akt and ERK 1/2 [58].

In mature human ECs, miR221/222 exhibit strictly antiangiogenic properties mediated by many targets. This mRNA cluster could display the antiangiogenic effects through inhibiting c-kit, transcription factors Ets1, Ets2 [25], zinc finger E-box binding homeobox 2 (ZEB2) [76], signal activator and transducer 5A (STAT5a) [60], and endothelial NO-synthase (eNOS) [77, 78]. Interestingly, Kaposi sarcoma-associated herpes virus (KSHV) oncogenes such as latent nuclear antigen (LANA) and kaposin B were shown to activate EC motility and proliferation essential for tumor neoangiogenesis by derepressing Ets1 and Ets2 [79, 80] through direct transcriptional suppression of miR-221/222. Ets1, a prototype of the Ets family of transcription factors, supports EC-mediated angiogenesis by inducing expression of MMPs and integrin-β3 in ECs [81] (Figure 2). eNOS, which plays a crucial role in the regulation of vascular function, contributes to angiogenesis by production of nitric oxide, a vasoactive molecule, in response to multiple stimuli [82].

In epithelial tissues, ZEB2 known as a regulator of epithelial-to-mesenchymal transition (EMT) acts as a stimulator of cell proliferation and mobility [83]. ZEB2 mRNA is directly targeted by miR-221 at the consensus site located at the 3′UTR [76]. On the other hand, ZEB2 acts as a translational repressor of mesenchyme homeobox 2 (MEOX2 or GAX), a transcriptional factor that is expressed in quiescent vascular ECs and inhibits EC transition to the angiogenic phenotype in response to proangiogenic growth factors [84]. In ECs, MEOX2 activates expression of $p21^{WAF1/CIP1}$ by binding directly to its promoter and enhancer [85] that causes cell cycle arrest in G0/G1. MEOX2 is also able to suppress EC activation through the downregulation of activity of the nuclear factor NF-κB [86].

STAT5A was shown to mediate angiogenic activation of ECs through several mechanisms including Src/Jak2-dependent stimulatory signals from fibroblast growth factors FGF2 and FGF8b [87]. Prolactin family members such as proliferin and prolactin could mediate proangiogenic properties of the FGF/STAT5A activating axis through autocrine mechanism [88]. FGF2-mediated activation leads to the recruitment of STAT1 and STAT5A and to a lesser content STAT3 in ECs followed by prolactin-induced production of VEGF, a potent proangiogenic factor able to promote angiogenesis via the positive autocrine feedback loop [89]. Interleukin- (IL-) 3

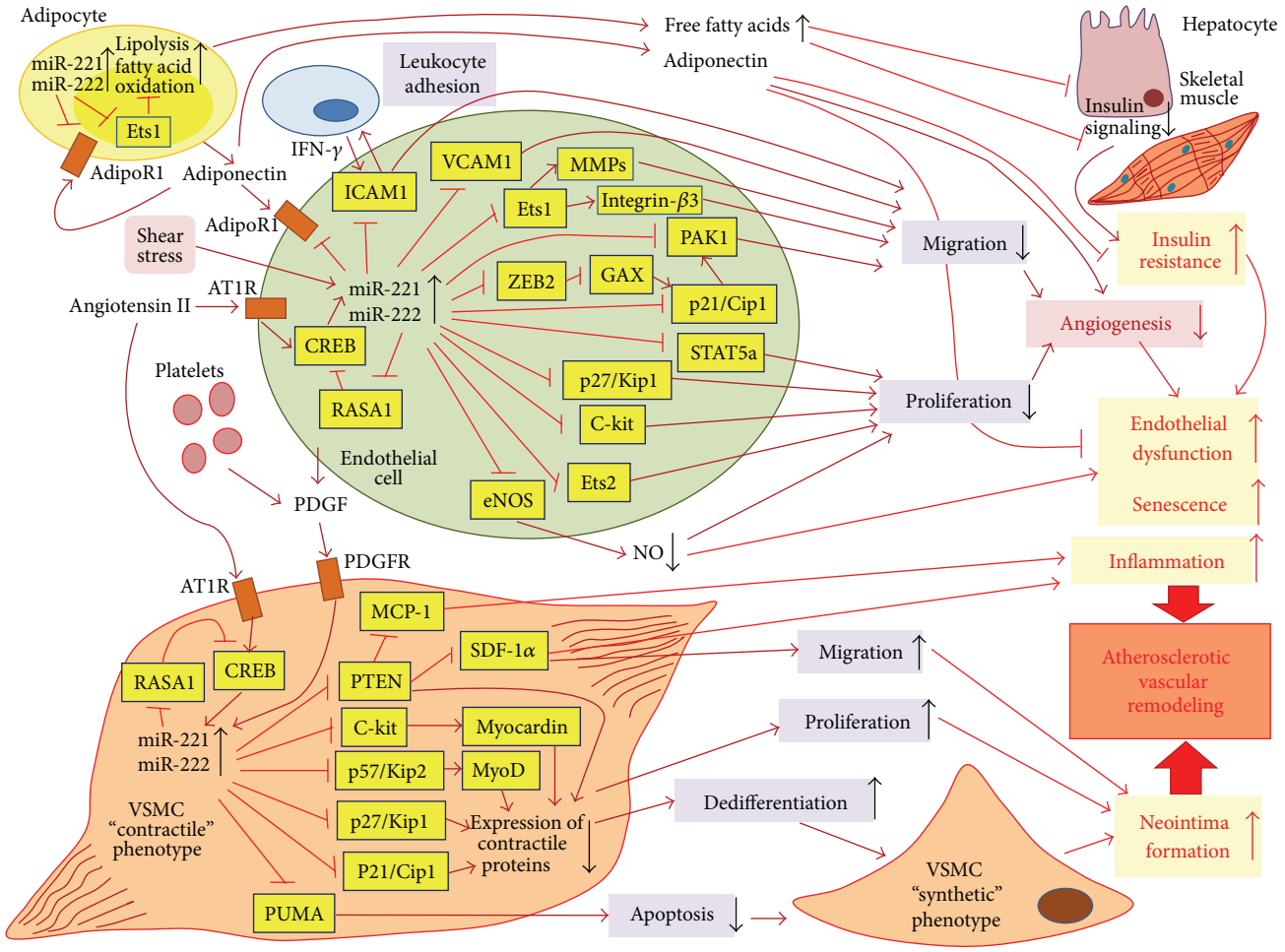


Figure 2: Effects of miR-221/222 on vascular endothelium and vascular smooth muscle cells (VSMCs) in atherosclerotic vascular remodeling. In arterial endothelial cells (ECs), expression of miR-221/222 could be upregulated by angiotensin II and shear stress. miR-221/222 are able to positively regulate expression through suppression of RAS p21 protein activator 1 (RASA1), an inhibitor of CREB (cAMP response element-binding protein) that drives angiotensin II induced expression of both miRNAs. Increased levels of miR-221/222 suppress angiogenic activation of quiescent terminally differentiated ECs through inhibiting endothelial proliferation and migration. Proliferation is suppressed via negative regulatory effects of the miR-221/222 cluster on several key genes such as cyclin-dependent kinase cell cycle regulators $p21^{Cip1}$ and $p27^{Kip1}$, transcription factors Ets1 and Ets2, signal transducer and activator STAT5a, and receptor for mast/stem cell growth factor c-kit. Notably, miR-221/222 downregulate expression of endothelial NO-synthase (eNOS) that lead to lowered production of nitric oxide (NO), an important modulator of function and proliferation of vascular ECs. Decreased NO production contributes to endothelial dysfunction and promotes EC senescence. miR-221/222 could downregulate $p21^{Cip1}$ either directly or through blocking of ZEB2 (zinc finger E-box binding homeobox 2), which represses translation of mesenchyme homeobox 2 (MEOX2 or GAX), a transcriptional activator of $p21^{Cip1}$. The miR-221/222 cluster attenuates EC migration by suppressing endothelial production of matrix metalloproteinases (MMPs) and several key adhesion modulators such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), integrin-β3, and serine/threonine-protein kinase PAK-1. The miR-221/222 cluster could probably diminish endothelial expression of adiponectin receptor AdipoR1. Adiponectin is produced by adipocytes and plays protective role for ECs by preventing endothelial dysfunction. In obesity, miR-221/222 are upregulated in the adipose tissue causing activation of lipid catabolism (lipolysis and fatty cell oxidation) through inhibition of Ets1, a transcription factor that controls expression of fatty acid synthase and other lipid-synthesizing enzymes. As a result, adipocytes release increased amounts of free fatty acids to blood that inhibit insulin signaling in liver and skeletal muscle inducing peripheral insulin resistance. Insulin resistance contributes to endothelial dysfunction. In VSMCs, expression of miR-221/222 is stimulated by angiotensin II and platelet-derived growth factor (PDGF) that is secreted by activated platelets and ECs in response to vascular injury. Upregulation of these miRNAs supports proliferation and increase mobility of VSMCs. In VSMCs, miR-221/222 inhibit several regulatory factors such as those of $p21^{Cip1}$, $p27^{Kip1}$, $p57^{Kip2}$, c-kit, and phosphatase and tensin homolog (PTEN) that are crucial for differentiation and establishment of the contractile phenotype of VSMCs. $p57^{Kip2}$ and c-kit activate MyoD and myocardin, two key transcription factors involved in myogenesis. Indeed, miR-221/222-dependent downregulation of expression of SMC-specific contractile proteins causes VSMC dedifferentiation and switch from the "contractile" to "synthetic" phenotype. By suppressing PTEN, miR-221/222 induce expression of several proinflammatory chemokines such as monocyte chemotactic protein-1 (MCP-1) and stromal cell-derived factor 1α (SDF-1α) that attract proinflammatory lymphocytes, dendritic cells, and macrophages to the inflamed site, for example, to the atherosclerotic plaque. In addition, miR-221/222 downregulate PUMA (p53 upregulated modulator of apoptosis), a critical apoptotic inducer thereby preventing apoptosis of VSMCs. Finally, dedifferentiated VSMCs become involved in neointima formation, an essential stage in atherosclerosis-associated remodeling of the arterial wall.

induces endothelial mitogenic proliferation by binding to the IL-3 receptor and recruitment of STAT5A and STAT5B [90]. IL-3-mediated activation of vascular ECs is accompanied with a secondary release of platelet-activating factor (PAF) that stimulates EC motility but not proliferation.

ECs were found to spontaneously respond to the vascular injury by induction of the proangiogenic transcriptional program and changes in the cellular phenotype and behavior. For example, exposure of human hepatic sinusoidal ECs to clinical doses of ionizing radiation results in upregulation of AMP-activated protein kinase (AMPK) and p38 mitogen-activated protein kinase- (MAPK-) mediated production of MMP-2 and VEGFR2 needed to degrade the extracellular matrix and basement membrane and activate ECs towards tube formation [91]. Irradiated HUVECs were observed to induce expression of angiogenesis-related miRNAs including the miR-221/222 cluster [92] that does not support neoangiogenesis itself but contributes to stabilization and integrity of already assembled neovessels. Indeed, in ECs, the miR-221/222 cluster shows antiangiogenic activity and prevents endothelial activation towards vascular remodeling and neovascularization. miR-221/222 are responsible for establishing quiescent phenotype of ECs and maintaining homeostasis of the vascular endothelium.

In consistence with this, senescent human aortic ECs were shown to express higher levels of antiangiogenic miR-221 and miR-222 [93] associated with reduced synthesis and activity of eNOS and increased production of caveolin-1, a negative eNOS regulator [30]. However, rhesus macaque mesenchymal stem cells (MSCs) derived from the bone marrow of aged animals displayed decreased expression of miRNA-221 compared with young monkeys [94]. In older humans, miR-221 expression in peripheral blood mononuclear cells (PMBCs) was also reduced while expression of its targets c-kit and PI3KR1 was increased with age [95]. Indeed, miR-221/222 could contribute to replicative senescence and cell aging but their lifespan-associated effects seem to be cell specific. Upregulation of miR221/222 in senescent ECs could be a consequence of age-related endothelial dysfunction and stimulatory effects of low-grade inflammation commonly persisted in the wall of aged vessels.

3.2. miR-221/222-Mediated Regulation of Phenotypic Plasticity of VSMCs. VSMCs are the prevalent cell type in the arterial tunica media. They are primarily involved in the regulation of vascular tone. A notable feature of VSMCs is their significant phenotypic plasticity and ability to differentiate/dedifferentiate in response to various physiological stimuli and pathological stresses such as vessel wounding or vascular pathology [29]. Quiescent VSMCs rarely proliferate and have a contractile phenotype characterized by expression of SMC-specific genes including smooth muscle α-actin (α-SMA), transgelin (TAGLN or SM22α), smooth muscle myosin heavy chain (SM-MHC), α- and β-tropomyosins, α1-integrin, caldesmon, and calponin [96]. During dedifferentiation, VSMCs downregulate expression of contractile markers and acquire a synthetic phenotype associated with enhanced synthesis of collagens and MMPs along with induction of proliferation and motility [97]. VSMC-mediated neointimal hyperplasia plays a central and universal role in response to vascular injury and wound healing. In neointimal hyperplasia, VSMCs actively proliferate and migrate from the tunica media primarily to the tunica intima causing arterial wall thickening [98]. However, an aberrant proliferation of VSMCs is frequently involved in the pathogenesis of vascular proliferative diseases such as atherosclerosis, postangioplasty or in-stent restenosis, and transplant vasculopathy [99]. In atherosclerotic intima, the structural and functional changes of VSMCs lead to the formation of so-called secretory phenotype of VSMCs [100, 101].

miR-221/222 are highly expressed in VSMCs [32, 102]. Knockdown of miR-221 and miR-222 reduces VSMC proliferation *in vitro* and inhibits neointimal hyperplasia-induced intimal thickening in rat carotid artery after vascular injury [103]. Interestingly, the miR-221/222 cluster exhibits opposite activities towards vascular ECs and VSMCs. In ECs, these miRNAs inhibit proliferation and migration and cause proapoptosis. In VSMCs, both miRNAs stimulate proliferation and cell mobility and induce antiapoptosis [102]. miR-221/222 play a key role in triggering VSMC dedifferentiation and switching from the contractile phenotype to synthetic phenotype [102]. The miR-221/222 cluster was reported to be markedly upregulated in quiescent VSMCs in response to vascular injury and in proliferative VSMCs [102]. In VSMCs, the miRNA cluster expression in response to injury is induced by platelet-derived growth factor (PDGF) [26].

PDGF is released from platelets and endothelial cells at sites of vascular injury [104]. In VSMCs, PDGF-induced miR-221/222 expression leads to the inhibition of several cell cycle regulators such as $p27^{Kip1}$, $p57^{Kip2}$, and c-kit [26, 102]. miR-221-dependent downregulation of $p27^{Kip1}$ is crucial for PDGF-mediated induction of cell proliferation. Interestingly, miR-221 and Skp2 (S-phase kinase-associated protein 2) were shown to temporally regulate $p27^{Kip1}$ in VSMCs: miR-221 in G1-phase while Skp2 in S-phase associated with cell growth [105]. Skp2 is a component of the ubiquitin protein ligase complex (SKP1-cullin-F-box) that is involved in the control of cell cycle progression via degradation of CDKs, cyclins, and CDK inhibitors including $p27^{Kip1}$ [106].

Like $p27^{Kip1}$, $p57^{Kip2}$ (cyclin-dependent kinase inhibitor 1C, CDKN1C) suppresses several G1 cyclin/Cdk complexes preventing cell proliferation [107]. In addition, $p57^{Kip2}$ stabilizes and activates MyoD, a myogenic regulatory transcription factor, essential for muscle differentiation [108]. miR-221-induced downregulation of $p57^{Kip2}$ results in inactivation of MyoD and prevention of VSMC differentiation towards the contractile phenotype (Figure 2). Decreased c-Kit causes inhibition of SMC-specific contractile gene transcription program by reducing expression of Myocardin (Myocd), a potent SMC-specific nuclear coactivator [109]. Indeed, miR-221 mediates PDGF-induced phenotypic changes in VSMCs by blocking expression of SMC contractile genes and stimulating VSMC dedifferentiation, proliferation, and migration.

4. Role of miR-221/222 in Atherosclerotic Vascular Remodeling

4.1. Inhibitory Effects of miR-221/222 on ECs in Atherosclerosis-Associated Vascular Remodeling. In mammals, the cardiovascular function is greatly influenced by the interaction between the hemodynamics and the vascular endothelium. Shear stress is created by blood flow when by blood flow affects the endothelium. Shear-induced mechanotransduction (e.g., conversion of mechanical stresses to biochemical responses) is especially influential in elastic vessels such as aorta and arteries where blood flow controls vascular tone and structure [110]. Mechanical stimulation of vascular endothelium with shear stress results in release of endothelial-derived vasoactive substances such as NO, growth factors, prostaglandins, and so forth. Sustained changes in local hemodynamics stimulate adaptive structural remodeling of the artery wall through coordinated changes in expression of multiple vascular proteins.

Shear stress induces phenotypic changes in the vascular endothelium in specific aortic and arterial sites that in turn specify whether this site will be resistant or prone to proatherogenic changes and progression to the atherosclerotic plaque [111, 112]. In principal, intensive shear stress is beneficial since it promotes adaptive dilatation or structural arterial remodeling through mechanisms mediated by endothelium [113]. In hypertension, hypercholesterolemia, or diabetes, endothelial dysfunction that is considered as a primary manifestation of cardiovascular disease is systemic and is widely attributed to impaired expression of eNOS [114]. Shear stress induces eNOS expression and hence results in greater availability of NO. Since NO is protected vessels from oxidative stress and inflammation, vascular zones with low NO production will be more susceptible to atherosclerosis.

Apolipoprotein E- (ApoE-) deficient mice spontaneously develop atherosclerosis when fed on high cholesterol diet. When a tapered cast was placed around the carotid artery in ApoE-deficient mice, low NO production and advanced lesions were observed in the atherosclerosis-prone flow separation region located immediately downstream of the cast [115]. Further studies on ApoE-deficient mice with implanted cast showed that shear stress type (such as lowered stress or oscillatory stress) is an essential condition for establishing plaque phenotype. Lowered shear stress induces larger lesions with a vulnerable plaque phenotype, whereas vortices with oscillatory shear stress induce stable lesions [116].

Atherosclerosis-resistant and atherosclerosis-susceptible arterial regions were shown to have differentiated endothelial-specific transcriptome patterns, with global expression shifted towards upregulation of proinflammatory and procoagulant genes in susceptible regions and towards upregulation of antioxidant and anticoagulant genes in resistant regions [111, 117]. Disturbed blood flow also contributes to the formation of atherosclerosis-prone vascular regions. Indeed, coordinated regulation of gene expression in ECs in response to local shear stresses should determine regional endothelial phenotypes that protect or predispose to atherosclerosis [118, 119]. Unidirectional and laminar shear stress correlates with transcript profiles considered protective (e.g., antioxidative, anti-inflammatory, and antiproliferative) [120].

Recently, researchers implemented comparative quantification of global miRNA transcriptome in endothelium derived from atheroresistant and atherosusceptible arterial regions in order to identify miRNAs whose expression could distinguish between resistant and susceptible regions. A role of miR-10a in establishing proinflammatory endothelial phenotype in atherosusceptible aortic endothelium was revealed [121]. In swine aorta, site-specific miR-92a regulation of expression of endothelial antiatherogenic transcription Krüppel-like factors- (KLF-) 2 and 4 was shown to contribute to endothelial phenotype heterogeneity associated with regional atherosusceptibility and protection *in vivo* [122].

Upregulation of the miR-221/222 cluster in arterial ECs appears to be proatherogenic since these miRNAs downregulate eNOS and inhibit angiogenesis essential for vascular repair. In addition, miR-221 targets PI3KR1 that is involved in the regulation of PI3K/Akt-mediated signaling that was shown to stimulate the atheroprotective transcription factor Nrf2 (nuclear factor (erythroid-derived 2)-like 2) [120]. Notably, expression of Nrf2 in ECs is induced by mechanical forces (e.g., by shear stress) [123] and contributes to formation of the endothelial regional atherosclerosis-resistant phenotype in vessels [120]. In ECs, Nrf2 drives expression of several antioxidant genes and downregulates several inflammatory mediators such as monocyte chemoattractant protein- (MCP-) 1 and vascular cell adhesion molecule-1 (VCAM-1) therefore enhancing antioxidant and anti-inflammatory properties of the arterial epithelium [124]. Furthermore, expression levels of circulating miR-221/222 are dynamically modulated by blood flow since they were shown to be upregulated by acute exercise before and after sustained training [107]. Indeed, miR-221/222 have a promising potential to be used for mapping atherosusceptible regions in apparently normal arteries.

miR-221 and miR-222 could be upregulated in the vascular epithelium and VSMCs in angiotensin II dependent manner [32, 39]. Pathological activation of the renin-angiotensin system is an established cardiovascular risk factor associated with the development of endothelial dysfunction and essential hypertension [125]. Vascular inflammation, an essential feature of atherosclerosis, also stimulates expression of both miRNAs in the vascular wall [30, 32, 60]. High glucose was shown to induce elevated levels of miR-221 in ECs that impairs normal endothelial function [37]. Furthermore, in blood serum of patients with metabolic syndrome, increased concentrations of circulating miR-221 were detected [126]. Given a prominent role of diabetic hyperglycemia and cardiometabolic stress in the development of cardiovascular pathology, increased expression of the miR-221/222 cluster in vascular tissues should predispose to atherosclerosis and increase risk of endothelial dysfunction and cardiovascular disease. In susceptible individuals, arterial expression of miR-221/222 may be enhanced even in early atherosclerotic stages such as preclinical atherosclerosis [127].

The initial stage of atherosclerosis is characterized by proinflammatory recruitment of leukocytes to activated ECs [128]. Since miR-221 was shown to suppress adhesiveness of ECs through inhibiting several adhesion-related molecules such as VCAM-1, CD47, and PAK1 [59, 129], it seems to initially play an atheroprotective role and is upregulated as a part of the adaptive inflammatory-driven response in order to maintain endothelial homeostasis and quiescent phenotype and prevent activation of arterial ECs. However, continuous proinflammatory stimulation of ECs in early atherosclerosis further upregulates miR-221/222 that in turn promotes switch in the adaptive reaction to the maladaptive proatherogenic response. In atherosclerosis, elevated miR-221/222 inhibits angiogenic activation and therefore limits recruitment and availability of EPCs for vascular repair [70, 130]. In patients with coronary artery disease (CAD), increased serum levels of miR-221 were reported to inversely correlate with numbers of EPCs [131]. Treatment with atorvastatin, a lipid-lowering agent, results in decreasing miR-221 levels and restoring EPC numbers in CAD patients [131]. In diabetes-related atherosclerosis, hyperglycemia and advanced glycation-end products (AGEs) contribute to endothelial dysfunction and cell cycle arrest. AGE-induced downregulation of miR-221/222 in vascular ECs leads to subsequent activation of their targets $p27^{Kip1}$ and $p57^{Kip2}$ that in turn suppress cell cycle progression [132].

Serum miR-221 was reported to be significantly increased in internal mammary arteries of diabetic subjects with coronary artery bypass grafts [133], in patients with carotid atherosclerosis and CAD patients [131]. However, some reports showed reduced levels of serum miR-221 in subjects with atherosclerosis obliterans [134] and atherosclerotic patients with subclinical hypothyroidism [135]. In advanced atherosclerosis, long-term chronic inflammation, which occurs parallel with remodeling of the intimal extracellular matrix [136], was found to downregulate expression of the miR-221/222 cluster in vascular ECs [60]. Probably, suppression of miR-221/222 could precede the neointima formation in atherosclerotic vessels when the recruitment of ECs is needed to support neoangiogenesis. Inflammatory factors suppressing miR-221/222 in ECs should be detected. Interferon-γ (IFN-γ), a proinflammatory cytokine, inhibits miR-221 expression in cholangiocytes but it is unknown whether IFN-γ is involved in miR-221 suppression in ECs [137]. Another inflammatory cytokine that is likely to regulate vascular expression of miR-221 in atherosclerosis is high-mobility group B1 (HMGB1; alarmin) [138]. HMGB1 was found to play a remarkable role in atherosclerosis-associated inflammation by signaling via receptor for advanced glycation end products (RAGE) and Toll-like receptors [139].

4.2. miR-221/222 Cluster Induces VSMC-Mediated Neointima Formation and Contributes to Vascular Calcification in Atherosclerosis. Like PDGF, angiotensin II is able to induce miR-221/222 [39], followed by VSMC proliferation and hypertrophy [140]. Angiotensin II induced activation of miR-221/222 expression in VSMCs is more attributable to vascular pathology such as hypertension, restenosis, or atherosclerosis and is accompanied with induction of the proinflammatory microenvironment [141]. In VCMCs, angiotensin II stimulates expression of miR-221 through binding to angiotensin II type 1 receptor (AT1R) that mediates activation of cAMP response element-binding protein (CREB), a transcription factor [36]. miR-221 was found to show a positive feedback on its own expression by suppressing RASA1 (RAS p21 protein activator 1), a CREB inhibitor. miR-221 also negatively regulates PTEN (phosphatase and tensin homolog), a suppressor of Akt/PI3K signaling that induces NF-κB-mediated production of inflammatory chemokine MCP-1 [36]. Indeed, angiotensin II dependent downregulation of PTEN results in increased expression of proinflammatory mediators and activates proliferation, hypertrophy, and migration of VSMCs [142].

PDGF production and PDGF-dependent signaling were shown to be activated in vascular proliferative diseases including atherosclerosis and restenosis and therefore could drive VSMC recruitment in vascular remodeling [143]. Indeed, PDGF-induced miR-221/222 upregulation followed by suppression of the cell cycle regulator $p27^{Kip1}$ in VSMCs could contribute to atherogenesis because loss of $p27^{Kip1}$ was reported to aggravate atherosclerosis in ApoE-deficient mice [144, 145]. In ApoE-deficient mice, expression of transgenic ApoE, an essential component of serum high density lipoproteins (HDL) and triglyceride-enriched lipoproteins that possess atheroprotective properties, negatively regulates miR-221/222 and restores $p27^{Kip1}$ expression in VSMCs thereby preventing pathological recruitment of these cells in atherosclerosis-related vascular remodeling. ApoE was found to downregulate miR-221 and miR-222 in a Cox-2 (prostaglandin-endoperoxide synthase)/prostacyclin/ inositol monophosphate-dependent manner [146]. ApoE could also prevent dedifferentiation of VSMCs by suppressing PDGF-mediated entry to S-phase through activation of inducible NOS [147] or interaction with perlecan, an extracellular matrix protein [148]. However, these mechanisms are dependent on specific ApoE isoforms while all three isoforms of ApoE are able to suppress miR-221/222 through Cox-2-dependent pathway [146]. Indeed, due to capacity of all ApoE isoforms to regulate miR-221/222-dependent input to control of VSMC phenotypic plasticity, this mechanism could represent the major way by which ApoE may limit proatherogenic entrance of VSMCs to arterial remodeling.

In the family of CDK protein inhibitors ($p15^{Ink4A}$, $p16^{Ink4B}$, $p18^{Ink4C}$, $p19^{Ink4D}$, $p21^{Waf1/Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$), three cell cycle regulators ($p21^{Waf1/Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$) play a central role in control of VSMC differentiation/dedifferentiation because $p57^{Kip2}$ regulates G1/S transition of cell cycle while $p21^{Waf1/Cip1}$ and $p27^{Kip1}$utilize different molecular mechanisms of CDK inhibition in G1-phase [149, 150]. Since miR-221 and miR-222 target two of them ($p27^{Kip1}$ and $p57^{Kip2}$), and this miRNA cluster may have a profound impact in the regulation of the role of VSMCs in proatherogenic neointimal hyperplasia [24, 127].

Furthermore, results recently presented by Mackenzie et al. [34, 151] about supportive effects of miR-221/222 on vascular calcification expand proatherogenic properties of this miRNA cluster. Calcification commonly affects the arterial tree in a variety of diseases and pathological conditions [152–155], with calcification process often being associated with VSMCs [152, 156]. In calcifying VSMCs transfected with miR-221/222 mimics, a significant increase in intracellular calcium accumulation associated with quantitative changes in expression of ectonucleotide phosphodiesterase 1 (Enpp1) and Pit-1 (sodium-dependent phosphate cotransporter) expression was observed [34]. Both proteins are essential for regulation of intracellular phosphate levels and hence are primarily involved in control of calcium phosphate deposits in osteogenesis and pathogenic ectopic mineralization including atherosclerosis-related arterial calcification [157]. Interestingly, when VSMCs were transfected with miR-221 and miR-222 mimics, an increase in calcium deposition was observed in combined treatment but not in individual miR treatments suggesting for synergistic effects of miR-221/222 on vascular calcification [151]. Both miRNAs were shown to contribute to VSMC calcification independently of osteogenic transcription factors Runx2 (runt-related transcription factor 2) and Msx2 (MSH homeobox 2) [34].

Calcification of VSMCs is associated with significant decrease in miR-221/222 levels [151]. Indeed, downregulation of miR-221/222 could induce osteogenic/chondrogenic changes in the VSMC phenotype. These observations are in agreement with the data presented by Li et al. [158] who detected reduced levels of miR-221/222 in sclerotic intima samples from patients with atherosclerosis obliterans compared to the normal vascular tissues. In advanced atherosclerosis, long-term inflammation decreases miR-221/222 in ECs [60]. It is likely that chronic vascular inflammation could also diminish miR-221/222 expression in VSMCs and therefore promote induction of phenotypic changes towards calcifying cells. However, molecular mechanisms by which the miR-221/222 cluster contributes to vascular calcification are widely unknown and indeed should be precisely evaluated in future studies.

4.3. Challenges for Analysis of the Roles of miR-221/222 in Functioning of Cells Types That Represent Minor Cell Populations in the Vascular Wall. Current appreciation of a remarkable role of miRNAs in vascular biology of VSMCs and ECs warrants investigation of possible contribution of miR-221/222 to functioning of other cell types residing in the vascular wall, in both homeostatic and pathological conditions. In particular, such a demand is relevant to investigation of vascular dendritic cells representing the most crucial cell type that regulates immune processes in atherogenesis and other vascular pathologies in which immune inflammation plays an important role [159–162]. Accumulating evidence indicates that miRNAs, including miR-221/222, are importantly involved in functioning of dendritic cells in other organs and other pathologies [163–165]. In particular, differentially expressed microRNAs have been reported to regulate plasmacytoid versus conventional dendritic cell development [164]. As vascular dendritic cells (VDCs) represent a minor cell population in the arterial wall [159], obviously there would be some methodological difficulties during the investigation. However, the recent achievements in the visualization of miRNA localization in cells within biological tissue sections by a combination of *in situ* hybridization with immunohistochemistry [166–168] along with the achievements in the development of protocols for the isolation of arterial cells by means of the use of laser capture-microdissection technique [169–171] would assist in investigation of the impact of miR-221/222 in controlling of behavior patterns of dendritic cells in various vascular pathologies.

5. Extravasal Functional Effects of miR-221/222 Increasing Cardiovascular Risk

In addition to the direct regulation of function and development of vascular cells, miR-221 and miR-222 exhibit their effects on nonvascular tissues. Some of these functional activities could be related to the development of atherosclerosis or cardiometabolic risk factors that promote atherogenesis. In the adipose tissue, miR-221 was recently found to control fat metabolism by affecting PPAR- (peroxisome proliferator-activated receptor-) dependent pathways and by directly targeting adiponectin receptor AdipoR1 and Ets1 [172]. miR-221 and RNA-binding protein polypyrimidine tract-binding protein (PTB) cooperate in negative posttranslational control of AdipoR1 in muscle and liver of genetic and dietary murine models of obesity that suggests a supportive role of miR-221 in insulin resistance in peripheral tissues [173].

AdipoR1 mediates biological effects of adiponectin, an adipocyte-specific cytokine, mainly focused on the regulation of glucose metabolism (increased glucose uptake, reduced gluconeogenesis) and fat metabolism (activation of lipid catabolism, oxidation of fatty acids, and utilization of triglycerides) [174]. Interestingly, in vascular ECs, AdipoR1 mediates vasculoprotective properties of adiponectin associated with anti-inflammation (inhibition of tumor necrosis factor-α- (TNF-α-) induced expression of ICAM-1 and NF-κB) and prevention of vascular dysfunction [175]. However, it is unclear whether miR-221 targets AdipoR1 in the vascular endothelium. In cultured preadipocytes, leptin and proinflammatory cytokine TNF-α suppress expression of miR-221 [172]. Similarly, Chou et al. [176] showed decreased levels of miR-221 in MSCs derived from the adipose tissue of obese women, and miR-221 expression was negatively correlated with levels of TNF-α mRNA in obese adipocytes. Indeed, TNF-α could downregulate miR-221 in adipocytes.

Overall, miR-221, which is upregulated in obese individuals [172], is involved in insulin resistance and metabolic syndrome, for example, two metabolic conditions that greatly increase atherosclerotic risk. In morbidly obese individuals, blood levels of miR221/222 are markedly increased [177].

In adipocytes, miR-221 contributes to the regulation of physiological network involved in fatty acid metabolism by targeting several proteins including fatty acid synthase (FASN), an enzyme overexpressed in the adipose tissue in obesity and type 2 diabetes [178]. In obese people, adipocytes have activated fat metabolism associated with lipolysis and enhanced release of free fatty acids to blood. Fatty acids promote insulin resistance via suppressing insulin signaling by stimulating serine protein kinases that phosphorylate insulin receptor substrates (IRS) and disrupt the downstream transduction of signal from the insulin receptor [179].

Serum miR-221 levels were shown to be elevated in women affected with metabolic syndrome although no significant correlations were observed between blood miR-221 concentrations and cardiometabolic risk factors [126]. Diabetic hyperglycemia and products of advanced nonenzymatic oxidative glycation that are significantly increased in diabetic blood were shown to stimulate expression of miR-221/222 in the vascular wall [37, 133, 180]. Indeed, upregulated expression of the miR-221/222 cluster in vessels of people with obesity, metabolic syndrome, insulin resistance, hypertension, and type 2 diabetes increases cardiovascular risk and promotes the development of atherosclerosis through endothelial dysfunction and neointimal hyperplasia. The proatherogenic role of miR-221/222 is supported by the fact of suppressing effects of lipid-lowering agents (atorvastatin), antidiabetic drugs (metformin), and garlic preparations on vascular expression of this miRNA cluster [58, 130, 131, 133].

6. Clinical Potential of miR-221/222

Serum levels of circulating miR-221 were shown to be significantly changed in several vascular and metabolic pathologies including hypertension, obesity, metabolic syndrome, CAD, and carotid atherosclerosis that make this miRNA a potential diagnostic biomarker. In patients with acute coronary syndrome, miR-221 was found to be significantly increased only in platelets and PMBCs of those with non-ST-segment elevation (NSTEMI) myocardial infarction but not with a ST-segment-elevation myocardial infarction (STEMI) suggesting a potential diagnostic value of this miRNA for distinguishing between STEMI and NSTEMI [181]. A phenomenon of the blood flow-mediated regulation of expression of miR-221 in the vascular wall should be studied in detail because differential expression of miR-221 in arterial wall harbors a promise of identification of atheroprone and atheroresistant vascular regions in asymptomatic patients or subjects with subclinical atherosclerosis that is important for early diagnosis and prophylaxis of cardiovascular disease.

To date, the major progress on the way of clinical utility of miR-221/222 in cardiovascular medicine was achieved in the field of stem cell therapy for cardiovascular diseases. The miR-221/222 cluster is critically involved in the regulation of myogenesis and function of myocardium [173, 182–184]. Both in myoblasts and myotubules, miR221/222 expression was shown to be under control of the Ras-MAPK pathway and inversely correlated with levels of $p27^{Kip1}$, a common target for these miRNAs [182]. The cluster could also contribute to several heart pathologies including myotonic dystrophy type 2 [185] and hypertrophic cardiomyopathy (through targeting $p27^{Kip1}$, a cardiac hypertrophic suppressor in cardiomyocytes) [35, 59, 186].

Treatment with a prosurvival cocktail consisting of lentivirus constructs carrying the precursor of miR-21, miR-24, and miR-221 significantly improved survival and engraftment of cardiomyocyte precursors after transplantation to the mouse cardiac muscle [187]. In part, the prosurvival effect of the miRNA cocktail was supported by inhibition of BIM/BCL2L11 (BCL2-like 11), a critical apoptotic activator, which is a common target for all three miRNAs [187]. In advanced oxidative stress, miR-221 was demonstrated to cooperate with superoxide dismutase-2 in unacylated ghrelin-driven muscle repair after oxidative injury by downregulation of $p57^{Kip1}$ that in turn promotes cell cycle progression [180]. In addition, miR-221 was found to support survival of cocultures of primary rat neonatal ventricle cardiomyocytes and MSCs transduced with GATA-4 (a critical transcription factor for proper mammalian cardiac development) in hypoxic conditions. The miR-221-mediated cardioprotection was achieved via downregulation of p53 upregulated modulator of apoptosis (PUMA). Interestingly, compared to cardiomyocytes, cultured MSCs developed significantly higher expression levels of miR-221 and supported cardiomyocyte viability by shedding miR-221-containing microvesicles [188]. Thus, miR-221 show marked cardioprotective and antiapoptotic properties that would be beneficial for increasing efficiency of cardiac cell transplantation and heart regeneration.

7. Conclusions

Likewise other miRNAs [189–194], the miR-221/222 cluster plays a remarkable role in vascular biology and vascular pathology. In the vascular tissue, these miRNAs exhibit cell-specific effects by supporting dedifferentiation proliferation and migration of VSMCs while inhibiting proliferation and motility of ECs. In different muscle lineage cells, miR-221 and miR-222 show similar effects by activating regenerative properties through targeting key cell cycle regulators $p21^{Waf1/Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$ involved in induction of expression of contractile proteins. In contrast to antiangiogenic activity in vascular ECs, miR-221/222 were shown to support tumor-associated neoangiogenesis and invasion in many cancers including hepatocellular carcinoma [195], breast cancer [196], lung cancer [197], colorectal cancer [198], and other epithelial cancers. Tumorigenic function of miR-221/222-mediated is mainly attributed to targeting several key tumor suppressors such as $p21^{Waf1/Cip1}$, $p27^{Kip1}$, $p57^{Kip2}$, PTEN, and PUMA [199, 200].

In epithelial cancers, the miR-221/222 cluster is involved in induction of EMT, a mechanism that leads to phenotypic changes in cancer cells and cancer-associated ECs towards increased dedifferentiation, mobility, adhesion, and invasiveness [201]. It seems that tumor progression to metastasis stage does not happen occasionally and chaotically but is induced

in a coordinated manner, with burst in tumor neovascularization. EMT was shown to play a central role in vasculogenic mimicry, a new pattern of tumor microcirculation, in which cancer cells acquire properties of vascular cells having tumor and endothelial phenotypes with maintenance of stem cell-like characteristics to form capillary-like structures in the tumor mass [202]. Hypoxia promotes vasculogenic mimicry formation by inducing EMT [203]. Indeed, activation of the proinvasive EMT mechanism should be involved in the control of tumor-associated neovascularization since tumor microvascular network facilitates mobilization of tumor cells to circulation and further expansion [204]. For example, miR-106b/93 and miR-221/222 are upregulated in gastric cancer tissues and control suppression of CDK protein inhibitors in a coordinated manner thereby stimulating tumor invasion and progression [199]. EMT is absent in normal and diseased noncancer vascular tissues and therefore does not influence neoangiogenesis. Therefore, upregulated production of miR-221/222 in terminally differentiated vascular ECs will inhibit angiogenic activation whereas downregulation of this miRNA cluster will favor induction of neoangiogenesis.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgment

The authors wish to thank the Russian Scientific Foundation (Grant no. 14-15-00112) for support of their work.

References

[1] M. K. Pugsley and R. Tabrizchi, "The vascular system: an overview of structure and function," *Journal of Pharmacological and Toxicological Methods*, vol. 44, no. 2, pp. 333–340, 2000.

[2] M. Lannoy, S. Slove, and M. P. Jacob, "The function of elastic fibers in the arteries: beyond elasticity," *Pathologie Biologie*, vol. 62, no. 2, pp. 79–83, 2014.

[3] V. A. Korshunov, S. M. Schwartz, and B. C. Berk, "Vascular remodeling: hemodynamic and biochemical mechanisms underlying Glagov's phenomenon," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 27, no. 8, pp. 1722–1728, 2007.

[4] D. B. Cowan and B. L. Langille, "Cellular and molecular biology of vascular remodeling," *Current Opinion in Lipidology*, vol. 7, no. 2, pp. 94–100, 1996.

[5] S. Gerecht-Nir, S. Osenberg, O. Nevo, A. Ziskind, R. Coleman, and J. Itskovitz-Eldor, "Vascular development in early human embryos and in teratomas derived from human embryonic stem cells," *Biology of Reproduction*, vol. 71, no. 6, pp. 2029–2036, 2004.

[6] J. L. Lucitti, E. A. V. Jones, C. Huang, J. Chen, S. E. Fraser, and M. E. Dickinson, "Vascular remodeling of the mouse yolk sac requires hemodynamic force," *Development*, vol. 134, no. 18, pp. 3317–3326, 2007.

[7] M. D. Garcia and I. V. Larina, "Vascular development and hemodynamic force in the mouse yolk sac," *Frontiers in Physiology*, vol. 5, article 308, 2014.

[8] E. Tzima, M. Irani-Tehrani, W. B. Kiosses et al., "A mechanosensory complex that mediates the endothelial cell response to fluid shear stress," *Nature*, vol. 437, no. 7057, pp. 426–431, 2005.

[9] D. E. Conway, M. T. Breckenridge, E. Hinde, E. Gratton, C. S. Chen, and M. A. Schwartz, "Fluid shear stress on endothelial cells modulates mechanical tension across VE-cadherin and PECAM-1," *Current Biology*, vol. 23, no. 11, pp. 1024–1030, 2013.

[10] L. Robb, I. Lyons, R. Li et al., "Absence of yolk sac hematopoiesis from mice with a targeted disruption of the scl gene," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 92, no. 15, pp. 7075–7079, 1995.

[11] N. Matsumoto, A. Kubo, H. Liu et al., "Developmental regulation of yolk sac hematopoiesis by Krüppel-like factor 6," *Blood*, vol. 107, no. 4, pp. 1357–1365, 2006.

[12] N. Krankel, T. F. Luscher, and U. Landmesser, "Novel insights into vascular repair mechanisms," *Current Pharmaceutical Design*, vol. 20, no. 14, pp. 2430–2438, 2014.

[13] M. Li, S. Li, L. Yu et al., "Bone mesenchymal stem cells contributed to the neointimal formation after arterial injury," *PLoS ONE*, vol. 8, no. 12, Article ID e82743, 2013.

[14] A. K. Wara, A. Manica, J. F. Marchini et al., "Bone marrow-derived kruppel-like factor 10 controls reendothelialization in response to arterial injury," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 33, no. 7, pp. 1552–1560, 2013.

[15] K. R. Stenmark, M. E. Yeager, K. C. El Kasmi et al., "The adventitia: essential regulator of vascular wall structure and function," *Annual Review of Physiology*, vol. 75, pp. 23–47, 2013.

[16] A. N. Orekhov, Y. V. Bobryshev, and D. A. Chistiakov, "The complexity of cell composition of the intima of large arteries: focus on pericyte-like cells," *Cardiovascular Research*, vol. 103, no. 4, pp. 438–451.

[17] P. Libby, P. M. Ridker, and G. K. Hansson, "Leducq Transatlantic Network on Atherothrombosis. Inflammation in atherosclerosis: from pathophysiology to practice," *Journal of the American College of Cardiology*, vol. 54, no. 23, pp. 2129–2138, 2009.

[18] P. Libby, A. Lichtman, and G. Hansson, "Immune effector mechanisms implicated in atherosclerosis: from mice to humans," *Immunity*, vol. 38, no. 6, pp. 1092–1104, 2013.

[19] S. Glagov, E. Weisenberg, C. K. Zarins, R. Stankunavicius, and G. J. Kolettis, "Compensatory enlargement of human atherosclerotic coronary arteries," *The New England Journal of Medicine*, vol. 316, no. 22, pp. 1371–1375, 1987.

[20] S. Lim and S. Park, "Role of vascular smooth muscle cell in the inflammation of atherosclerosis," *BMB Reports*, vol. 47, no. 1, pp. 1–7, 2014.

[21] P. Puddu, A. Muscari, G. M. Puddu, E. Cravero, C. Giannoni, and M. Zoli, "The complexity of platelet metabolism and its contribution to atherothrombosis," *Acta Cardiologica*, vol. 64, no. 2, pp. 157–165, 2009.

[22] F. Xu, Y. Sun, Y. Chen et al., "Endothelial cell apoptosis is reponsible for the formation of coronary thrombotic atherosclerotic plaques," *Tohoku Journal of Experimental Medicine*, vol. 218, no. 1, pp. 25–33, 2009.

[23] F. Otsuka, K. Sakakura, K. Yahagi, M. Joner, and R. Virmani, "Has our understanding of calcification in human coronary atherosclerosis progressed?" *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 34, no. 4, pp. 724–736, 2014.

[24] Y. Wei, A. Schober, and C. Weber, "Pathogenic arterial remodeling: the good and bad of microRNAs," *American Journal of Physiology: Heart and Circulatory Physiology*, vol. 304, no. 8, pp. H1050–H1059, 2013.

[25] L. Poliseno, A. Tuccoli, L. Mariani et al., "MicroRNAs modulate the angiogenic properties of HUVECs," *Blood*, vol. 108, no. 9, pp. 3068–3071, 2006.

[26] B. N. Davis, A. C. Hilyard, P. H. Nguyen, G. Lagna, and A. Hata, "Induction of microRNA-221 by platelet-derived growth factor signaling is critical for modulation of vascular smooth muscle phenotype," *Journal of Biological Chemistry*, vol. 284, no. 6, pp. 3728–3738, 2009.

[27] A. Kuehbacher, C. Urbich, and S. Dimmeler, "Targeting microRNA expression to regulate angiogenesis," *Trends in Pharmacological Sciences*, vol. 29, no. 1, pp. 12–15, 2008.

[28] S. Albinsson and W. C. Sessa, "Can microRNAs control vascular smooth muscle phenotypic modulation and the response to injury?" *Physiological Genomics*, vol. 43, no. 10, pp. 529–533, 2011.

[29] Z. Song and G. Li, "Role of specific MicroRNAs in regulation of vascular smooth muscle cell differentiation and the response to injury," *Journal of Cardiovascular Translational Research*, vol. 3, no. 3, pp. 246–250, 2010.

[30] C. Rippe, M. Blimline, K. A. Magerko et al., "MicroRNA changes in human arterial endothelial cells with senescence: relation to apoptosis, eNOS and inflammation," *Experimental Gerontology*, vol. 47, no. 1, pp. 45–51, 2012.

[31] P. K. Santhekadur, S. K. Das, R. Gredler et al., "Multifunction protein staphylococcal nuclease domain containing 1 (SND1) promotes tumor angiogenesis in human hepatocellular carcinoma through novel pathway that involves nuclear factor κB and miR-221," *The Journal of Biological Chemistry*, vol. 287, no. 17, pp. 13952–13958, 2012.

[32] N. Zhu, D. Zhang, S. Chen et al., "Endothelial enriched microRNAs regulate angiotensin II-induced endothelial inflammation and migration," *Atherosclerosis*, vol. 215, no. 2, pp. 286–293, 2011.

[33] T. Ogawa, M. Enomoto, H. Fujii et al., "MicroRNA-221/222 upregulation indicates the activation of stellate cells and the progression of liver fibrosis," *Gut*, vol. 61, no. 11, pp. 1600–1609, 2012.

[34] N. C. Mackenzie, K. A. Staines, D. Zhu, P. Genever, and V. E. Macrae, "miRNA-221 and miRNA-222 synergistically function to promote vascular calcification," *Cell Biochemistry and Function*, vol. 32, no. 2, pp. 209–216, 2014.

[35] C. Wang, S. Wang, P. Zhao et al., "MiR-221 promotes cardiac hypertrophy in vitro through the modulation of p27 expression," *Journal of Cellular Biochemistry*, vol. 113, no. 6, pp. 2040–2046, 2012.

[36] W. Jin, M. A. Reddy, Z. Chen et al., "Small RNA sequencing reveals MicroRNAs that modulate angiotensin II effects in vascular smooth muscle cells," *Journal of Biological Chemistry*, vol. 287, no. 19, pp. 15672–15683, 2012.

[37] Y. Li, Y.-H. Song, F. Li, T. Yang, Y. W. Lu, and Y.-J. Geng, "microRNA-221 regulates high glucose-induced endothelial dysfunction," *Biochemical and Biophysical Research Communications*, vol. 381, no. 1, pp. 81–83, 2009.

[38] G. di Leva, P. Gasparini, C. Piovan et al., "MicroRNA cluster 221-222 and estrogen receptor α interactions in breast cancer," *Journal of the National Cancer Institute*, vol. 102, no. 10, pp. 706–721, 2010.

[39] A. Leung, C. Trac, W. Jin et al., "Novel long noncoding RNAs are regulated by angiotensin II in vascular smooth muscle cells," *Circulation Research*, vol. 113, no. 3, pp. 266–278, 2013.

[40] M. Landthaler, A. Yalcin, and T. Tuschl, "The human DiGeorge syndrome critical region gene 8 and its *D. melanogaster* homolog are required for miRNA biogenesis," *Current Biology*, vol. 14, no. 23, pp. 2162–2167, 2004.

[41] M. T. Bohnsack, K. Czaplinski, and D. Görlich, "Exportin 5 is a RanGTP-dependent dsRNA-binding protein that mediates nuclear export of pre-miRNAs," *RNA*, vol. 10, no. 2, pp. 185–191, 2004.

[42] T. P. Chendrimada, R. I. Gregory, E. Kumaraswamy et al., "TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing," *Nature*, vol. 436, no. 7051, pp. 740–744, 2005.

[43] V. N. Kim, "MicroRNA biogenesis: coordinated cropping and dicing," *Nature Reviews Molecular Cell Biology*, vol. 6, no. 5, pp. 376–385, 2005.

[44] B. Yao, S. Li, and E. K. L. Chan, "Function of GW182 and GW bodies in siRNA and miRNA pathways," *Advances in Experimental Medicine and Biology*, vol. 768, pp. 71–96, 2013.

[45] J. Yang, T. Välineva, J. Hong et al., "Transcriptional co-activator protein p100 interacts with snRNP proteins and facilitates the assembly of the spliceosome," *Nucleic Acids Research*, vol. 35, no. 13, pp. 4485–4494, 2007.

[46] X. Meng, K. W. Thiel, and K. K. Leslie, "Drug resistance mediated by AEG-1/MTDH/LYRIC," *Advances in Cancer Research*, vol. 120, pp. 135–157, 2013.

[47] A. Khvorova, A. Reynolds, and S. D. Jayasena, "Functional siRNAs and miRNAs exhibit strand bias," *Cell*, vol. 115, no. 2, pp. 209–216, 2003.

[48] M. Kulkarni, S. Ozgur, and G. Stoecklin, "On track with P-bodies," *Biochemical Society Transactions*, vol. 38, no. 1, pp. 242–251, 2010.

[49] G. L. Sen and H. M. Blau, "Argonaute 2/RISC resides in sites of mammalian mRNA decay known as cytoplasmic bodies," *Nature Cell Biology*, vol. 7, no. 6, pp. 633–636, 2005.

[50] L.-A. MacFarlane and P. R. Murphy, "MicroRNA: biogenesis, function and role in cancer," *Current Genomics*, vol. 11, no. 7, pp. 537–561, 2010.

[51] J. Brennecke, A. Stark, R. B. Russell, and S. M. Cohen, "Principles of microRNA-target recognition," *PLoS Biology*, vol. 3, no. 3, article e85, 2005.

[52] L. Maillet, C. Tu, Y. K. Hong, E. O. Shuster, and M. A. Collart, "The essential function of Not1 lies within the Ccr4-Not complex," *Journal of Molecular Biology*, vol. 303, no. 2, pp. 131–143, 2000.

[53] J. Coller and R. Parker, "Eukaryotic mRNA decapping," *Annual Review of Biochemistry*, vol. 73, pp. 861–890, 2004.

[54] P. A. Maroney, Y. Yu, J. Fisher, and T. W. Nilsen, "Evidence that microRNAs are associated with translating messenger RNAs in human cells," *Nature Structural and Molecular Biology*, vol. 13, no. 12, pp. 1102–1107, 2006.

[55] J. A. Arribere, J. A. Doudna, and W. V. Gilbert, "Reconsidering movement of eukaryotic mRNAs between polysomes and P bodies," *Molecular Cell*, vol. 44, no. 5, pp. 745–758, 2011.

[56] A. Mugge, U. Forstermann, and P. R. Lichtlen, "Endothelial functions in cardiovascular diseases," *Zeitschrift für Kardiologie*, vol. 78, no. 3, pp. 147–160, 1989.

[57] B. G. Schwartz, C. Economides, G. S. Mayeda, S. Burstein, and R. A. Kloner, "The endothelial cell in health and disease: its function, dysfunction, measurement and therapy," *International Journal of Impotence Research*, vol. 22, no. 2, pp. 77–90, 2010.

[58] E.-P. I. Chiang, S.-C. Chiu, M.-H. Pai et al., "Organosulfur garlic compounds induce neovasculogenesis in human endothelial progenitor cells through a modulation of MicroRNA 221 and

the PI3-K/Akt signaling pathways," *Journal of Agricultural and Food Chemistry*, vol. 61, no. 20, pp. 4839–4849, 2013.

[59] M. Duan, H. Yao, G. Hu, X. Chen, A. K. Lund, and S. Buch, "HIV Tat induces expression of ICAM-1 in HUVECs: implications for miR-221/-222 in HIV-associated cardiomyopathy," *PLoS ONE*, vol. 8, no. 3, Article ID e60170, 2013.

[60] P. Dentelli, A. Rosso, F. Orso, C. Olgasi, D. Taverna, and M. F. Brizzi, "MicroRNA-222 controls neovascularization by regulating signal transducer and activator of transcription 5A expression," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 30, no. 8, pp. 1562–1568, 2010.

[61] M. T. Aye, S. Hashemi, B. Leclair et al., "Expression of stem cell factor and c-kit mRNA in cultured endothelial cells, monocytes and cloned human bone marrow stromal cells (CFU-RF)," *Experimental Hematology*, vol. 20, no. 4, pp. 523–527, 1992.

[62] V. C. Broudy, N. L. Kovach, L. G. Bennett, N. Lin, F. W. Jacobsen, and P. G. Kidd, "Human umbilical vein endothelial cells display high-affinity c-kit receptors and produce a soluble form of the c-kit receptor," *Blood*, vol. 83, no. 8, pp. 2145–2152, 1994.

[63] N. Felli, L. Fontana, E. Pelosi et al., "MicroRNAs 221 and 222 inhibit normal erythropoiesis and erythroleukemic cell growth via kit receptor down-modulation," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 50, pp. 18081–18086, 2005.

[64] S. Nicoli, C.-P. Knyphausen, L. J. Zhu, A. Lakshmanan, and N. D. Lawson, "miR-221 is required for endothelial tip cell behaviors during vascular development," *Developmental Cell*, vol. 22, no. 2, pp. 418–429, 2012.

[65] S. Nicoli, C. Standley, P. Walker, A. Hurlstone, K. E. Fogarty, and N. D. Lawson, "MicroRNA-mediated integration of haemodynamics and Vegf signalling during angiogenesis," *Nature*, vol. 464, no. 7292, pp. 1196–1200, 2010.

[66] K. Marcelo, T. Sills, S. Coskun et al., "Hemogenic endothelial cell specification requires c-Kit, notch signaling, and p27-mediated cell-cycle control," *Developmental Cell*, vol. 27, no. 5, pp. 504–515, 2013.

[67] M. Graupera, J. Guillermet-Guibert, L. C. Foukas et al., "Angiogenesis selectively requires the p110α isoform of PI3K to control endothelial cell migration," *Nature*, vol. 453, no. 7195, pp. 662–666, 2008.

[68] B. Vanhaesebroeck, J. Guillermet-Guibert, M. Graupera, and B. Bilanges, "The emerging mechanisms of isoform-specific PI3K signalling," *Nature Reviews Molecular Cell Biology*, vol. 11, no. 5, pp. 329–341, 2010.

[69] J. F. Wang, X. Zhang, and J. E. Groopman, "Activation of vascular endothelial growth factor receptor-3 and its downstream signaling promote cell survival under oxidative stress," *Journal of Biological Chemistry*, vol. 279, no. 26, pp. 27088–27097, 2004.

[70] X. Zhang, H. Mao, J.-Y. Chen et al., "Increased expression of microRNA-221 inhibits PAK1 in endothelial progenitor cells and impairs its function via c-Raf/MEK/ERK pathway," *Biochemical and Biophysical Research Communications*, vol. 431, no. 3, pp. 404–408, 2013.

[71] S. Zhang, J. Han, M. A. Sells et al., "Rho family GTPases regulate p38 mitogen-activated protein kinase through the downstream mediator Pak1," *Journal of Biological Chemistry*, vol. 270, no. 41, pp. 23934–23936, 1995.

[72] N. Soga, N. Namba, S. McAllister et al., "Rho family GTPases regulate VEGF-stimulated endothelial cell motility," *Experimental Cell Research*, vol. 269, no. 1, pp. 73–87, 2001.

[73] B. H. Fryer, C. Wang, S. Vedantam et al., "cGMP-dependent protein kinase phosphorylates p21-activated kinase (Pak) 1, inhibiting Pak/Nck binding and stimulating Pak/vasodilator-stimulated phosphoprotein association," *The Journal of Biological Chemistry*, vol. 281, no. 17, pp. 11487–11495, 2006.

[74] E. R. Park, S. T. Eblen, and A. D. Catling, "MEK1 activation by PAK: A novel mechanism," *Cellular Signalling*, vol. 19, no. 7, pp. 1488–1496, 2007.

[75] M. Zang, C. Hayne, and Z. Luo, "Interaction between active Pak1 and Raf-1 is necessary for phosphorylation and activation of Raf-1," *Journal of Biological Chemistry*, vol. 277, no. 6, pp. 4395–4405, 2002.

[76] Y. Chen, M. Banda, C. L. Speyer, J. S. Smith, A. B. Rabson, and D. H. Gorski, "Regulation of the expression and activity of the antiangiogenic homeobox gene *GAX/MEOX2* by ZEB2 and MicroRNA-221," *Molecular and Cellular Biology*, vol. 30, no. 15, pp. 3902–3913, 2010.

[77] A. Kuehbacher, C. Urbich, A. M. Zeiher, and S. Dimmeler, "Role of Dicer and Drosha for endothelial microRNA expression and angiogenesis," *Circulation Research*, vol. 101, no. 1, pp. 59–68, 2007.

[78] Y. Suárez, C. Fernández-Hernando, J. S. Pober, and W. C. Sessa, "Dicer dependent microRNAs regulate gene expression and functions in human endothelial cells," *Circulation Research*, vol. 100, no. 8, pp. 1164–1173, 2007.

[79] A. J. O'Hara, P. Chugh, L. Wang et al., "Pre-micro rna signatures delineate stages of endothelial cell transformation in kaposi sarcoma," *PLoS Pathogens*, vol. 5, no. 4, Article ID e1000389, 2009.

[80] Y.-H. Wu, T.-F. Hu, Y.-C. Chen et al., "The manipulation of miRNA-gene regulatory networks by KSHV induces endothelial cell motility," *Blood*, vol. 118, no. 10, pp. 2896–2905, 2011.

[81] Y. Sato, K. Teruyama, T. Nakano et al., "Role of transcription factors in angiogenesis: Ets-1 promotes angiogenesis as well as endothelial apoptosis," *Annals of the New York Academy of Sciences*, vol. 947, pp. 117–123, 2001.

[82] U. Förstermann and T. Münzel, "Endothelial nitric oxide synthase in vascular disease: from marvel to menace," *Circulation*, vol. 113, no. 13, pp. 1708–1714, 2006.

[83] E. Sánchez-Tilló, L. Siles, O. de Barrios et al., "Expanding roles of ZEB factors in tumorigenesis and tumor progression," *The American Journal of Cancer Research*, vol. 1, no. 7, pp. 897–912, 2011.

[84] D. H. Gorski and A. J. Leal, "Inhibition of endothelial cell activation by the homeobox gene Gax," *Journal of Surgical Research*, vol. 111, no. 1, pp. 91–99, 2003.

[85] Y. Chen, A. D. Leal, S. Patel, and D. H. Gorski, "The homeobox gene *GAX* activates $p21^{WAF1/CIP1}$ expression in vascular endothelial cells through direct interaction with upstream AT-rich sequences," *Journal of Biological Chemistry*, vol. 282, no. 1, pp. 507–517, 2007.

[86] S. Patel, A. D. Leal, and D. H. Gorski, "The homeobox gene *Gax* inhibits angiogenesis through inhibition of nuclear factor-κB-dependent endothelial cell gene expression," *Cancer Research*, vol. 65, no. 4, pp. 1414–1424, 2005.

[87] X. Yang, D. Qiao, K. Meyer, and A. Friedl, "Signal transducers and activators of transcription mediate fibroblast growth factor-induced vascular endothelial morphogenesis," *Cancer Research*, vol. 69, no. 4, pp. 1668–1677, 2009.

[88] X. Yang, D. Qiao, K. Meyer, T. Pier, S. Keles, and A. Friedl, "Angiogenesis induced by signal transducer and activator of transcription 5A (STAT5A) is dependent on autocrine activity of proliferin," *The Journal of Biological Chemistry*, vol. 287, no. 9, pp. 6490–6502, 2012.

[89] X. Yang, K. Meyer, and A. Friedl, "STAT5 and prolactin participate in a positive autocrine feedback loop that promotes angiogenesis," *Journal of Biological Chemistry*, vol. 288, no. 29, pp. 21184–21196, 2013.

[90] P. Dentelli, L. del Sorbo, A. Rosso et al., "Human IL-3 stimulates endothelial cell motility and promotes in vivo new vessel formation," *The Journal of Immunology*, vol. 163, no. 4, pp. 2151–2159, 1999.

[91] M.-T. Park, E.-T. Oh, M.-J. Song, H. Lee, and H. J. Park, "Radiosensitivities and angiogenic signaling pathways of irradiated normal endothelial cells derived from diverse human organs," *Journal of Radiation Research*, vol. 53, no. 4, pp. 570–580, 2012.

[92] S. Vincenti, N. Brillante, V. Lanza et al., "HUVEC respond to radiation by inducing the expression of pro-angiogenic MicroRNAs," *Radiation Research*, vol. 175, no. 5, pp. 535–546, 2011.

[93] H. Dellago, B. Preschitz-Kammerhofer, L. Terlecki-Zaniewicz et al., "High levels of oncomiR-21 contribute to the senescence-induced growth arrest in normal human cells and its knockdown increases the replicative lifespan," *Aging Cell*, vol. 12, no. 3, pp. 446–458, 2013.

[94] J. M. Yu, X. Wu, J. M. Gimble, X. Guan, M. A. Freitas, and B. A. Bunnell, "Age-related changes in mesenchymal stem cells derived from rhesus macaque bone marrow," *Aging Cell*, vol. 10, no. 1, pp. 66–79, 2011.

[95] N. N. Hooten, K. Abdelmohsen, M. Gorospe, N. Ejiogu, A. B. Zonderman, and M. K. Evans, "microRNA expression patterns reveal differential expression of target genes with age," *PLoS ONE*, vol. 5, no. 5, Article ID e10724, 2010.

[96] K. Sobue, K. Hayashi, and W. Nishida, "Expressional regulation of smooth muscle cell-specific genes in association with phenotypic modulation," *Molecular and Cellular Biochemistry*, vol. 190, no. 1-2, pp. 105–118, 1999.

[97] K. Kawai-Kowase and G. K. Owens, "Multiple repressor pathways contribute to phenotypic switching of vascular smooth muscle cells," *American Journal of Physiology: Cell Physiology*, vol. 292, no. 1, pp. C59–C69, 2007.

[98] C. Purcell, M. Tennant, and J. McGeachie, "Neo-intimal hyperplasia in vascular grafts and its implications for autologous arterial grafting," *Annals of the Royal College of Surgeons of England*, vol. 79, no. 3, pp. 164–168, 1997.

[99] G. K. Owens, "Molecular control of vascular smooth muscle cell differentiation and phenotypic plasticity," *Novartis Foundation Symposia*, vol. 283, pp. 174–191, 2007.

[100] V. R. Babaev, Y. V. Bobryshev, O. V. Stenina, E. M. Tararak, and G. Gabbiani, "Heterogeneity of smooth muscle cells in atheromatous plaque of human aorta," *The American Journal of Pathology*, vol. 136, no. 5, pp. 1031–1042, 1990.

[101] V. R. Babaev, Y. V. Bobryshev, G. K. Sukhova, and I. A. Kasantseva, "Monocyte/macrophage accumulation and smooth muscle cell phenotypes in early atherosclerotic lesions of human aorta," *Atherosclerosis*, vol. 100, no. 2, pp. 237–248, 1993.

[102] X. Liu, Y. Cheng, J. Yang, L. Xu, and C. Zhang, "Cell-specific effects of miR-221/222 in vessels: molecular mechanism and therapeutic application," *Journal of Molecular and Cellular Cardiology*, vol. 52, no. 1, pp. 245–255, 2012.

[103] X. Liu, Y. Cheng, S. Zhang, Y. Lin, J. Yang, and C. Zhang, "A necessary role of miR-221 and miR-222 in vascular smooth muscle cell proliferation and neointimal hyperplasia," *Circulation Research*, vol. 104, no. 4, pp. 476–486, 2009.

[104] C. A. McNamara, I. J. Sarembock, B. G. Bachhuber et al., "Thrombin and vascular smooth muscle cell proliferation: implications for atherosclerosis and restenosis," *Seminars in Thrombosis and Hemostasis*, vol. 22, no. 2, pp. 139–144, 1996.

[105] P. Castagnino, D. Kothapalli, E. A. Hawthorne et al., "miR-221/222 compensates for skp2-mediated p27 degradation and is a primary target of cell cycle regulation by prostacyclin and cAMP," *PLoS ONE*, vol. 8, no. 2, Article ID e56140, 2013.

[106] C. A. Auld, K. M. Fernandes, and R. F. Morrison, "Skp2-mediated p27(Kip1) degradation during S/G_2 phase progression of adipocyte hyperplasia," *Journal of Cellular Physiology*, vol. 211, no. 1, pp. 101–111, 2007.

[107] S. Matsuoka, M. C. Edwards, C. Bai et al., "P57KIP2, a structurally distinct member of the p21CIP1 Cdk inhibitor family, is a candidate tumor suppressor gene," *Genes & Development*, vol. 9, no. 6, pp. 650–662, 1995.

[108] E. G. Reynaud, M. P. Leibovitch, L. A. J. Tintignac, K. Pelpel, M. Guillier, and S. A. Leibovitch, "Stabilization of MyoD by direct binding to $p57^{Kip2}$," *The Journal of Biological Chemistry*, vol. 275, no. 25, pp. 18767–18776, 2000.

[109] Z. Wang, D.-Z. Wang, G. C. T. Pipes, and E. N. Olson, "Myocardin is a master regulator of smooth muscle gene expression," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 12, pp. 7129–7134, 2003.

[110] P. F. Davies, "Hemodynamic shear stress and the endothelium in cardiovascular pathophysiology," *Nature Clinical Practice Cardiovascular Medicine*, vol. 6, no. 1, pp. 16–26, 2009.

[111] A. G. Passerini, D. C. Polacek, C. Shi et al., "Coexisting proinflammatory and antioxidative endothelial transcription profiles in a disturbed flow region of the adult porcine aorta," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 8, pp. 2482–2487, 2004.

[112] A. R. Brooks, P. I. Lelkes, and G. M. Rubanyi, "Gene expression profiling of vascular endothelial cells exposed to fluid mechanical forces: relevance for focal susceptibility to atherosclerosis," *Endothelium*, vol. 11, no. 1, pp. 45–57, 2004.

[113] E. J. R. Mattsson, T. R. Kohler, S. M. Vergel, and A. W. Clowes, "Increased blood flow induces regression of intimal hyperplasia," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 17, no. 10, pp. 2245–2249, 1997.

[114] T. M. Griffith, "Endothelial control of vascular tone by nitric oxide and gap junctions: a haemodynamic perspective," *Biorheology*, vol. 39, no. 3-4, pp. 307–318, 2002.

[115] C. Cheng, R. van Haperen, M. de Waard et al., "Shear stress affects the intracellular distribution of eNOS: direct demonstration by a novel in vivo technique," *Blood*, vol. 106, no. 12, pp. 3691–3698, 2005.

[116] C. Cheng, D. Tempel, R. van Haperen et al., "Atherosclerotic lesion size and vulnerability are determined by patterns of fluid shear stress," *Circulation*, vol. 113, no. 23, pp. 2744–2753, 2006.

[117] G. Dai, M. R. Kaazempur-Mofrad, S. Natarajan et al., "Distinct endothelial phenotypes evoked by arterial waveforms derived from atherosclerosis-susceptible and -resistant regions of human vasculature," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 41, pp. 14871–14876, 2004.

[118] J. Suo, D. E. Ferrara, D. Sorescu, R. E. Guldberg, W. R. Taylor, and D. P. Giddens, "Hemodynamic shear stresses in mouse aortas: implications for atherogenesis," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 27, no. 2, pp. 346–351, 2007.

[119] L. Rouleau, J. Rossi, and R. L. Leask, "The response of human aortic endothelial cells in a stenotic hemodynamic environment: effect of duration, magnitude, and spatial gradients in

wall shear stress," *Journal of Biomechanical Engineering*, vol. 132, no. 7, Article ID 071015, 2010.

[120] G. Dai, S. Vaughn, Y. Zhang, E. T. Wang, G. Garcia-Cardena, and M. A. Gimbrone Jr., "Biomechanical forces in atherosclerosis-resistant vascular regions regulate endothelial redox balance via phosphoinositol 3-kinase/Akt-dependent activation of Nrf2," *Circulation Research*, vol. 101, no. 7, pp. 723–733, 2007.

[121] Y. Fang, C. Shi, E. Manduchi, M. Civelek, and P. F. Davies, "MicroRNA-10a regulation of proinflammatory phenotype in athero-susceptible endothelium in vivo and in vitro," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 30, pp. 13450–13455, 2010.

[122] Y. Fang and P. F. Davies, "Site-specific microRNA-92a regulation of Krüppel-like factors 4 and 2 in atherosusceptible endothelium," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 32, no. 4, pp. 979–987, 2012.

[123] C.-Y. Hsieh, H.-Y. Hsiao, W.-Y. Wu et al., "Regulation of shear-induced nuclear translocation of the Nrf2 transcription factor in endothelial cells," *Journal of Biomedical Science*, vol. 16, no. 1, article 12, 2009.

[124] X. L. Chen, G. Dodd, S. Thomas et al., "Activation of Nrf2/ARE pathway protects endothelial cells from oxidant injury and inhibits inflammatory gene expression," *The American Journal of Physiology—Heart and Circulatory Physiology*, vol. 290, no. 5, pp. H1862–H1870, 2006.

[125] J. Y. Moon, "Recent update of renin-angiotensin-aldosterone system in the pathogenesis of hypertension," *Electrolytes & Blood Pressure*, vol. 11, no. 2, pp. 41–45, 2013.

[126] Y.-T. Wang, P.-C. Tsai, Y.-C. Liao, C.-Y. Hsu, and S.-H. H. Juo, "Circulating microRNAs have a sex-specific association with metabolic syndrome," *Journal of Biomedical Science*, vol. 20, no. 1, article 72, 2013.

[127] I. Jovanović, M. Živković, J. Jovanović, T. Djurić, and A. Stanković, "The co-inertia approach in identification of specific microRNA in early and advanced atherosclerosis plaque," *Medical Hypotheses*, vol. 83, no. 1, pp. 11–15, 2014.

[128] Y. V. Bobryshev, "Monocyte recruitment and foam cell formation in atherosclerosis," *Micron*, vol. 37, no. 3, pp. 208–222, 2006.

[129] A.-Y. Gong, G. Hu, R. Zhou et al., "MicroRNA-221 controls expression of intercellular adhesion molecule-1 in epithelial cells in response to *Cryptosporidium parvum* infection," *International Journal for Parasitology*, vol. 41, no. 3-4, pp. 397–403, 2011.

[130] Q. Zhang, I. Kandic, and M. J. Kutryk, "Dysregulation of angiogenesis-related microRNAs in endothelial progenitor cells from patients with coronary artery disease," *Biochemical and Biophysical Research Communications*, vol. 405, no. 1, pp. 42–46, 2011.

[131] Y. Minami, M. Satoh, C. Maesawa et al., "Effect of atorvastatin on microRNA 221/222 expression in endothelial progenitor cells obtained from patients with coronary artery disease," *European Journal of Clinical Investigation*, vol. 39, no. 5, pp. 359–367, 2009.

[132] G. Togliatto, A. Trombetta, P. Dentelli, A. Rosso, and M. F. Brizzi, "MIR221/MIR222-driven post-transcriptional regulation of P27KIP1 and P57KIP2 is crucial for high-glucose- and AGE-mediated vascular cell damage," *Diabetologia*, vol. 54, no. 7, pp. 1930–1940, 2011.

[133] C. B. Coleman, D. J. Lightell, S. C. Moss, M. Bates, P. E. Parrino, and T. C. Woods, "Elevation of miR-221 and -222 in the internal mammary arteries of diabetic subjects and normalization with metformin," *Molecular and Cellular Endocrinology*, vol. 374, no. 1-2, pp. 125–129, 2013.

[134] T. Li, H. Cao, J. Zhuang et al., "Identification of miR-130a, miR-27b and miR-210 as serum biomarkers for atherosclerosis obliterans," *Clinica Chimica Acta*, vol. 412, no. 1-2, pp. 66–70, 2011.

[135] X. Zhang, S. Shao, H. Geng et al., "Expression profiles of six circulating microRNAs critical to atherosclerosis in patients with subclinical hypothyroidism: a clinical study," *Journal of Clinical Endocrinology and Metabolism*, vol. 99, no. 5, pp. E766–E774, 2014.

[136] D. A. Chistiakov, I. A. Sobenin, and A. N. Orekhov, "Vascular extracellular matrix in atherosclerosis," *Cardiology in Review*, vol. 21, no. 6, pp. 270–288, 2013.

[137] G. Hu, A.-Y. Gong, J. Liu, R. Zhou, C. Deng, and X.-M. Chen, "miR-221 suppresses ICAM-1 translation and regulates interferon-γ-induced ICAM-1 expression in human cholangiocytes," *American Journal of Physiology—Gastrointestinal and Liver Physiology*, vol. 298, no. 4, pp. G542–G550, 2010.

[138] S. Mardente, E. Mari, F. Consorti et al., "HMGB1 induces the overexpression of miR-222 and miR-221 and increases growth and motility in papillary thyroid cancer cells," *Oncology Reports*, vol. 28, no. 6, pp. 2285–2289, 2012.

[139] Y. Luo, S.-J. Li, J. Yang, Y.-Z. Qiu, and F.-P. Chen, "HMGB1 induces an inflammatory response in endothelial cells via the RAGE-dependent endoplasmic reticulum stress pathway," *Biochemical and Biophysical Research Communications*, vol. 438, no. 4, pp. 732–738, 2013.

[140] G. H. Gibbons, R. E. Pratt, and V. J. Dzau, "Vascular smooth muscle cell hypertrophy vs. hyperplasia: autocrine transforming growth factor-β, expression determines growth response to angiotensin II," *Journal of Clinical Investigation*, vol. 90, no. 2, pp. 456–461, 1992.

[141] P. K. Mehta and K. K. Griendling, "Angiotensin II cell signaling: physiological and pathological effects in the cardiovascular system," *The American Journal of Physiology—Cell Physiology*, vol. 292, no. 1, pp. C82–C97, 2007.

[142] X. Dong, L.-G. Yu, R. Sun et al., "Inhibition of PTEN expression and activity by angiotensin II induces proliferation and migration of vascular smooth muscle cells," *Journal of Cellular Biochemistry*, vol. 114, no. 1, pp. 174–182, 2013.

[143] J. Andrae, R. Gallini, and C. Betsholtz, "Role of platelet-derived growth factors in physiology and medicine," *Genes and Development*, vol. 22, no. 10, pp. 1276–1312, 2008.

[144] A. Díez-Juan and V. Andrés, "The growth suppressor $p27^{Kip1}$ protects against diet-induced atherosclerosis," *FASEB Journal*, vol. 15, no. 11, pp. 1989–1995, 2001.

[145] L. M. Akyürek, M. Boehm, M. Olive, A.-X. Zhou, H. San, and E. G. Nabel, "Deficiency of cyclin-dependent kinase inhibitors $p21^{Cip1}$ and $p27^{Kip1}$ accelerates atherogenesis in apolipoprotein E-deficient mice," *Biochemical and Biophysical Research Communications*, vol. 396, no. 2, pp. 359–363, 2010.

[146] D. Kothapalli, P. Castagnino, D. J. Rader, M. C. Phillips, S. Lund-Katz, and R. K. Assoian, "Apolipoprotein E-mediated cell cycle arrest linked to p27 and the Cox2-dependent repression of miR221/222," *Atherosclerosis*, vol. 227, no. 1, pp. 65–71, 2013.

[147] M. Ishigami, D. K. Swertfeger, M. S. Hui, N. A. Granholm, and D. Y. Hui, "Apolipoprotein E inhibition of vascular smooth muscle cell proliferation but not the inhibition of migration is mediated through activation of inducible nitric oxide synthase," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 20, no. 4, pp. 1020–1026, 2000.

[148] L. Paka, I. J. Goldberg, J. C. Obunike et al., "Perlecan mediates the antiproliferative effect of apolipoprotein E on smooth muscle cells. An underlying mechanism for the modulation of smooth muscle cell growth?" *The Journal of Biological Chemistry*, vol. 274, no. 51, pp. 36403–36408, 1999.

[149] F. C. Tanner, M. Boehm, L. M. Akyürek et al., "Differential effects of the cyclin-dependent kinase inhibitors p27(Kip1), p21(Cip1) and p16(Ink4) on vascular smooth muscle cell proliferation," *Circulation*, vol. 101, no. 17, pp. 2022–2025, 2000.

[150] N. Nakano, K. Urasawa, Y. Takagi et al., "Downregulation of cyclin-dependent kinase inhibitor; $p57^{kip2}$, is involved in the cell cycle progression of vascular smooth muscle cells," *Biochemical and Biophysical Research Communications*, vol. 338, no. 3, pp. 1661–1667, 2005.

[151] N. Mackenzie, D. Zhu, P. Genever, and V. MacRae, "The miR-221/222 family regulates vascular smooth muscle cell calcification," *Bone Abstracts*, vol. 1, article PP496, 2013.

[152] Y. V. Bobryshev, "Targeting vascular calcification: up-date," *Current Pharmaceutical Design*, vol. 20, no. 37, p. 5799, 2014.

[153] Y. V. Bobryshev, R. S. A. Lord, and B. A. Warren, "Calcified deposit formation in intimal thickenings of the human aorta," *Atherosclerosis*, vol. 118, no. 1, pp. 9–21, 1995.

[154] N. Niederhoffer, Y. V. Bobryshev, I. Lartaud-Idjouadiene, P. Giummelly, and J. Atkinson, "Aortic calcification produced by vitamin D_3 plus nicotine," *Journal of Vascular Research*, vol. 34, no. 5, pp. 386–398, 1997.

[155] D. A. Chistiakov, I. A. Sobenin, A. N. Orekhov, and Y. V. Bobryshev, "Mechanisms of medial arterial calcification in diabetes," *Current Pharmaceutical Design*, vol. 20, no. 37, pp. 5870–5883, 2014.

[156] Y. V. Bobryshev, "Transdifferentiation of smooth muscle cells into chondrocytes in atherosclerotic arteries in situ: implications for diffuse intimal calcification," *Journal of Pathology*, vol. 205, no. 5, pp. 641–650, 2005.

[157] R. Villa-Bellosta, X. Wang, J. L. Millán, G. R. Dubyak, and W. C. O'Neill, "Extracellular pyrophosphate metabolism and calcification in vascular smooth muscle," *American Journal of Physiology: Heart and Circulatory Physiology*, vol. 301, no. 1, pp. H61–H68, 2011.

[158] T. Li, H. Cao, J. Zhuang et al., "Identification of miR-130a, miR-27b and miR-210 as serum biomarkers for atherosclerosis obliterans," *Clinica Chimica Acta*, vol. 412, no. 1-2, pp. 66–70, 2011.

[159] Y. V. Bobryshev, "Dendritic cells in atherosclerosis: current status of the problem and clinical relevance," *European Heart Journal*, vol. 26, no. 17, pp. 1700–1704, 2005.

[160] K. J. Woollard, "Immunological aspects of atherosclerosis," *Clinical Science*, vol. 125, no. 5, pp. 221–235, 2013.

[161] D. A. Chistiakov, I. A. Sobenin, A. N. Orekhov, and Y. V. Bobryshev, "Dendritic cells: a double-edge sword in atherosclerotic inflammation," *Atherosclerosis*, 2013.

[162] H. Ait-Oufella, A. P. Sage, Z. Mallat, and A. Tedgui, "Adaptive (T and B Cells) immunity and control by dendritic cells in atherosclerosis," *Circulation Research*, vol. 114, no. 10, pp. 1640–1660, 2014.

[163] C. Lu, X. Huang, X. Zhang et al., "miR-221 and miR-155 regulate human dendritic cell development, apoptosis, and IL-12 production through targeting of p27 kip1, KPC1, and SOCS-1," *Blood*, vol. 117, no. 16, pp. 4293–4303, 2011.

[164] H. Kuipers, F. M. Schnorfeil, and T. Brocker, "Differentially expressed microRNAs regulate plasmacytoid vs. conventional dendritic cell development," *Molecular Immunology*, vol. 48, no. 1–3, pp. 333–340, 2010.

[165] S. Montagner, E. M. Orlandi, S. Merante, and S. Monticelli, "The role of miRNAs in mast cells and other innate immune cells," *Immunological Reviews*, vol. 253, no. 1, pp. 12–24, 2013.

[166] B. S. Nielsen and K. Holmstrøm, "Combined microRNA in situ hybridization and immunohistochemical detection of protein markers," *Methods in Molecular Biology*, vol. 986, pp. 353–365, 2013.

[167] M. Schneider, D. C. Andersen, A. Silahtaroglu et al., "Cell-specific detection of microRNA expression during cardiomyogenesis by combined in situ hybridization and immunohistochemistry," *Journal of Molecular Histology*, vol. 42, no. 4, pp. 289–299, 2011.

[168] A. D. Chaudhuri, S. V. Yelamanchili, and H. S. Fox, "Combined fluorescent in situ hybridization for detection of microRNAs and immunofluorescent labeling for cell-type markers," *Frontiers in Cellular Neuroscience*, vol. 7, article 160, 2013.

[169] Y. V. Bobryshev, "Intracellular localization of oxidized low-density lipoproteins in atherosclerotic plaque cells revealed by electron microscopy combined with laser capture microdissection," *Journal of Histochemistry and Cytochemistry*, vol. 53, no. 6, pp. 793–797, 2005.

[170] J. E. Feig and E. A. Fisher, "Laser capture microdissection for analysis of macrophage gene expression from atherosclerotic lesions," *Methods in Molecular Biology*, vol. 1027, pp. 123–135, 2013.

[171] R. Malgor, P. M. Bhatt, B. A. Connolly et al., "Wnt5a, TLR2 and TLR4 are elevated in advanced human atherosclerotic lesions," *Inflammation Research*, vol. 63, no. 4, pp. 277–285, 2014.

[172] A. Meerson, M. Traurig, V. Ossowski, J. M. Fleming, M. Mullins, and L. J. Baier, "Human adipose microRNA-221 is upregulated in obesity and affects fat metabolism downstream of leptin and TNF-α," *Diabetologia*, vol. 56, no. 9, pp. 1971–1979, 2013.

[173] Y. Lustig, E. Barhod, R. Ashwal-Fluss et al., "RNA-binding protein PTB and MicroRNA-221 coregulate AdipoR1 translation and adiponectin signaling," *Diabetes*, vol. 63, no. 2, pp. 433–445, 2014.

[174] J. Nedvídková, K. Smitka, V. Kopský, and V. Hainer, "Adiponectin, an adipocyte-derived protein," *Physiological Research*, vol. 54, no. 2, pp. 133–140, 2005.

[175] P. Zhang, Y. Wang, Y. Fan, Z. Tang, and N. Wang, "Overexpression of adiponectin receptors potentiates the antiinflammatory action of subeffective dose of globular adiponectin in vascular endothelial cells," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 29, no. 1, pp. 67–74, 2009.

[176] W.-W. Chou, Y.-T. Wang, Y.-C. Liao, S.-C. Chuang, S.-N. Wang, and S.-H. H. Juo, "Decreased MicroRNA-221 is associated with high levels of TNF-α in human adipose tissue-derived mesenchymal stem cells from obese woman," *Cellular Physiology and Biochemistry*, vol. 32, no. 1, pp. 127–137, 2013.

[177] F. J. Ortega, J. M. Mercader, V. Catalán et al., "Targeting the circulating microRNA signature of obesity," *Clinical Chemistry*, vol. 59, no. 5, pp. 781–792, 2013.

[178] J. Berndt, P. Kovacs, K. Ruschke et al., "Fatty acid synthase gene expression in human adipose tissue: association with obesity and type 2 diabetes," *Diabetologia*, vol. 50, no. 7, pp. 1472–1480, 2007.

[179] C. Capurso and A. Capurso, "From excess adiposity to insulin resistance: the role of free fatty acids," *Vascular Pharmacology*, vol. 57, no. 2–4, pp. 91–97, 2012.

[180] G. Togliatto, A. Trombetta, P. Dentelli et al., "Unacylated ghrelin promotes skeletal muscle regeneration following hindlimb ischemia via SOD-2-mediated miR-221/222 expression," *Journal of the American Heart Association*, vol. 2, no. 6, Article ID e000376, 2013.

[181] J. A. Ward, N. Esa, R. Pidikiti et al., "Circulating cell and plasma microRNA profiles differ between non-ST-segment and ST-segment-elevation myocardial infarction," *Family Medicine & Medical Science Research*, vol. 2, no. 2, article 108, 2013.

[182] B. Cardinalli, L. Castellani, P. Fasanaro et al., "Microrna-221 and microrna-222 modulate differentiation and maturation of skeletal muscle cells," *PLoS ONE*, vol. 4, no. 10, Article ID e7607, 2009.

[183] M. W. Hamrick, S. Herberg, P. Arounleut et al., "The adipokine leptin increases skeletal muscle mass and significantly alters skeletal muscle miRNA expression profile in aged mice," *Biochemical and Biophysical Research Communications*, vol. 400, no. 3, pp. 379–383, 2010.

[184] A. P. D. Andreote, M. F. Rosario, M. C. Ledur et al., "Identification and characterization of microRNAs expressed in chicken skeletal muscle," *Genetics and Molecular Research*, vol. 13, no. 1, pp. 1465–1479, 2014.

[185] S. Greco, A. Perfetti, P. Fasanaro et al., "Deregulated microRNAs in myotonic dystrophy type 2," *PLoS ONE*, vol. 7, no. 6, Article ID e39732, 2012.

[186] H. J. Feng, W. Ouyang, J. H. Liu et al., "Global microRNA profiles and signaling pathways in the development of cardiac hypertrophy," *Brazilian Journal of Medical and Biological Research*, vol. 47, no. 5, pp. 361–368, 2014.

[187] S. Hu, M. Huang, P. K. Nguyen et al., "Novel MicroRNA prosurvival cocktail for improving engraftment and function of cardiac progenitor cell transplantation," *Circulation*, vol. 124, no. 11, pp. S27–S34, 2011.

[188] B. Yu, M. Gong, Y. Wang et al., "Cardiomyocyte protection by GATA-4 gene engineered mesenchymal stem cells is partially mediated by translocation of miR-221 in microvesicles," *PLoS ONE*, vol. 8, no. 8, Article ID e73304, 2013.

[189] X. Zhou, P. Yuan, and Y. He, "Role of microRNAs in peripheral artery disease," *Molecular Medicine Reports*, vol. 6, no. 4, pp. 695–700, 2012.

[190] D. A. Chistiakov, I. A. Sobenin, and A. N. Orekhov, "Strategies to deliver microRNAs as potential therapeutics in the treatment of cardiovascular pathology," *Drug Delivery*, vol. 19, no. 8, pp. 392–405, 2012.

[191] C. Fernández-Hernando and A. Baldán, "MicroRNAs and cardiovascular disease," *Current Genetic Medicine Reports*, vol. 1, no. 1, pp. 30–38, 2013.

[192] T. Imanishi and T. Akasaka, "MicroRNAs in peripheral artery disease," *Current Topics in Medicinal Chemistry*, vol. 13, no. 13, pp. 1589–1595, 2013.

[193] H. Toba, D. Cortez, M. L. Lindsey, and R. J. Chilton, "Applications of miRNA technology for atherosclerosis," *Current Atherosclerosis Reports*, vol. 16, no. 2, ar, 2014.

[194] X. Lu and V. Kakkar, "The roles of microRNAs in atherosclerosis," *Current Medicinal Chemistry*, vol. 21, no. 13, pp. 1531–1543, 2014.

[195] L. Gramantieri, F. Fornari, E. Callegari et al., "MicroRNA involvement in hepatocellular carcinoma: microRNA review series," *Journal of Cellular and Molecular Medicine*, vol. 12, no. 6A, pp. 2189–2204, 2008.

[196] R. Nassirpour, P. P. Mehta, S. M. Baxi, and M. J. Yin, "miR-221 promotes tumorigenesis in human triple negative breast cancer cells," *PLoS ONE*, vol. 8, no. 4, Article ID e62170, 2013.

[197] Q. Lin, W. Mao, Y. Shu et al., "A cluster of specified microRNAs in peripheral blood as biomarkers for metastatic non-small-cell lung cancer by stem-loop RT-PCR," *Journal of Cancer Research and Clinical Oncology*, vol. 138, no. 1, pp. 85–93, 2012.

[198] K. Sun, W. Wang, J.-J. Zeng, C.-T. Wu, S.-T. Lei, and G.-X. Li, "MicroRNA-221 inhibits CDKN1C/p57 expression in human colorectal carcinoma," *Acta Pharmacologica Sinica*, vol. 32, no. 3, pp. 375–384, 2011.

[199] Y.-K. Kim, J. Yu, T. S. Han et al., "Functional links between clustered microRNAs: suppression of cell-cycle inhibitors by microRNA clusters in gastric cancer," *Nucleic Acids Research*, vol. 37, no. 5, pp. 1672–1681, 2009.

[200] S. Sarkar, H. Dubaybo, S. Ali et al., "Down-regulation of miR-221 inhibits proliferation of pancreatic cancer cells through up-regulation of PTEN, p27(kip1), p57(kip2), and PUMA," *American Journal of Cancer Research*, vol. 3, no. 5, pp. 465–477, 2013.

[201] E. N. Howe, D. R. Cochrane, and J. K. Richer, "The miR-200 and miR-221/222 microRNA families: opposing effects on epithelial identity," *Journal of Mammary Gland Biology and Neoplasia*, vol. 17, no. 1, pp. 65–77, 2012.

[202] Y.-L. Fan, M. Zheng, Y.-L. Tang, and X.-H. Liang, "A new perspective of vasculogenic mimicry: EMT and cancer stem cells," *Oncology Letters*, vol. 6, no. 5, pp. 1174–1180, 2013.

[203] J. Du, B. Sun, X. Zhao et al., "Hypoxia promotes vasculogenic mimicry formation by inducing epithelial-mesenchymal transition in ovarian carcinoma," *Gynecologic Oncology*, vol. 133, no. 3, pp. 575–583, 2014.

[204] S. M. Ehsan, K. M. Welch-Reardon, M. L. Waterman, C. C. W. Hughes, and S. C. George, "A three-dimensional in vitro model of tumor cell intravasation," *Integrative Biology (United Kingdom)*, vol. 6, no. 6, pp. 603–610, 2014.

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 732397, 12 pages
http://dx.doi.org/10.1155/2015/732397

Research Article

MicroRNA Clusters in the Adult Mouse Heart: Age-Associated Changes

Xiaomin Zhang,[1,2] Gohar Azhar,[1,2] Emmanuel D. Williams,[1,2] Steven C. Rogers,[1,2] and Jeanne Y. Wei[1,2]

[1]*Reynolds Institute on Aging and Department of Geriatrics, University of Arkansas for Medical Science, 4301 West Markham Street, No. 748, Little Rock, AR 72205, USA*
[2]*Geriatric Research Education and Clinical Center, Central Arkansas Veterans Healthcare System, Little Rock, AR 72205, USA*

Correspondence should be addressed to Jeanne Y. Wei; weijeanne@uams.edu

Received 19 September 2014; Revised 10 December 2014; Accepted 13 December 2014

Academic Editor: Ling-Qing Yuan

The microRNAs and microRNA clusters have been implicated in normal cardiac development and also disease, including cardiac hypertrophy, cardiomyopathy, heart failure, and arrhythmias. Since a microRNA cluster has from two to dozens of microRNAs, the expression of a microRNA cluster could have a substantial impact on its target genes. In the present study, the configuration and distribution of microRNA clusters in the mouse genome were examined at various inter-microRNA distances. Three important microRNA clusters that are significantly impacted during adult cardiac aging, the miR-17-92, miR-106a-363, and miR-106b-25, were also examined in terms of their genomic location, RNA transcript character, sequence homology, and their relationship with the corresponding microRNA families. Multiple microRNAs derived from the three clusters potentially target various protein components of the cdc42-SRF signaling pathway, which regulates cytoskeleton dynamics associated with cardiac structure and function. The data indicate that aging impacted the expression of both guide and passenger strands of the microRNA clusters; nutrient stress also affected the expression of the three microRNA clusters. The miR-17-92, miR-106a-363, and miR-106b-25 clusters are likely to impact the Cdc42-SRF signaling pathway and thereby affect cardiac morphology and function during pathological conditions and the aging process.

1. Introduction

The regulation of cardiomyocyte proliferation, hypertrophy, and function during normal development, maturation, and adult aging is a continuous and progressive process that results in changes in the structure and function of the myocardium and blood vessels, which in turn lead to an increased incidence of cardiovascular diseases with advancing age [1]. It has been documented that the altered expressions of a number of cardiac genes are involved in response to stress and in a number of pathological conditions, as well as in the aging process. These genes include the muscle-specific genes (ANF, β-MHC, skeletal α-actin, α-MHC, cardiac α-actin, and SERCA2) [2–5] and transcription factors and cofactors (SRF, myocardin, p49) [6–10], as well as genes regulating metabolism and the extracellular matrix (ECM) [11–13]. Recent studies indicate that noncoding RNAs, especially microRNAs (miRNAs), play an important role in the regulation of messenger RNA (mRNA) expression [14–18].

MicroRNAs (miRNAs) are relatively short (20 to 23 nucleotides), endogenous, and single-stranded RNA molecules that regulate gene expression usually by hybridizing to messenger RNAs (mRNAs) with the consequence of mRNA degradation or translational inhibition of targeted transcripts. Genes that encode for miRNA are transcribed by either RNA polymerase II or RNA polymerase III into primary miRNA (pri-miRNA) transcripts, which are then cleaved by the nuclear microprocessor complex formed by the RNase III enzyme Drosha (RNASEN) and the DGCR8 (DiGeorge critical region 8) protein. The RNase III Dicer cleaves off the loop of the pre-miRNA to generate a roughly

22-nucleotide miRNA duplex [19]. Genes that encode for miRNAs are distributed across chromosomes either individually or in clusters, in which two or more miRNA genes are located within a short distance on the same segment of a chromosome [20, 21]. In the mouse genome, each miRNA cluster contains from two to as many as 71 miRNA genes [22]. The miRNAs within the same cluster are likely to be in the same RNA transcript. In addition, the miRNAs within a single gene cluster may share the same "seed" sequence or may have high sequence homology [21]. Therefore, the increase or decrease in the expression of a miRNA cluster could potentially have a substantial regulatory effect on the posttranscriptional regulation of protein-encoding genes.

In our previous study, we reported altered expression of miRNAs with aging in the adult mouse heart, in which approximately half of the age-related miRNAs were in miRNA clusters, suggesting that these miRNA clusters likely play important roles in the process of cardiac aging [22]. In the present study, we utilized the newly released miRBase database version 21 to examine the miRNA clusters in the mouse genome and observed that 30% of the mouse miRNAs in the current database were clustered miRNAs. We also studied the miRNA clusters that were previously reported to be significantly altered in the old versus young adult mouse heart, especially three related miRNA clusters: the miR-17-92 cluster and its two paralogs, the miR-106a-363 cluster and miR-106b-25 cluster. The expressions of miRNA strands from both the 5′ arm and 3′ arm were considered, and the potential DNA methylation regions were identified. Since the metabolic response to stress may change during the aging process, the effect of nutrient change on miRNA expression was evaluated.

2. Materials and Methods

2.1. Bioinformatics Analysis. The miRBase (http://www.mirbase.org/) database was used to obtain the 1193 mouse miRNAs from the miRBase database version 21 (June 2014) [23]. The miRBase search tools were used to locate the miRNAs on each chromosome and to search the miRNA clusters with various inter-miRNA distances (1 Kb, 2 Kb, 3 Kb, 5 Kb, and 10 Kb). TargetScan (http://www.targetscan.org/) and http://www.microrna.org/ databases were used to search target mRNAs [24, 25]. The miRNA gene promoter sequence was analyzed using EMBOSS CpGPlot software [26].

2.2. Animal Tissues. Healthy C57BL/6 mice were obtained from colonies maintained by the National Institute of Aging (NIA) of the National Institutes of Health, under contractual agreement with Harlan Sprague-Dawley, Inc. (Harlan, IN). After euthanasia, the hearts were removed from mice and subjected to standard RNA isolation and histological procedures. Some heart tissue samples (4 months and 24 months) were obtained from the Aged Rodent Tissue Bank at NIA. For each time point, there were three independent biological replicates. The studies were conducted with the approval of the Institutional Animal Care and Use Committee (IACUC) at Central Arkansas Veterans Healthcare System (IACUC# 4-02-03) and in accordance with the NIH Guiding Principles for Research Involving Animals and Human Beings.

2.3. Total RNA Isolation. All RNA samples were first isolated from the mouse cardiac ventricles using UltraSpec RNA Isolation Reagent as previously described. To minimize mouse DNA contamination and enrich the small RNA fraction, the total RNA samples were purified using miRNeasy Mini Kit (Qiagen) and RNase-free DNase I according to the manufacture's instruction manual [10, 27].

2.4. miRNA Arrays. The ventricular tissue samples used for the miRNA array analysis were obtained from healthy young adult (4-month-old) and healthy old (24-month-old) C57BL6 mice. The RNA sample isolation and miRNA array were performed in triplicate for young adult and old animals. A total of six RNA samples of ventricular tissue representing three young adult and three old mice were shipped on dry ice to Exiqon, Inc., which provided the service for RNA quality verification, miRNA array hybridization, and comprehensive statistical analysis. The microarray data have been deposited in the NCBI Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE32935. Briefly, each pair of young adult and old mouse samples were labeled with Hy3 and Hy5 fluorescent dyes, respectively, and hybridized to a miRCURY LNA Mouse miRNA Array (version 11.0), which held 648 mature miRNA probes, as well as perfectly matched and mismatched probes for quality control. After signal amplification, the background was subtracted and normalized using LOWESS (locally weighted scatter plot smoothing) regression algorithm. This within-slide normalization was performed to minimize potential differences between the colors in an intensity-dependent manner.

The array output was received in Excel spreadsheets containing the normalized miRNA expression profiles in each heart sample; the expression comparison between old versus young adult heart samples and "expression matrix" containing normalized Hy3/Hy5 ratios (log2 transformed) from all hybridizations was also included. The list was sorted based on the most variant expressed miRNAs comparing the two sample types. 65 miRNAs passed the filtering criteria with an average "log median ratio" > 0.58, which represents at least > 1.5-fold change in miRNA expression, and the differential miRNA expression in all three pairs (young adult versus old) being in the same direction.

2.5. Nutrient Stress Assay. The DMEM media containing 10% newborn bovine serum (Invitrogen) with three different glucose concentrations were used in the present study, the normal glucose (100 mg/dL) medium, the low glucose (30 mg/dL) medium, and the high glucose (400 mg/dL) medium. The muscle cell line C2C12 cells (ATCC CRL-1772) were cultured in six-well plates to 80% confluence in normal glucose medium, after which the cells either remained in normal glucose medium (control) or were subjected to high (400 mg/dL) or low (30 mg/dL) glucose for six hours,

respectively. The cells were then harvested and the total RNA was isolated using miRNeasy Mini Kit (Qiagen) and RNase-free DNase I according to the manufacture's instruction manual. Individual experiments were carried out in triplicate, and the results were reported as averages (mean ± SD) from representative experiments [28].

2.6. Real-Time RT-PCR Quantitation of Pri-miRNAs and Mature miRNAs. To quantitate the expression of the miRNA primary transcripts and the miRNA mature forms, real-time RT-PCR was performed. To select a proper internal loading control for RT-PCR, we examined the expression of 5S ribosomal RNA (5S RNA) and U6 snRNA in young adult versus old hearts. We found that the expression of 5S RNA remained unchanged in young adult versus old hearts, while U6 snRNA expression changed significantly in young adult versus old hearts. Therefore, 5S RNA was used as an internal loading control.

2.6.1. Detection of Pri-miRNAs. The primers for the detection of pri-miRNAs were designed using PRISM Primer Express 3.0 software (Applied Biosystems) and synthesized at Integrated DNA Technologies Inc. The first-strand cDNA synthesis was carried out using random hexamer primer, and the PCR was performed using the following primers: pri-mir-17 forward 5′-GCTTTGGCTTTTTCCTTTTTG-3′, pri-mir-17 reverse 5′-CCTCACTGCAGTAGATGCACA-3′; pri-mir-20a forward 5′-CGTGGTGTGTGTGATGTG-AC-3′, pri-mir-20a reverse 5′-GCTCGTAATGCAGTA-GATGGC-3′; pri-mir-25 forward 5′-CAGTGTTGAGAG-GCGGAGAC-3′, pri-mir-25 reverse 5′-TCAGACCGAGAC-AAGTGCAA-3′; pri-mir-27a forward 5′-TTTGATGCC-AGTCACAAATCA-3′, pri-mir-27a reverse 5′-AGCCAC-TGTGAACACGACTTT-3′; pri-mir-92a-1 forward 5′-GGG-ATTTGTCGCAATGCTGT-3′, pri-mir-92a-1 reverse 5′-GGTCACAATCCCCACCAAAC-3′; pri-mir-93 forward 5′-CACCTCACCTAATGACCCTCA-3′, pri-mir-93 reverse 5′-CAAGTCCTAGCCCTCATGGAT-3′; pri-mir-106a forward 5′-TAAATGCCCCTTCTCGCACA-3′, pri-mir-106a reverse 5′-GGCGAAACACTGAAAGAGCC-3′; pri-mir-106b forward 5′-CTTCCCTCCTACCAGCCCT-3′, pri-mir-106b reverse 5′-GAGCAGCAAGTACCCACAGT-3′; pri-mir-363 forward 5′-TCTGCATCGTAATGGACACCT-3′, pri-mir-363 reverse 5′-TAATGCCACCAATCCCCACC-3′.

2.6.2. Detection of Mature miRNAs. To detect the mature miRNA strand of either miR or miR*, the first-strand cDNA synthesis was carried out using a universal reverse primer and the Universal RT miRNA PCR System (Exiqon). The RT-PCR reagents, the primers for mature miRNAs, and the 5S RNA reference primers that were used as endogenous controls were purchased from Exiqon.

The PCR amplification was performed in a 7900HT Fast Sequence Detector System (Applied Biosystems) with the following program: Cycle 1, 95°C for 10 minutes; Cycle 2, 40 cycles of 95°C for 15 seconds, 60°C for 60 seconds; Cycle 3, 95°C for 15 seconds, 60°C for 15 seconds, 95°C for 15 seconds. CT values were automatically obtained. Relative expression values were obtained by normalizing CT values of the miRNA genes in comparison with CT values of the endogenous control (5S RNA) using the CT method [29, 30].

2.7. Statistical Analysis. Data are given as mean values ± SD, with n denoting the number of experiments unless otherwise indicated. The differentially expressed miRNAs with at least a 1.5-fold change were identified using a t-test with a cut-off P value of $P < 0.05$.

3. Results

3.1. An Overview of miRNA and miRNA Clusters in Mouse Genome. The number of miRNAs that have been deposited in the miRNA database has progressively increased over the past several years. The recently released (June 2014) miRBase database version 21 hosts 1193 mouse miRNA sequences. To examine the potential genomic distribution of the miRNAs in the mouse genome, we utilized the web tool in miRBase (http://www.mirbase.org/) database to search the miRNAs by genomic location and the miRNA clusters based on base pair distance between neighboring miRNAs. As shown in Table 1, 1193 miRNAs were distributed across the mouse chromosomes, with chromosome 2 hosting the highest number of miRNAs. When using 10 Kb as a default distance for clustering neighboring miRNAs, 94 miRNA clusters (containing 372 miRNAs) were found, which account for 31% of the total miRNAs (Table 1). To get an in-depth view of the relationship between miRNA cluster distribution and the distance of neighboring miRNAs, we also used distances of 5 Kb, 3 Kb, 2 Kb, and 1 Kb to explore the miRNA clusters. As shown in Table 1 and Figure 1, more than 300 miRNAs (approximately 1/4 of mouse miRNAs) were distributed in clusters within a 2 Kb distance. Compared to the previous miRBase version 20, the current version 21 had a 39% increase of miRNAs in the mouse genome (Table 2).

3.2. The Genomic Loci of miR-17-92 and Its Paralogs and Their Gene Families. In our previous study, we observed that 11 miRNA clusters were significantly impacted in the cardiac aging process; among them were three clusters that belong to miR-17-92 cluster and its paralogs. They are miR-17-92, miR-106a-363, and miR-106b-25 clusters [22]. These data indicate that miR-17-92 and its paralogs may play an important role in the regulation of physiological and structural changes in the adult heart during aging. Due to the similarity of gene sequences of these miRNA clusters, it is speculated that the paralogs, clusters miR-106b-25 and miR-106a-363, are likely to have been derived from the gene duplication of the miR-17-92 cluster [31].

In the mouse genome, the miR-17-92 cluster is located on chromosome 14, the miR-106a-363 cluster is on chromosome X, and miR-106b-25 cluster is on chromosome 5 (Figure 2). The gene loci of all 3 clusters span less than 1 Kb, indicating that the miRNAs in each cluster are likely to be transcribed within one transcript.

The entire mouse miR-17-92 cluster is transcribed within a RNA transcript of 2339 bp in length, termed "Mir17 host

Table 1: The number of mouse miRNAs and miRNA clusters distributed on each chromosome. A total of 1193 miRNAs were observed in the mouse miRBase database release version 21. Six miRNAs were not assigned to any of the chromosomes. Various inter-miRNA distances (1 Kb, 2 Kb, 3 Kb, 5 Kb, and 10 Kb) were used in an effort to define the potential miRNA clusters. Approximately 25% of the miRNA clusters are located within a 2 Kb distance.

Chromosome	Total miRs	Cluster number				
		<10 Kb	<5 Kb	<3 Kb	<2 Kb	<1 Kb
1	61	12	8	6	6	6
2	147	90	88	88	86	83
3	46	9	9	9	9	9
4	56	8	8	8	7	7
5	54	5	5	5	3	3
6	43	11	11	10	10	10
7	85	19	16	15	11	11
8	57	12	8	8	8	8
9	50	8	6	6	6	4
10	52	6	4	4	2	2
11	86	18	16	16	16	14
12	94	60	56	55	55	47
13	33	10	10	10	10	8
14	42	10	10	10	10	10
15	48	6	6	4	4	4
16	34	8	8	6	6	6
17	40	15	9	7	5	5
18	32	7	7	5	4	2
19	36	2	2	2	2	2
X	91	56	56	47	42	34
Other*	6					
Cluster number		**94**	**87**	**84**	**81**	**93**
Total miRs	1193	372	343	321	302	275
Cluster %		**31%**	**29%**	**27%**	**25%**	**23%**

*Six miRNAs have not been localized into any of the above chromosome.

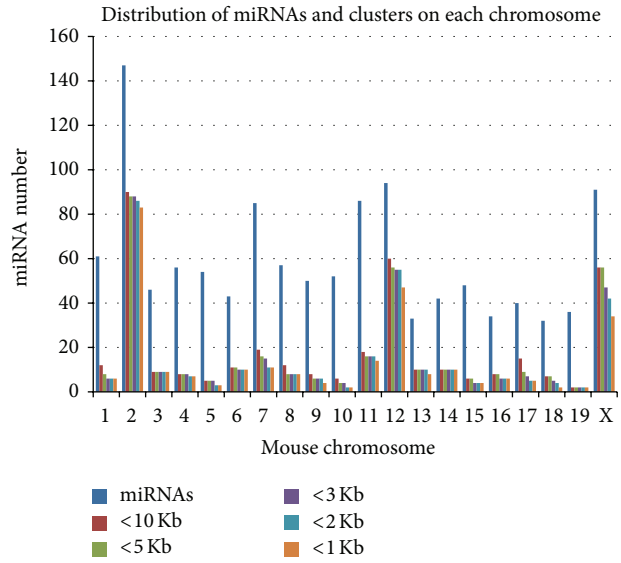


Figure 1: The distribution of mouse microRNAs (miRNAs) and miRNA clusters in each chromosome (chr), based on miRBase version 21. The distribution of miRNA clusters was compared at various inter-miRNA distances (1 Kb, 2 Kb, 3 Kb, 5 Kb, and 10 Kb). Three chromosomes, chr 2, chr 12, and chr X, host most (2/3) of the miRNA clusters, indicating that miRNA clusters are not randomly distributed but rather are enriched at certain genomic locations.

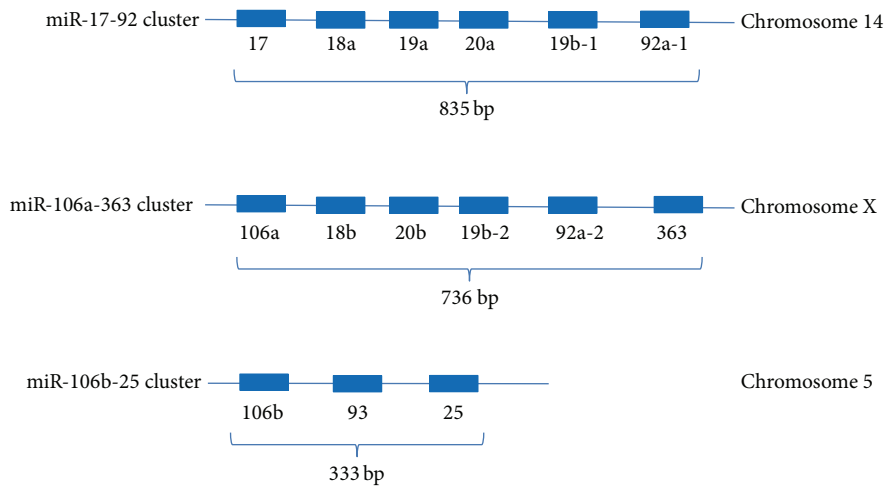


Figure 2: Genomic loci of miR-17-92, miR-106a, and miR-106b-25 clusters. The miR-17-92 cluster spans approximately 825 bp on chromosome 14. The miR-106a-363 cluster spans approximately 736 bp on chromosome X. The miR-106b-25 cluster spans 333 bp on chromosome 5.

Table 2: The increased identification of miRNAs and miRNA clusters in the mouse genome, according to miRBase database version 20 versus version 21.

miRBase	Release 20	Release 21	Increase
Total miRs	855	1193	39%
Total clusters	71	94	32%
Seq. in clusters	318	372	17%

gene 1" (GenBank accession number NR_029382), which is the only documented transcript variant/isoform. In contrast, the human miR-17-92 cluster has two reported host gene variants/isoforms: (1) the "miR-17 host gene 1," which is 5018 bp in length (NR_027350) and contains all 6 miRNAs of the miR-17-92 cluster, and (2) the "miR-17-92 cluster host gene variant 2," which is 927 bp in length (NR_027349) and apparently does not have perfect sequence alignment with any of the miRNAs in the cluster.

The three miR-17-92-related clusters hosted 15 miRNA genes (Figure 2) and produced 15 mature miRNAs (guide strand) and 15 passenger strands (Table 3). These miRNAs were categorized into four miRNA families in the miRBase database, based on their sequence similarity of the "seed" sequence. As shown in Table 4, eight miRNAs were observed to belong to the miR-17 family; three miRNAs were identified to belong to the miR-19 family and three miRNAs to the miR-25 family; the miR-363-5p was found to belong to the miR-363 family, but its sequence was similar in part to that of miR-25 family members (Table 3).

3.3. The Expression of miR-17-92 and Its Paralogs in the Old Heart. The miR-17-92 cluster and its paralogs are usually first transcribed as primary transcripts (pri-miRNA) which contain the miRNA genes. The pri-miRNAs are cleaved by the microprocessor complex into pre-miRNA, which is a short stem loop ~70 nucleotides in length. The pre-miRNA is then cleaved by the RNase III enzyme Dicer and becomes mature miRNAs as a guide strand (miR) and a passenger strand (miR*) [32]. During typical miRNA biogenesis, the miR strand is preferentially selected for entry into a RISC complex, whereas the miR* strand has been thought to be degraded [33–35]. However, growing evidence now indicates that the miRNA passenger strand may also have important biological functions [36]. Therefore, the expression of both the guide and passenger strands was examined in the hearts of old versus young adult mice. Exiqon miRNA chip covered all the 15 guide strands from the three miRNA gene clusters (Figure 3(a)) and covered more than half of the passenger strands (Figure 3(b)). Most of the 15 guide strands were upregulated in the aging heart, where 11 guide strands were expressed in the same direction, but four guide strands (miR-18a-5p, miR-18b-5p, miR-20b-5p, and miR-363-5p) were increased in some old mice while decreased in other old mice (Figure 3(a)). The expression of the eight passenger strands was more complex, where only one, miR-106a-3p, was increased in all three old versus young adult mice, while seven passenger strands were increased in some old mice and decreased in other old mice (Figure 3(b)).

3.4. miRNA Expression in Response to Stress. The metabolic response to stress tends to change during adult aging. To examine whether nutrient stress could have an effect on miRNA expression, miRNA expression was measured in cultured cells in response to high and low glucose versus normal glucose (control) treatment. The expression of two or three microRNAs was measured in each of the miR-17-92-related clusters. As shown in Figure 4, after six hours of high glucose (400 mg/dL) and low glucose (30 mg/dL) treatment, the miR-17, miR-20a, and miR-92a-1 from miR-17-92 cluster were decreased in response to high glucose but increased in response to low glucose. The miR-106a and miR-363 from miR-106a-363 cluster were increased in response to both high and low glucose stresses. The miR-106b, miR-93, and miR-25 from miR-106b-25 cluster were decreased in response to high glucose while increased in response to low glucose treatment.

Table 3: The "seed" sequence similarity and miRNA families of miR-17-92 and paralogs. The "seed" sequences of the 15 miRNAs (underlined) are derived from miR-17-92, miR-106a-363, and miR-106b-25 clusters. The mature miRNAs are grouped into four miRNA families, based on their "seed" sequence similarity (miRBase version 21).

ID	5′ arm strand	ID	3′ arm strand
miR-17 family			
mmu-miR-17-5p	CAAAGUGCUUACAGUGCAGGUAG	mmu-miR-17-3p	ACUGCACUGCAGUGAGGGCACUUGUAG
mmu-miR-20a-5p	UAAAGUGCUUAUAGUGCAGGUAG	mmu-miR-20a-3p	ACUGCAUUACGAGCACUUAAAG
mmu-miR-93-5p	CAAGUGCUGUUCGUGCAGGUAG	mmu-miR-93-3p	ACUGCUGAGCUAGCACUUCCCG
mmu-miR-106b-5p	UAAGUGCUGACAGUGCAGAU	mmu-miR-106b-3p	CCGCACGUGGGUACUUGCUGC
mmu-miR-20b-5p	CAAGUGCUCAUAGUGCAGGUAG	mmu-miR-20b-3p	ACUGCAGUGUGAGCACUUCUAG
mmu-miR-106a-5p	CAAGUGCUAACAGUGCAGGUAG	mmu-miR-106a-3p	ACUGCAGUGCCAGCACUUCUUAC
mmu-miR-18a-5p	UAAGGUGCAUCUAGUGCAGAUAG	mmu-miR-18a-3p	ACUGCCCUAAGUGCUCCUUCUG
mmu-miR-18b-5p	UAGGUGCAUCUAGUGCUGUUAG	mmu-miR-18b-3p	UACUGCCCUAAAUGCCCCUUCU
miR-19 family			
mmu-miR-19a-5p	UAGUUUUGCAUAGUUGCACUAC	mmu-miR-19a-3p	UGUGCAAAUCUAUGCAAAACUGA
mmu-miR-19b-1-5p	AUUUUGCAGGUUUGCAUCCAGC	mmu-miR-19b-3p	UGUGCAAAUCCAUGCAAAACUGA
mmu-miR-19b-2-5p	AUUUUGCAGAUUUGCAGUUCAGC	mmu-miR-19b-3p	UGUGCAAAUCCAUGCAAAACUGA
miR-25 family			
mmu-miR-25-5p	AGGCGGAGACUUGGGCAAUUGC	mmu-miR-25-3p	CAUUGCACUUGUCUCGGUCUGA
mmu-miR-92a-1-5p	AGUUGGUGGGAUUUGUCGCAAUGCU	mmu-miR-92a-3p	UAUUGCACUUGUCCCGGCCUG
mmu-miR-92a-2-5p	AGGUGGUGGGGAUUGGUGGCAUUAC	mmu-miR-92a-3p	UAUUGCACUUGUCCCGGCCUG
mir-363 family			
mmu-miR-363-5p	CAGGUGGAACACGAUGCAAUUU	mmu-miR-363-3p	AAUUGCACGGUAUCCAUCUGUA

Table 4: The miRNA families and locations of the miR-17-92 cluster and paralogs. The miRNA families of the miR-17-92 cluster and its paralogs. The miRNAs in the miR-17-92 cluster and its paralogs are grouped into four families based on the data in the miRBase database. The mouse miR-17 family has eight members, which are from three clusters. The mouse miR-19 family has three members, which are from two clusters. Three members of the miR-17-92 cluster and its paralogs belong to the miR-25 family. The miR-363 family currently has only one mouse miRNA member.

miR ID	Cluster	Chromosome	Accession
mir-17 family			
mmu-mir-17	miR-17-92 cluster	chr14	MI0000687
mmu-mir-18a	miR-17-92 cluster	chr14	MI0000567
mmu-mir-18b	miR-106a-363 cluster	chrX	MI0005483
mmu-mir-20a	miR-17-92 cluster	chr14	MI0000568
mmu-mir-20b	miR-106a-363 cluster	chrX	MI0003536
mmu-mir-93	miR-106b-25 cluster	chr5	MI0000581
mmu-mir-106a	miR-106a-363 cluster	chrX	MI0000406
mmu-mir-106b	miR-106b-25 cluster	chr5	MI0000407
miR-19 family			
mmu-mir-19a	miR-17-92 cluster	chr14	MI0000688
mmu-mir-19b-1	miR-17-92 cluster	chr14	MI0000718
mmu-mir-19b-2	miR-106a-363 cluster	chrX	MI0000546
miR-25 family			
mmu-mir-25	miR-106b-25 cluster	chr5	MI0000689
mmu-mir-92a-1	miR-17-92 cluster	chr14	MI0000719
mmu-mir-92a-2	miR-106a-363 cluster	chrX	MI0000580
mir-363 family			
mmu-mir-363	miR-106a-363 cluster	chrX	MI0000765

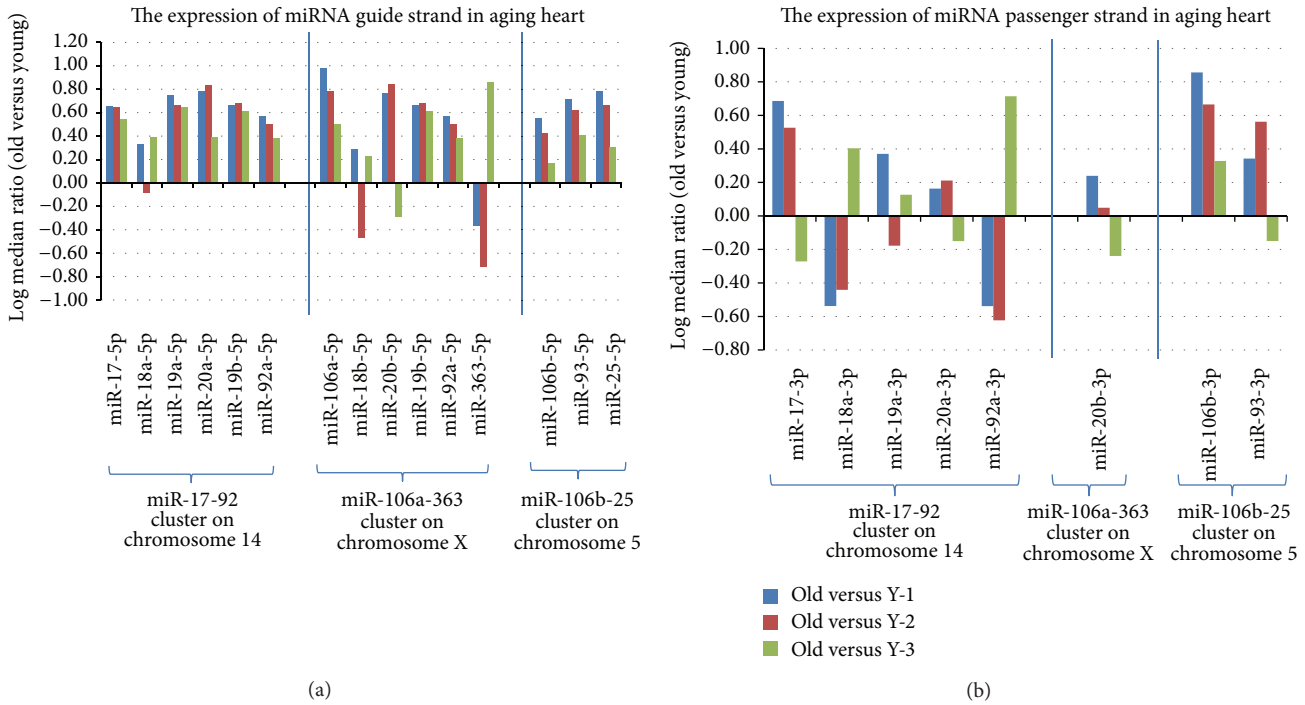


Figure 3: Expression of miR-17-92, miR-106a-363, and miR-106b-25 clusters in old versus young adult mouse hearts. (a) The expression of miRNA guide strands with aging in the heart. Most of the 15 guide strands were upregulated with aging in the heart, where 11 guide strands were expressed in the same direction, but four guide strands (miR-18a-5p, miR-18b-5p, miR-20b-5p, and miR-363-5p) were increased in some old mice while decreased in other old mice. (b) The expression of miRNA passenger strands with aging in the heart. The expression of the eight passenger strands was complex, where only one, miR-106a-3p, was increased in all three old versus young adult mice, while seven passenger strands were increased in some old mice and decreased in other old mice.

The miR-27, which does not belong to any of the miR-17-92-related clusters, was increased in response to both high and low glucose stresses.

3.5. Analysis of the miRNA Gene Promoter and miRNA Target Genes. It has been reported that miRNA gene promoters are frequent targets of DNA methylation under various physiological and pathological conditions [37–39]. Since altered DNA methylation has been reported in cardiac disease and during the adult aging process, the GC-rich region in the promoter of miR-17-92 cluster was further examined. As shown in Figure 5, the miR-17-92 promoter has a high GC-rich region, suggesting that the miR-17-92 cluster could quite possibly also be regulated by DNA methylation.

The Cdc42-SRF signaling pathway is important for the development and maintenance of the cardiovascular system. Potential signaling proteins that are likely targeted by the miR-17-92 cluster and its paralogs were analyzed using TargetScan and http://www.microrna.org/ web tools. The schematic diagram shown in Figure 6 is a simplified presentation of the Cdc42-SRF pathway that has been reported to be targeted by miRNAs based on the present study and other published reports in the literature.

4. Discussion

In the present study, the miRNA clusters in the mouse genome were examined based on their chromosomal location and various inter-miRNA distances. Three important miRNA clusters that were identified to be significantly impacted during the cardiac aging process, the miR-17-92 cluster and its paralogs, miR-106a-363 and miR-106b-25, were also examined in terms of their genomic location, RNA transcript character, sequence homology, and their relationship with the corresponding miRNA families. The expression of both the guide and passenger strands of the miRNAs was evaluated in the old compared to that of young adult mouse hearts. Since response to various stresses is implicated in the process of aging and in the development of disease in the heart, the effect of glucose stresses on the expression of miRNA clusters was also evaluated. In addition, a high GC-rich region in the promoter region of miR-17-92 cluster was identified, which indicates that DNA methylation could also be a potential mechanism of regulation of miRNA cluster expression during aging in the heart. The data indicate that miR17-92 cluster and its paralogs, miR-106a-363 and miR-106b-25, potentially target the cd242-SRF signaling pathway, thereby regulating the cardiac response to pathophysiological conditions, including hypertension, cardiac hypertrophy, heart failure, and arrhythmias.

The number of miRNAs collected in miRNA databases has been growing steadily over the past several years, with both increased number of miRNA entries and increased coverage of species. For instance, miRBase released its miRNA database version 1.0 in December 2002, which had only 218 entries. However, the version 20 had 24,521 entries, while the

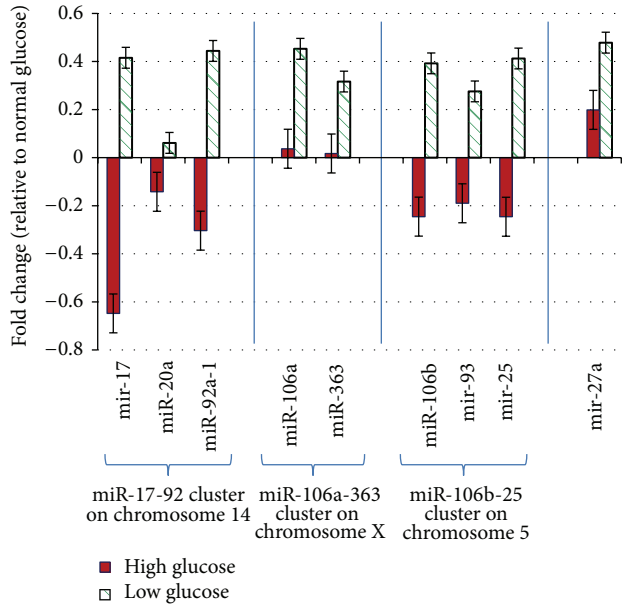


Figure 4: The miRNA expression in response to glucose stress. The C2C12 cells (ATCC CRL-1772) were cultured in normal glucose (100 mg/dL) medium to 80% confluence and then treated with either high glucose (400 mg/dL) or low glucose (30 mg/dL) for six hours, respectively. The miR-17-92 cluster is represented by three microRNAs; the miR-106a-363 cluster is represented by two microRNAs; the miR-106b-25 cluster is represented by three microRNAs. The mircroRNAs in each of the clusters either increased or decreased in the same direction in response to the stresses. Please note that miR-27a does not belong to any of the miR-17-92-related clusters but has been included here for comparison only.

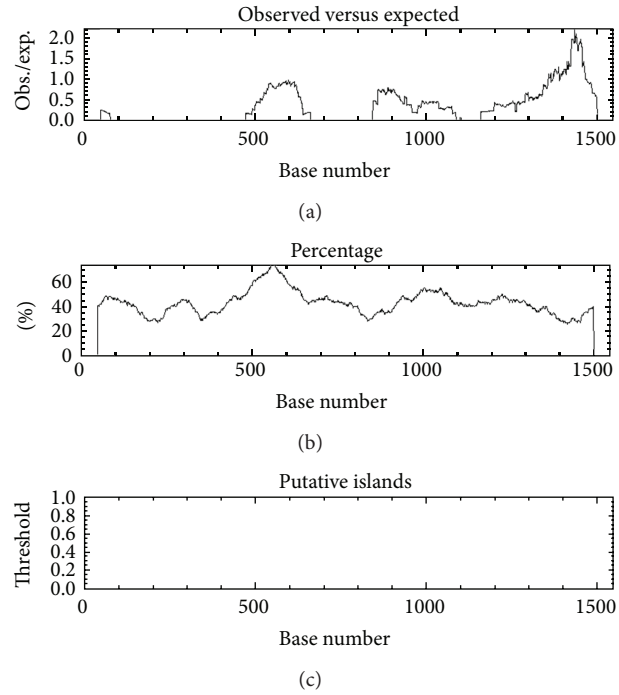


Figure 5: Analysis of the miR-17-92 cluster promoter region using "cpgplot" web tool (http://www.ebi.ac.uk/Tools/emboss/cpgplot/) to search the CpG island/GC-rich region, which revealed the GC-rich region in the promoter region.

most recently released (June 2014) version 21 has increased to up to 28,645 entries. Similar to many other species, the miRNAs listed in the mouse genome have also increased over time. There were 855 mouse miRNAs reported in version 20, but this number has significantly increased to 1193 miRNAs in version 21. Similarly, the number of miRNA clusters has also increased over time (Table 2).

The inter-miRNA distance used for grouping miRNA clusters has varied among different reports, with ranges from 1 Kb to 50 Kb [21, 22, 40]. However, most miRNA clusters are located within 1–3 Kb inter-miRNA distance [21, 41]. In the present study, we observed that more than 20–25% of the miRNAs were within 1-2 Kb distance from each other, and more than 30% of the miRNAs were within a 10 Kb distance from each other. Since the length of the RNA transcript typically varies from 1 Kb to over 10 Kb, many of these miRNA clusters may have one core promoter region and the same transcriptional start site and are perhaps expressed within a single RNA transcript [42]. We also observed that the miRNAs and miRNA clusters are not evenly distributed across the chromosomes. For instance, three chromosomes, chromosome 2, chromosome 12, and chromosome X, hosted over 2/3 of the miRNA clusters, suggesting that the distribution of miRNA clusters is not random but rather is enriched at certain genomic locations.

The miRNAs have been implicated in the processes of cardiac hypertrophy and heart failure [43–48]. Left ventricular hypertrophy and heart failure are known to increase significantly in prevalence with advancing age. The older heart is characterized by a progressive loss of myocytes with subsequent hypertrophy of the remaining cells and increased fibrosis with collagen deposition, as well as calcification involving the conduction and valvular apparatus [1, 49, 50]. The old heart is more vulnerable to environmental changes, including dynamic stress, hypoxia, ischemia-reperfusion, hyperglycemia, and/or hypoglycemia.

Multiple signaling pathways are likely involved in the lifelong process of cardiac aging, one of which is the Rho-SRF signaling pathway [51–53]. Serum response factor (SRF) has been shown to be a major transcription factor in the regulation of the genes involved in the maintenance of cardiac structure and function [54–57]. Cardiac-specific overexpression of SRF at a mild level caused an accelerated cardiac aging phenotype in mice [9]. A number of other mouse models also highlight the importance of SRF and its related signaling pathway in the regulation of cardiac structure and function during embryogenesis, maturation, and aging [8, 9, 56–59].

SRF is a downstream effector of the Rho family GTPases [60–62]. The Rho GTPases (Cdc42, Rac1, and RhoA) signaling pathways regulate cytoskeletal genes that are important in the development and maintenance of cardiovascular structure and function as well as the processes of pathological conditions [63, 64]. The miRNAs in the three miR-17-92-related clusters apparently target multiple components of

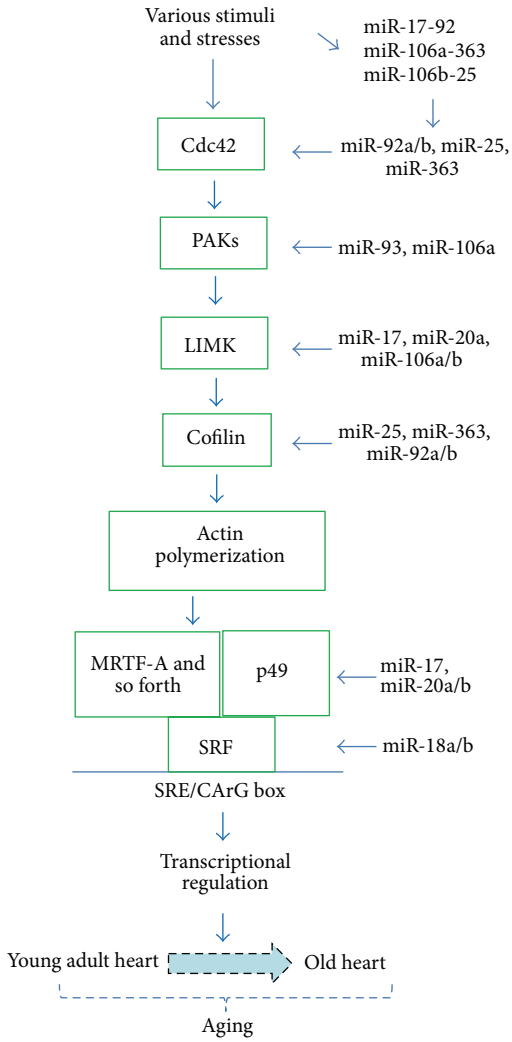


Figure 6: Cdc42-SRF signaling pathway regulates cytoskeletal genes that are important in the development and maintenance of cardiovascular structure and function. The schematic here is a simplified presentation of the cdc42 pathway with some of the main proteins, which are potential targets of the miRNAs from the miR-17-92 cluster and its paralogs, the miR-106a-363 and miR-106b-25 clusters. The targeting information has been obtained from the literature and from the bioinformatics search with TargetScan and http://www.microrna.org/ databases.

the Rho GTPase-related signaling pathways, including the Cdc42-SRF signaling pathway (Figure 6) [65].

Cdc42 is an upstream regulator in the pathway, which has been implicated in regulating myofibrillar architecture, cell polarity, and morphology, as well as sarcomere assembly in the cardiac myocyte [64, 66–68]. Cdc42 is a target of miR-92a and miR-92b and miR-25 and miR-363. The p21 protein (Cdc42/Rac) activated kinases (PAKs) are targets of Cdc42/Rac and play a critical role in proper morphogenesis, including normal electrical conductance of the heart, cardiac contractility, and development and maintaining of the integrity of the vasculature [65]. PAK2 is a target of miR-93 and miR-106a; PAK6 is a target of miR-19a and miR-19b [69]. LIM-kinases (LIMKs) are serine/threonine-protein kinases that play an essential role in the regulation of actin filament dynamics by phosphorylating cofilins. Cofilins are actin binding/depolymerizing factors which can polymerize and depolymerize F-actin and G-actin [70, 71]. LIM-kinase 1 (LIMK1) is a target of miR-20a and other miRNAs including miR-106a, miR-106b, miR-17, miR-20a, miR-20b, and miR-93 [72]. Cofilin 2 is a target of miR-25, miR-363, miR-92a, and miR-92b. The p49/STRAP (SRFBP1) gene is a target of miR-106a, miR-106b, miR-17, miR-20a, miR-20b, and miR-93. The SRF gene is a target of miR-18a and miR-18b. Therefore, altered expression of the miR-17-92, miR-106a-363, and miR-106b-25 clusters is likely to sufficiently and significantly impact the Cdc42-SRF signaling pathway and thereby affect cardiac structure and function during pathological conditions and the aging process.

The heart comprises cardiomyocytes, fibroblasts, endothelial cells, and vascular smooth muscle cells. The cardiomyocytes account for 30–35% of cell population, while noncardiomyocytes account for 65–70% of the cell population of the heart in human and rat [73–75]. Due to the species difference, however, the young adult murine myocardium is composed of 56% myocytes, 27% fibroblasts, 7% endothelial cells, and 10% vascular smooth muscle cells [76]. The cardiac fibroblasts tend to increase to cause cardiac fibrosis and induce collagen deposition in the aging heart as well as in the hypertrophied heart. Cardiac fibroblasts can also secrete miRNA-enriched exosomes which contain a relatively high abundance of many miRNA passenger strands, including miR-21*. The miR-21* has been shown to induce cardiomyocyte hypertrophy [36]. Therefore, the microRNAs may serve as paracrine signaling mediators of cellular responses, including cardiomyocyte hypertrophy. The analysis of microRNAs from each of the cell populations of the heart in response to cardiac stress would be of interest, as would the consideration of promoter regions of the microRNA cluster in terms of GC-rich regions. These questions warrant future investigation.

5. Conclusions

It is becoming increasingly clear that the miRNAs and miRNA clusters are important in regulating an animal's response to environmental stimuli, including nutrient stress, the maintenance of normal function, and the development of pathological conditions, as well as the process of aging. Inasmuch as Cdc42-SRF signaling pathway, including serum response factor (SRF) and its cofactors, has been observed to mediate the cellular responses to a variety of external and internal stimuli, those miRNAs that modulate and/or are modulated by components of the Rho GTPase-related signaling pathways are likely to be attractive targets for future therapeutic considerations.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

This study was supported in part by NIH Grants R01 AG-018388, R01 AG-026091, and P30 AG-028718 from the

Department of Health and Human Services, The Geriatric Research, Education, and Clinical Center (GRECC), and the Central Arkansas Veterans Healthcare System. No additional external funding was received for this study. The authors thank S. Foster for his helpful input.

References

[1] J. Y. Wei, "Age and the cardiovascular system," *The New England Journal of Medicine*, vol. 327, no. 24, pp. 1735–1739, 1992.

[2] S. Argentin, A. Ardati, S. Tremblay et al., "Developmental stage-specific regulation of atrial natriuretic factor gene transcription in cardiac cells," *Molecular and Cellular Biology*, vol. 14, no. 1, pp. 777–790, 1994.

[3] R. Chien, "Signaling mechanisms for the activation of an embryonic gene program during the hypertrophy of cardiac ventricular muscle," *Basic Research in Cardiology*, vol. 87, supplement 2, pp. 49–58, 1992.

[4] W. S. Colucci, "Molecular and cellular mechanisms of myocardial failure," *The American Journal of Cardiology*, vol. 80, no. 11, pp. 15L–25L, 1997.

[5] J.-B. Durand, "Genetic basis of cardiomyopathy," *Current Opinion in Cardiology*, vol. 14, no. 3, pp. 225–229, 1999.

[6] T. J. Nelson, R. Balza Jr., Q. Xiao, and R. P. Misra, "SRF-dependent gene expression in isolated cardiomyocytes: regulation of genes involved in cardiac hypertrophy," *Journal of Molecular and Cellular Cardiology*, vol. 39, no. 3, pp. 479–489, 2005.

[7] D.-Z. Wang, P. S. Chang, Z. Wang et al., "Activation of cardiac gene expression by myocardin, a transcriptional cofactor for serum response factor," *Cell*, vol. 105, no. 7, pp. 851–862, 2001.

[8] X. Zhang, G. Azhar, J. Chai et al., "Cardiomyopathy in transgenic mice with cardiac-specific overexpression of serum response factor," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 280, no. 4, pp. H1782–H1792, 2001.

[9] X. Zhang, G. Azhar, M. C. Furr, Y. Zhong, and J. Y. Wei, "Model of functional cardiac aging: young adult mice with mild overexpression of serum response factor," *American Journal of Physiology—Regulatory Integrative and Comparative Physiology*, vol. 285, no. 3, pp. R552–R560, 2003.

[10] X. Zhang, G. Azhar, Y. Zhong, and J. Y. Wei, "Identification of a novel serum response factor cofactor in cardiac gene regulation," *The Journal of Biological Chemistry*, vol. 279, no. 53, pp. 55626–55632, 2004.

[11] H. B. Kwak, "Aging, exercise, and extracellular matrix in the heart," *Journal of Exercise Rehabilitation*, vol. 9, no. 3, pp. 338–347, 2013.

[12] J.-S. Silvestre, D. M. Smadja, and B. I. Lévy, "Postischemic revascularization: from cellular and molecular mechanisms to clinical applications," *Physiological Reviews*, vol. 93, no. 4, pp. 1743–1802, 2013.

[13] M. A. Horn, H. K. Graham, M. A. Richards et al., "Age-related divergent remodeling of the cardiac extracellular matrix in heart failure: Collagen accumulation in the young and loss in the aged," *Journal of Molecular and Cellular Cardiology*, vol. 53, no. 1, pp. 82–90, 2012.

[14] S. Daubman, "MicroRNAs in angiogenesis and vascular smooth muscle cell function," *Circulation Research*, vol. 106, no. 3, pp. 423–425, 2010.

[15] M. Abonnenc, A. A. Nabeebaccus, U. Mayr et al., "Extracellular matrix secretion by cardiac fibroblasts: role of MicroRNA-29b and MicroRNA-30c," *Circulation Research*, vol. 113, no. 10, pp. 1138–1147, 2013.

[16] J. Wang and J. F. Martin, "Macro advances in microRNAs and myocardial regeneration," *Current Opinion in Cardiology*, vol. 29, no. 3, pp. 207–213, 2014.

[17] N. Liu and E. N. Olson, "MicroRNA regulatory networks in cardiovascular development," *Developmental Cell*, vol. 18, no. 4, pp. 510–525, 2010.

[18] X.-B. Liao, Z.-Y. Zhang, K. Yuan et al., "MiR-133a modulates osteogenic differentiation of vascular smooth muscle cells," *Endocrinology*, vol. 154, no. 9, pp. 3344–3352, 2013.

[19] J. Winter, S. Jung, S. Keller, R. I. Gregory, and S. Diederichs, "Many roads to maturity: microRNA biogenesis pathways and their regulation," *Nature Cell Biology*, vol. 11, no. 3, pp. 228–234, 2009.

[20] B. Kaczkowski, E. Torarinsson, K. Reiche, J. H. Havgaard, P. F. Stadler, and J. Gorodkin, "Structural profiles of human miRNA families from pairwise clustering," *Bioinformatics*, vol. 25, no. 3, pp. 291–294, 2009.

[21] Y. Altuvia, P. Landgraf, G. Lithwick et al., "Clustering and conservation patterns of human microRNAs," *Nucleic Acids Research*, vol. 33, no. 8, pp. 2697–2706, 2005.

[22] X. Zhang, G. Azhar, and J. Y. Wei, "The expression of microRNA and microRNA clusters in the aging heart," *PLoS ONE*, vol. 7, no. 4, Article ID e34688, 2012.

[23] A. Kozomara and S. Griffiths-Jones, "MiRBase: annotating high confidence microRNAs using deep sequencing data," *Nucleic Acids Research*, vol. 42, no. 1, pp. D68–D73, 2014.

[24] R. C. Friedman, K. K.-H. Farh, C. B. Burge, and D. P. Bartel, "Most mammalian mRNAs are conserved targets of microRNAs," *Genome Research*, vol. 19, no. 1, pp. 92–105, 2009.

[25] D. Betel, A. Koppal, P. Agius, C. Sander, and C. Leslie, "Comprehensive modeling of microRNA targets predicts functional non-conserved and non-canonical sites," *Genome Biology*, vol. 11, article R90, 2010.

[26] P. Rice, L. Longden, and A. Bleasby, "EMBOSS: the European molecular biology open software suite," *Trends in Genetics*, vol. 16, no. 6, pp. 276–277, 2000.

[27] X. Zhang, G. Azhar, S. A. Helms, and J. Y. Wei, "Regulation of cardiac microRNAs by serum response factor," *Journal of Biomedical Science*, vol. 18, no. 1, article 15, 2011.

[28] S. C. Rogers, X. Zhang, G. Azhar, S. Luo, and J. Y. Wei, "Exposure to high or low glucose levels accelerates the appearance of markers of endothelial cell senescence and induces dysregulation of nitric oxide synthase," *The Journals of Gerontology. Series A, Biological Sciences and Medical Sciences*, vol. 68, no. 12, pp. 1469–1481, 2013.

[29] T. D. Schmittgen and K. J. Livak, "Analyzing real-time PCR data by the comparative C_T method," *Nature Protocols*, vol. 3, no. 6, pp. 1101–1108, 2008.

[30] K. J. Livak and T. D. Schmittgen, "Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}$T method," *Methods*, vol. 25, no. 4, pp. 402–408, 2001.

[31] J. T. Mendell, "miRiad roles for the miR-17–92 cluster in development and disease," *Cell*, vol. 133, no. 2, pp. 217–222, 2008.

[32] B. R. Cullen, "Transcription and processing of human microRNA precursors," *Molecular Cell*, vol. 16, no. 6, pp. 861–865, 2004.

[33] N. Shomron and C. Levy, "MicroRNA-biogenesis and pre-mRNA splicing crosstalk," *Journal of Biomedicine and Biotechnology*, vol. 2009, Article ID 594678, 6 pages, 2009.

[34] J. Winter and S. Diederichs, "Argonaute proteins regulate microRNA stability: increased microRNA abundance by Argonaute proteins is due to microRNA stabilization," *RNA Biology*, vol. 8, no. 6, pp. 1149–1157, 2011.

[35] B. Wang, S. Li, H. H. Qi, D. Chowdhury, Y. Shi, and C. D. Novina, "Distinct passenger strand and mRNA cleavage activities of human Argonaute proteins," *Nature Structural and Molecular Biology*, vol. 16, no. 12, pp. 1259–1266, 2009.

[36] C. Bang, S. Batkai, S. Dangwal et al., "Cardiac fibroblast-derived microRNA passenger strand-enriched exosomes mediate cardiomyocyte hypertrophy," *The Journal of Clinical Investigation*, vol. 124, no. 5, pp. 2136–2146, 2014.

[37] J. Dickstein, V. Senyuk, K. Premanand et al., "Methylation and silencing of miRNA-124 by *EVI1* and self-renewal exhaustion of hematopoietic stem cells in murine myelodysplastic syndrome," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 21, pp. 9783–9788, 2010.

[38] M. Skårn, T. Barøy, E. W. Stratford, and O. Myklebost, "Epigenetic regulation and functional characterization of microRNA-142 in mesenchymal cells," *PLoS ONE*, vol. 8, no. 11, Article ID e79231, 2013.

[39] D. Dakhlallah, K. Batte, Y. Wang et al., "Epigenetic regulation of *mir-17~92* contributes to the pathogenesis of pulmonary fibrosis," *American Journal of Respiratory and Critical Care Medicine*, vol. 187, no. 4, pp. 397–405, 2013.

[40] W.-C. Chan, M.-R. Ho, S.-C. Li et al., "MetaMirClust: discovery of miRNA cluster patterns using a data-mining approach," *Genomics*, vol. 100, no. 3, pp. 141–148, 2012.

[41] A. Mathelier and A. Carbone, "Large scale chromosomal mapping of human microRNA structural clusters," *Nucleic Acids Research*, vol. 41, no. 8, pp. 4392–4408, 2013.

[42] K. Woods, J. M. Thomson, and S. M. Hammond, "Direct regulation of an oncogenic micro-RNA cluster by E2F transcription factors," *The Journal of Biological Chemistry*, vol. 282, no. 4, pp. 2130–2134, 2007.

[43] R. Kumarswamy and T. Thum, "Non-coding RNAs in cardiac remodeling and heart failure," *Circulation Research*, vol. 113, no. 6, pp. 676–689, 2013.

[44] G. C. van Almen, W. Verhesen, R. E. W. van Leeuwen et al., "MicroRNA-18 and microRNA-19 regulate CTGF and TSP-1 expression in age-related heart failure," *Aging Cell*, vol. 10, no. 5, pp. 769–779, 2011.

[45] S. Heymans, M. F. Corsten, W. Verhesen et al., "Macrophage MicroRNA-155 promotes cardiac hypertrophy and failure," *Circulation*, vol. 128, no. 13, pp. 1420–1432, 2013.

[46] E. M. Small, R. J. A. Frost, and E. N. Olson, "MicroRNAs add a new dimension to cardiovascular disease," *Circulation*, vol. 121, no. 8, pp. 1022–1032, 2010.

[47] T. Thum, C. Gross, J. Fiedler et al., "MicroRNA-21 contributes to myocardial disease by stimulating MAP kinase signalling in fibroblasts," *Nature*, vol. 456, no. 7224, pp. 980–984, 2008.

[48] L. S. Danielson, D. S. Park, N. Rotllan et al., "Cardiovascular dysregulation of miR-17-92 causes a lethal hypertrophic cardiomyopathy and arrhythmogenesis," *The FASEB Journal*, vol. 27, no. 4, pp. 1460–1467, 2013.

[49] D. E. Forman, A. Cittadini, G. Azhar, P. S. Douglas, and J. Y. Wei, "Cardiac morphology and function in senescent rats: gender-related differences," *Journal of the American College of Cardiology*, vol. 30, no. 7, pp. 1872–1877, 1997.

[50] K. G. Pugh and J. Y. Wei, "Clinical implications of physiological changes in the aging heart," *Drugs and Aging*, vol. 18, no. 4, pp. 263–276, 2001.

[51] T. Takahashi, H. Schunkert, S. Isoyama et al., "Age-related differences in the expression of proto-oncogene and contractile protein genes in response to pressure overload in the rat myocardium," *The Journal of Clinical Investigation*, vol. 89, no. 3, pp. 939–946, 1992.

[52] H. Tsou, G. Azhar, X. G. Lu, S. Kovacs, M. Peacocke, and J. Y. Wei, "Age-associated changes in basal c-fos transcription factor binding activity in rat hearts," *Experimental Cell Research*, vol. 229, no. 2, pp. 432–437, 1996.

[53] X.-G. Lu, G. Azhar, L. Liu, H. Tsou, and J. Y. Wei, "SRF binding to SRE in the rat heart: influence of age," *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, vol. 53, no. 1, pp. B3–B10, 1998.

[54] J. M. Miano, N. Ramanan, M. A. Georger et al., "Restricted inactivation of serum response factor to the cardiovascular system," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 49, pp. 17132–17137, 2004.

[55] K. Kuwahara, T. Barrientos, G. C. T. Pipes, S. Li, and E. N. Olson, "Muscle-specific signaling mechanism that links actin dynamics to serum response factor," *Molecular and Cellular Biology*, vol. 25, no. 8, pp. 3173–3181, 2005.

[56] A. Parlakian, D. Tuil, G. Hamard et al., "Targeted inactivation of serum response factor in the developing heart results in myocardial defects and embryonic lethality," *Molecular and Cellular Biology*, vol. 24, no. 12, pp. 5281–5289, 2004.

[57] G. Azhar, X. Zhang, S. Wang, Y. Zhong, C. M. Quick, and J. Y. Wei, "Maintaining serum response factor activity in the older heart equal to that of the young adult is associated with better cardiac response to isoproterenol stress," *Basic Research in Cardiology*, vol. 102, no. 3, pp. 233–244, 2007.

[58] S. Arsenian, B. Weinhold, M. Oelgeschläger, U. Rüther, and A. Nordheim, "Serum response factor is essential for mesoderm formation during mouse embryogenesis," *The EMBO Journal*, vol. 17, no. 21, pp. 6289–6299, 1998.

[59] X. Zhang, J. Chai, G. Azhar et al., "Early postnatal cardiac changes and premature death in transgenic mice overexpressing a mutant form of serum response factor," *The Journal of Biological Chemistry*, vol. 276, no. 43, pp. 40033–40040, 2001.

[60] C. S. Hill, J. Wynne, and R. Treisman, "The Rho family GTPases RhoA, Rac1, and CDC42Hs regulate transcriptional activation by SRF," *Cell*, vol. 81, no. 7, pp. 1159–1170, 1995.

[61] R. Treisman, A. S. Alberts, and E. Sahai, "Regulation of SRF activity by Rho family GTPases," *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 63, pp. 643–651, 1998.

[62] C. Esnault, A. Stewart, F. Gualdrini et al., "Rho-actin signaling to the MRTF coactivators dominates the immediate transcriptional response to serum in fibroblasts," *Genes and Development*, vol. 28, no. 9, pp. 943–958, 2014.

[63] D. Swope, J. Kramer, T. R. King, Y. S. Cheng, and S. G. Kramer, "Cdc42 is required in a genetically distinct subset of cardiac cells during *Drosophila* dorsal vessel closure," *Developmental Biology*, vol. 392, no. 2, pp. 221–232, 2014.

[64] A. Clerk and P. H. Sugden, "Small guanine nucleotide-binding proteins and myocardial hypertrophy," *Circulation Research*, vol. 86, no. 10, pp. 1019–1023, 2000.

[65] M. L. Kelly, A. Astsaturov, and J. Chernoff, "Role of p21-activated kinases in cardiovascular development and function," *Cellular and Molecular Life Sciences*, vol. 70, no. 22, pp. 4223–4228, 2013.

[66] T. Nagai, M. Tanaka-Ishikawa, R. Aikawa et al., "Cdc42 plays a critical role in assembly of sarcomere units in series of cardiac myocytes," *Biochemical and Biophysical Research Communications*, vol. 305, no. 4, pp. 806–810, 2003.

[67] J. Cooley, S. Whitaker, S. Sweeney, S. Fraser, and B. Davidson, "Cytoskeletal polarity mediates localized induction of the heart progenitor lineage," *Nature Cell Biology*, vol. 13, no. 8, pp. 952–957, 2011.

[68] L. Qian, J. D. Wythe, J. Liu et al., "Tinman/Nkx2-5 acts via miR-1 and upstream of Cdc42 to regulate heart function across species," *Journal of Cell Biology*, vol. 193, no. 7, pp. 1181–1196, 2011.

[69] A. Henrici, R. Montalbano, D. Neureiter et al., "The pan-deacetylase inhibitor panobinostat suppresses the expression of oncogenic miRNAs in hepatocellular carcinoma cell lines," *Molecular Carcinogenesis*, 2013.

[70] S. Arber, F. A. Barbayannis, H. Hanser et al., "Regulation of actin dynamics through phosphorylation of cofilin by LIM-kinase," *Nature*, vol. 393, no. 6687, pp. 805–809, 1998.

[71] O. Bernard, "Lim kinases, regulators of actin dynamics," *International Journal of Biochemistry and Cell Biology*, vol. 39, no. 6, pp. 1071–1076, 2007.

[72] J. Zhou, R. Liu, C. Luo et al., "MiR-20a inhibits cutaneous squamous cell carcinoma metastasis and proliferation by directly targeting LIMK1," *Cancer Biology & Therapy*, vol. 15, no. 10, pp. 1340–1349, 2014.

[73] D. Tirziu, F. J. Giordano, and M. Simons, "Cell communications in the heart," *Circulation*, vol. 122, no. 9, pp. 928–937, 2010.

[74] M. Xin, E. N. Olson, and R. Bassel-Duby, "Mending broken hearts: cardiac development as a basis for adult heart regeneration and repair," *Nature Reviews Molecular Cell Biology*, vol. 14, no. 8, pp. 529–541, 2013.

[75] A. C. Nag, "Study of non-muscle cells of the adult mammalian heart: a fine structural analysis and distribution," *Cytobios*, vol. 28, no. 109, pp. 41–61, 1980.

[76] I. Banerjee, J. W. Fuseler, R. L. Price, T. K. Borg, and T. A. Baudino, "Determination of cell types and numbers during cardiac development in the neonatal and adult rat and mouse," *American Journal of Physiology: Heart and Circulatory Physiology*, vol. 293, no. 3, pp. H1883–H1891, 2007.

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 497280, 11 pages
http://dx.doi.org/10.1155/2015/497280

Research Article

miR-126 Is Involved in Vascular Remodeling under Laminar Shear Stress

Ana Mondadori dos Santos,[1,2] Laurent Metzinger,[1,3] Oualid Haddad,[2] Eléonore M'baya-Moutoula,[1] Fatiha Taïbi,[1] Nathalie Charnaux,[2] Ziad A. Massy,[1,4] Hanna Hlawaty,[2] and Valérie Metzinger-Le Meuth[1,5]

[1] *INSERM U1088, Faculty of Pharmacy and Medicine, University of Picardie Jules Verne, Rue des Louvels, 80037 Amiens, France*
[2] *INSERM U1148, LVTS, UFR SMBH, University Paris 13 Sorbonne Paris Cité, 74 rue Marcel Cachin, 93000 Bobigny, France*
[3] *Centre de Biologie Humaine (CBH), Amiens University Hospital, 80054 Amiens, France*
[4] *Division of Nephrology, Ambroise Pare Hospital, Paris Ile de France Ouest (UVSQ) University, 09 avenue Charles de Gaulle, 92100 Boulogne Billancourt Cedex, France*
[5] *University Paris 13 Sorbonne Paris Cité, UFR SMBH, 74 rue Marcel Cachin, 93017 Bobigny, France*

Correspondence should be addressed to Valérie Metzinger-Le Meuth; valerie.metzinger@u-picardie.fr

Received 22 July 2014; Revised 2 October 2014; Accepted 5 October 2014

Academic Editor: Ling-Qing Yuan

Morphology and changes in gene expression of vascular endothelium are mainly due to shear stress and inflammation. Cell phenotype modulation has been clearly demonstrated to be controlled by small noncoding micro-RNAs (miRNAs). This study focused on the effect of laminar shear stress (LSS) on human endothelial cells (HUVECs), with an emphasis on the role of miRNA-126 (miR-126). Exposure of HUVECs *in vitro* to LSS modified the shape of HUVECs and concomitantly regulated the expression of miR-126, vascular cell adhesion molecule 1 (VCAM-1), and syndecan-4 (SDC-4). A significant upregulation of miR-126 during long-term exposure to flow was shown. Interestingly, LSS enhanced SDC-4 expression on the HUVEC membranes. Overexpression of miR-126 in HUVECs decreased the levels of targets stromal cell-derived factor-1 SDF-1/CXCL12 and VCAM-1 but increased the expression of RGS16, CXCR4, and SDC-4. No significant difference in terms of cell proliferation and apoptosis was observed between scramble, anti-miR-126, and pre-miR-126 transfected HUVECs. In Apo-E KO/CKD mice aortas expressing a high level of miR-126, SDC-4 was concomitantly increased. In conclusion, our results suggest that miR-126 (i) is overexpressed by long-term LSS, (ii) has a role in up- and downregulation of genes involved in atherosclerosis, and (iii) affects SDC-4 expression.

1. Introduction

Morphologic changes are observed in large vessels during the formation of atherosclerotic lesions, inducing substantial alterations in the phenotype of vascular wall cells, including endothelial and smooth muscle cells [1]. These changes recruit leukocytes that roll along the endothelial monolayer, thereby increasing the production of chemokines and cytokines. Chemokines are chemoattractant cytokines for leukocytes, including monocytes/macrophages, lymphocytes, and dendritic cells [2]. A chemokine such as SDF-1/CXCL12 is a CXC chemokine which binds to a family of specific G protein-coupled receptors (GPCRs) CXCR4 and plays an important role in hematopoiesis, development, and organization of the immune system [3]. SDF-1/CXCL12 induces the expression of adhesion molecules between endothelial cells and leukocytes, which transmigrate to the site of injury across the endothelial monolayer and initiate the inflammatory atherosclerotic process [4].

Vascular endothelial cells are submitted to various types of blood flow exerting tangential and perpendicular forces, the intensity of which plays an important role in endothelial cell homeostasis [5]. Straight parts of arteries are subjected to a unidirectional laminar shear stress (LSS). Atherosclerosis preferentially develops in arterial branches and curvatures, where the endothelial cell matrix layer comprising the cell

surface heparan sulfates (HSs) is scarcely expressed. Syndecan (SDC) represents a large family of proteoglycans on the cell membrane and is the major source of heparan sulfate chains (HS). SDC use their HS chains to bind a variety of soluble and insoluble ligands, such as extracellular matrix components, growth factors, cytokines, and proteinases, all interacting with endothelial-specific receptors [6–8]. This interaction occurs either *via* direct binding of biomolecules to increase their local concentration or by blocking other biomolecules to prevent them from reaching the endothelial surface [9].

Modulation of cellular phenotypes is known to be controlled by a regulatory system of small noncoding microRNAs (miRNAs) with an approximate length of 22 nucleotides [10]. Mature miRNA binds by partial complementary base-pairing to mRNA and is therefore theoretically able to target a large number of mRNA, conferring a great versatility in the modulation of gene expression. Conversely, one mRNA can also be regulated by several miRNAs [11].

miR-126 is a strongly expressed microRNA specific to endothelial cells which fine-tunes their phenotype [12]. miR-126 expression is also affected in the course of several physiological and pathological processes, such as angiogenesis, atherosclerosis, and the proinflammatory process [13, 14]. Deletion of miR-126 causes loss of vascular integrity and produces defects in endothelial cell proliferation, migration, and angiogenesis [15].

We recently studied the expression of several miRNAs, including miR-126, in large vessels during various stages of chronic kidney disease (CKD) and atherosclerosis [16] and showed that miR-126 is increased in the aorta in murine models of these diseases. This miRNA is of special interest in the study of the endothelial phenotype, as it has been recently described that miR-126 directly targets both SDF-1/CXCL12 [14] and vascular cell adhesion molecule 1 (VCAM-1) [12]. On the other hand, Zernecke et al. [17] showed that the atheroprotective effects of human endothelial cell apoptotic bodies are mediated by miR-126, which inhibits the negative regulator RGS16, thereby enabling CXCR4 to stimulate enhanced expression of SDF-1/CXCL12 via ERK1/2.

The aim of the present study was (1) to observe the impact of laminar flow on human endothelial cell morphology and cytoskeleton distribution, (2) to analyze its effect on miR-126, adhesion molecule (VCAM-1), and syndecans (SDC-1, SDC-4), and (3) to study the miR-126 and SDC-4 levels in mice model of atherosclerosis with CKD. These pieces of information are important to determine the altered interactions between monocytes in the blood and endothelial cells of the blood-vessels in early phase of atherosclerosis. We show that morphologic and genetic changes due to LSS induce the production of endogenous miR-126, which in turn affects the regulation of chemokines and proteoglycans.

2. Materials and Methods

2.1. Cell Culture. Human umbilical vein endothelial cells (HUVEC, CRL-1730, ATCC) were grown in ECBM culture medium (Endothelial Cell Basal Medium, PromoCell) supplemented with 12% fetal bovine serum (FBS), 5 ng/mL EGF (Epidermal Growth Factor), 0.2 mg/mL hydrocortisone, 0.5 ng/mL Vascular Endothelial Growth Factor (VEGF), 10 ng/mL basic Fibroblast Growth Factor (bFGF), 20 ng/mL R3 Insulin-like Growth Factor (IGF-1), 1 g/mL ascorbic acid, 1% penicillin-streptomycin (Invitrogen), and 1% L-glutamine (Invitrogen). The medium was changed twice a week. For the various experiments, the cells were maintained in either plastic T25-plates (25 cm^2, 1.2×10^6 cells/plate), 6-well glass plates (Labtek) (5×10^3/well), 96-well plastic plates (5×10^3 cells/well), 6-well plastic plates (2×10^5 cells/well), or Ibidi μ-Slides (I 0.4 Luer, ibiTreat) (3×10^5 cells/slide) under controlled humid atmosphere at 37°C with 5% CO_2.

2.2. Laminar Shear Stress. To analyze the influence of shear stress on cell shape, LSS studies were performed on HUVECs. Cells were seeded in Ibidi slides (μ-Slide I 0.4 Luer, Ibidi, Biovalley, France). After 16 h of treatment, cells were submitted to flow conditions in complete culture medium with a shear stress at 0.8 dyne/cm^2 (speed 0.6 mL/min) and two different flow times, 10 min and 24 h. For the 10 min flow time, the Ibidi slides were connected to the syringe pump system. For the long flow time (24 h), the Ibidi slides were connected to a closed-loop perfusion system with a peristaltic pump and maintained at 37°C with 5% CO_2.

2.3. Immunolabeling. To identify the presence of SDC-1 and SDC-4 proteoglycans on the surface of human endothelial cells at the end of LSS, cells were fixed with 1% paraformaldehyde (PFA) for 30 min at 4°C. Cells were incubated for 1 h at 4°C with primary antibodies directed against SDC-1 (10 μg/mL, monoclonal mouse IgG1, DL-101 Santa Cruz, TEBU, Biotechnology, Inc.), SDC-4 (10 μg/mL, polyclonal rabbit IgG H-140, Santa Cruz, TEBU, Biotechnology, Inc.), and VCAM-1 (10 μg/mL, Rat monoclonal IgG1 M/K-2, Santa Cruz,) or their isotypes. Anti-SDC-1 and anti-SDC-4 immunolabeling were revealed by Alexa Fluor 555 goat anti-mouse (IgG (γ2b)) or Alexa Fluor 555 goat anti-rabbit (IgG (H+L)) and Alexa Fluor 488 goat anti-rat (IgG (H+L)), respectively (both 1/100, Molecular Probes, Invitrogen, Cergy-Pontoise, France). All samples were also incubated with 1 mg/mL 4,6-diamidino-2-phenylindole hydrochloride (DAPI) solution (Sigma-Aldrich). Representative immunohistochemistry photomicrographs were taken using a Zeiss Axiophot microscope (Zeiss, AXIOPHOT, MicMac, Le Pecq, France) and fluorescence intensity was analyzed with IMAGE J software.

2.4. qRT-PCR. Some HUVECs were submitted to LSS for 10 min and 24 h. RNAs were then isolated with the mirVana miRNA Isolation Kit (Applied Biosystems) according to the manufacturer's instruction. DNAse was used to digest DNA, and reverse transcription was performed using High Capacity cDNA Synthesis Kit (Applied Biosystems). PCR reactions were performed with TaqMan Universal Master Mix (Applied

Biosystems) and the following target probes (Applied Biosystems): hsa-miR-126 (UCGUACCGUGAGUAAUAAUGCG). Hs.00896423_ml (SDC-1), Hs.01120909_ml (SDC-4), Hs.00607978_s1 (CXCR4), and Hs.413297 (RGS16), hs.00171022_ml (SDF-1/CXCL12), Hs.107740 (Krüppel-like Factor, KLF-2), and Hs.109225 (VCAM-1) were measured using Power SYBR Green Mix (Bio-Rad) according to the manufacturer's instruction. The U6 small nuclear RNA (for miRNAs) and GAPDH (for mRNA targets) were used as endogenous controls.

2.5. miR-126 Transfection. HUVECs were detached, quantified, and transfected in suspension with anti-miR-126 or pre-miR-126 or control scramble in the presence of siPort NeoFX Transfection agent according to the manufacturer's instruction (Ambion). Transfected cells were seeded in 6-well plastic plates (2×10^5/well) and cultured for 48 h at 37°C with 5% CO_2. Cells then were trypsinized, and RNAs were isolated and stored at −80°C for further analysis by qRT-PCR.

2.6. MTT Assay. HUVECs cell metabolic activity was measured by reduction of MTT (Sigma-Aldrich). Cells (5×10^3/well) were transfected with anti-miR-126 or pre-miR-126 or control scramble for 48 h, as previously described [18]. Cells were then incubated with 0.5 mg/mL MTT for 1 h at 37°C. After MTT withdrawal, the resulting blue formazan crystals were solubilized in DMSO (Merck, Fontenay-sous-Bois, France) and absorbance was determined at 595 nm.

2.7. Flow Cytometry Analysis. The Annexin V-FITC ApoptosisDetection Kit(BD Biosciences, USA) was used to verify the apoptosis level of HUVECs transfected by pre-miRNA.HUVECs were transfected with anti-miR-126 or pre-miR-126 or control scramble for 48 h, as previously described. Cells were then incubated with Annexin V-FITC for 30 min according to the manufacturer's instruction and flow cytometry was performed with a Becton-Dickinson FACS Calibur flow cytometer.

2.8. Animals: Diet and Surgical Procedures. All animal studies complied with the principles of Directive 2010/63/EU of the European Parliament and all protocols were approved by our Institution's Animal Care and Use Committee (*Comité Régional d'Ethique en Matière d'Expérimentation Animale de Picardie*, CREMEAP). The experiments were performed in female C57/BL6 mice purchased from Charles Rivers (Lyon, France). Animals were housed in polycarbonate cages in temperature- and humidity-controlled rooms with a 12:12-hour light-dark cycle and were given standard chow (Harlan Teklad Global Diet 2016, Harlan, Oxon, UK) and tap water *ad libitum*. Chow composition was 4.2% (wt/wt) fat, 16.7% protein, 60.89% carbohydrates, 0.98% calcium, 0.25% sodium, and 0.65% phosphorus. Mice were anesthetized with ketamine and xylazine (80 mg/kg and 8 mg/kg, resp.), and every effort was made to minimize suffering. At 8 weeks of age, mice were assigned to the following groups: wild-type (WT) mice which were submitted to sham operations (WT SHAM); WT mice with chronic kidney disease (WT CKD); Apolipoprotein-E knockout (Apo-E KO) mice which were submitted to sham operations (Apo-E KO SHAM); and Apolipoprotein-E knockout mice with CKD (Apo-E KO CKD). CKD was induced by applying cortical electrocautery to the right kidney and left total nephrectomy was then performed 2 weeks after the first operation [16]. After 10 weeks of uremia, animals were sacrificed and the whole aorta was removed surgically and stored at −80°C until further use.

2.9. Statistical Analysis. Data are expressed as mean ± S.D. Statistical comparisons were performed globally by one-way ANOVA and between two groups with a two-tailed Student's t-test. A P value <0.05 was considered significant ($^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.0001$).

3. Results

3.1. LSS Modified Morphology and F-Actin Distribution in HUVECs. LSS has been described to be a factor that does not cause damage to the vessel wall. However, altered morphology of endothelial cells exposed to LSS has only been described after long-term exposure [19]. In order to validate LSS in this endothelial cell model, HUVECs were exposed to three different durations of laminar flow culturein the Ibidi flow chamber: 10 min and 24 h with the same physiological wall shear stress at 0.8 dyne/cm^2 [20]. The first step of our experiment was to optimize the cell quantity at day 1 of cell culture in IBIDI chamber to obtain a homogeneous endothelial cell monolayer without excessive cell condensation. The second step was to optimize the shear stress (dyne/cm^2) which has been applied during all time of experiment, in our study for 10 min and 24 h, without any degradation of cell monolayer (data not shown). We decided to use IBIDI chambers without any additional layer on it (fibronectin, or collagen), and the highest shear stress (flow velocity) obtained without any cell detachment for 24 hours was 0.8 dyne/cm^2 which was used throughout the paper. Before LSS, (under static-control conditions), HUVECs presented physiological morphology with a homogenous F-actin distribution throughout the cytoplasm. No changes in F-actin distribution and cell shape were observed after 10 min of LSS. Interestingly, after 24 h of LSS, the cells presented a cobblestone appearance with F-actin distribution confined to the membrane (Figure 1).

3.2. Long-Term LSS Increased Syndecan-1 and Syndecan-4 but Not VCAM-1 Expression in HUVECs. Syndecan-1, syndecan-4 (SDC-1 and SDC-4), and VCAM-1 expressions were analyzed at the beginning of the experience, under static condition, and after 10 min and 24 h of LSS (Figures 2(a) and 2(b)). Then, HUVECs were incubated with anti-SDC-1, anti-SDC-4, and anti-VCAM-1 antibodies to study its membrane expression by fluorescent microscopy. The total fluorescence intensity was normalized by cell number in each field. The results are presented in arbitrary units (AU)/nuclei and

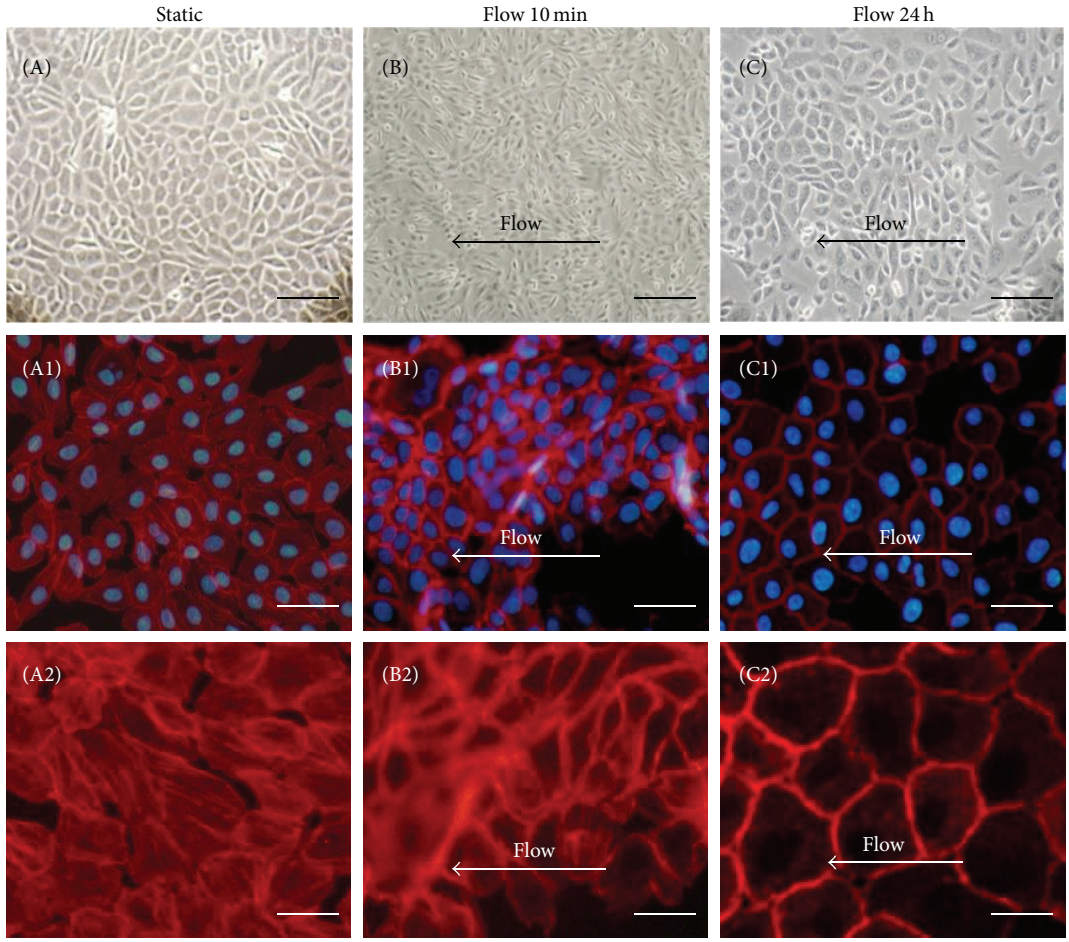


Figure 1: LSS alters the morphology of HUVEC cells. Representative phase contrast (A–C) or fluorescence (A1–C2) photographs of endothelial cells. HUVECs were grown under static conditions (A–A2) or LSS conditions for 10 min (B–B2) or 24 h (C–C2). Cells were then stained with Phalloidin (F-actin: red) and DAPI (Nucleus: blue). Global distribution of F-actin and cellular shape was analyzed for static conditions (A1), 10 min of LSS (B1) or 24 h of LSS (C1). High resolution zoom images of F-actin distribution in endothelial cells (A2-C2). Direction of flow is indicated by white arrows. For A–C scale bar = 40 μm, for A1–C1 scale bar = 20 μm, and for A2–C2 scale bar = 5 μm.

shown in Figure 2(b) There was a significant increase of SDC-1 (0.03 ± 0.005 versus 0.06 ± 0.007), SDC-4 (0.05 ± 0.005 versus 0.09 ± 0.006), and VCAM-1 (0.04 ± 0.003 versus 0.08 ± 0.009) expression observed after 10 min of LSS, as compared to static condition (Figure 2(b)). In addition, a significant increase of SDC-1 (0.03 ± 0.005 versus 0.12 ± 0.018), SDC-4 (0.05 ± 0.005 versus 0.096 ± 0.01), and VCAM-1 (0.04 ± 0.003 versus 0.049 ± 0.004) expression was observed after 24 h of LSS, as compared to static conditions (Figure 2(b), $P < 0.05$). Interestingly, only SDC-1 level was significantly increased by 1.8-fold (0.06±0.007 versus 0.12±0.018) between 10 min and 24 h of LSS. There were no changes of SDC-4 level (0.09 ± 0.006 versus 0.096 ± 0.001) and there was a 1.5-fold decrease of VCAM-1 level (0.08 ± 0.009 versus 0.049 ± 0.004) between 10 min and 24 h of LSS (Figure 2(b)). For these analyses we used the ANOVA statistic tests using Stat View software ($^{*}P < 0.05$, $^{**}P < 0.001$). Interestingly, only SDC-1 level was significantly increased by 1.8-fold (0.06 versus 0.12) between 10 min and 24 h of LSS. There were no changes of SDC-4 level (0.09 versus 0.096) and there was a 1.5-fold decrease of VCAM-1 level (0.08 versus 0.049) between 10 min and 24 h of LSS (Figure 2(b)). For these analyses we used the ANOVA/ANCOVA statistic tests using Stat View software ($^{*}P < 0.05$, $^{**}P < 0.001$).

3.3. LSS Modulated KLF-2 Level in HUVECs. Krüppel-like factor-2 (KLF-2) expression was studied, as KLF-2 is a transcriptional factor known to be induced by LSS [21, 22]. Interestingly, a significant decrease of KLF-2 mRNA expression was observed after 24 h of LSS, (0.37±0.21) (Figure 3(a)). A significant increase of KLF-2 mRNA expression was also observed at 10 min (1 versus 16.78 ± 0.84) but not at 24 h. A significant decrease of KLF-2 mRNA expression was also observed after 24 h of LSS, compared to 10 min of LSS (16.8 ± 0.84 versus 1.64 ± 0.07) (Figure 3(a)).

3.4. Regulation of miR-126 Levels in Endothelial Cells under Static and LSS Conditions. To assess miR-126 changes after

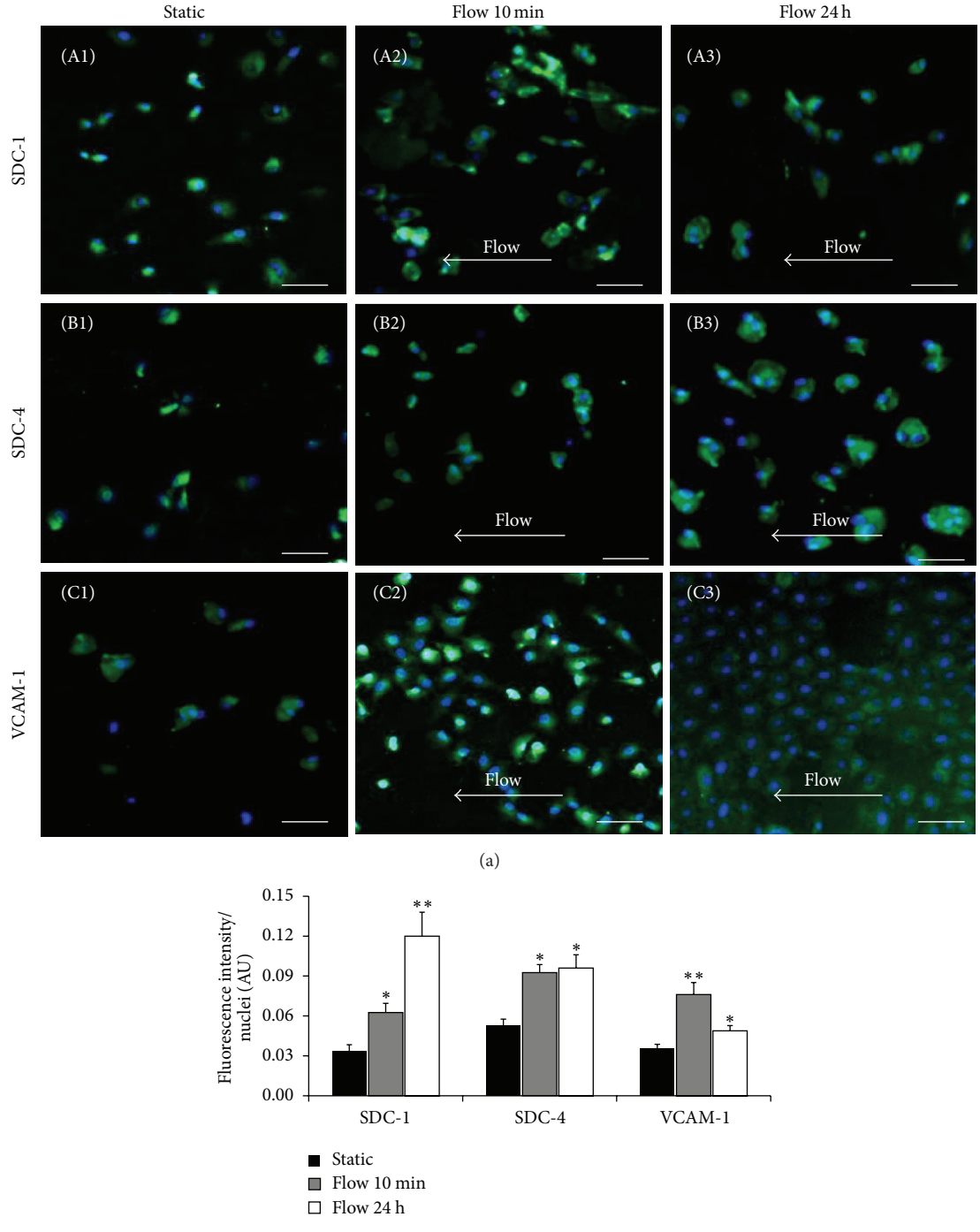


Figure 2: LSS induces SDC-1, SDC-4, and VCAM-1 expression in HUVECs. (a) Representative immunofluorescence staining of SDC-1, SDC-4, and VCAM-1 protein on endothelial cells cultured under static or LSS conditions for 10 min and 24 h. HUVEC cells were immunolabeled with anti-SDC-1 (A1–3), anti-SDC-4 (B1–3), and anti-VCAM-1 (C1–3) antibodies (green) or with their respective control isotypes. The nuclei were stained with DAPI (blue). High resolution zoom images of cells were added to each figure. Scale bar = 20 μm. (b) Immunofluorescence quantification of proteins normalized with the nuclei number ($n = 3$) was done and 5 different fields in HUVECs submitted to static conditions and LSS (10 min and 24 h) were counted and presented as fluorescence intensity/nuclei (AU: arbitrary units). A progressive increase of SDC-1 and SDC-4 expression up to 24 h and an increase at 10 min and then a decrease of VCAM-1 expression were observed. One-way Global ANOVA and Student's t-test, for SDC-1: $^{*}P < 0.05$ (10 min of LSS versus static), $^{**}P < 0.001$ (24 h of LSS versus static); for SDC-4: $^{*}P < 0.05$ (10 min of LSS versus static), $^{*}P < 0.05$ (24 h of LSS versus static); for VCAM-1: $^{**}P < 0.001$ (10 min of LSS versus static), $^{*}P < 0.05$ (24 h of LSS versus static).

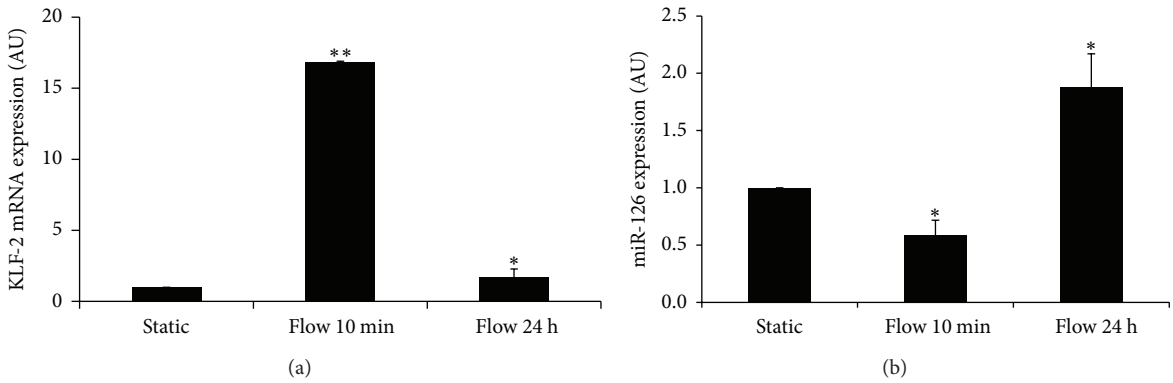


Figure 3: LSS increases KLF-2 mRNA expression and regulates miRNA expression in HUVECs. HUVECs cells were exposed to various durations of laminar shear stress (10 min and 24 h). (a) LSS increased KLF-2 mRNA expression. Values are expressed as mean ± SD of 3 experiments. (b) 24 h of LSS increased miR-126 levels in HUVECs. Values are expressed as mean ± SD of 3 independent experiments (AU: arbitrary units). Student's t-test, for KLF-2: $^{**}P < 0.001$ (10 min of LSS versus static), $^{*}P < 0.05$ (24 h of LSS versus static); for miR-126: $^{*}P < 0.05$ (10 min of LSS versus static), $^{*}P < 0.05$ (24 h of LSS versus static).

LSS treatment, miRNAs were quantified by qRT-PCR (Figure 3(b)). A significant decrease of miR-126 expression in HUVECs was demonstrated after 10 min of LSS, compared to control static conditions (1 versus 0.58 ± 0.13). A significant increase of miR-126 expression was also observed after 24 h of LSS, compared to 10 min of LSS (0.58 ± 0.13 versus 1.88 ± 0.29). Our data also demonstrated that 24 h of LSS significantly decreased SDF-1/CXCL12 by 98% (1 versus 0.12 ± 0.06). In order to assess the specificity of miR-126 response to LSS, we studied another miR from endothelial cells, miR-222 (data not shown) and found that it was unaffected by a 24 h flow.

3.5. Upregulation or Downregulation of miR-126 Does Not Affect HUVEC Proliferation or Apoptosis. Pre-miR-126 and anti-miR-126 sequences were initially transfected into HUVECs and compared to the scramble control (Figure 4(a)). A marked decrease of miR-126 expression was observed after anti-miR-126 transfection (1 versus 0.23 ± 0.034) and a marked increase of miR-126 expression was observed after pre-miR-126 transfection (1 versus 7000 ± 1168), compared to controls (Figure 4(a)).

Secondly, the effect of anti-miR-126 or pre-miR-126 or scramble transfection on cell proliferation was analyzed by means of the MTT assay and apoptosis was studied by flow cytometry-Annexin V assays. No significant difference in terms of cell proliferation was observed between scramble, anti-miR-126, and pre-miR-126 transfected cells, compared to nontransfected control cells (Figure 4(b), left panel). No significant changes in HUVEC apoptosis measured by flow cytometry were observed after anti-miR-126 or pre-miR-126 or scramble transfection (Figure 4(b), right panels).

3.6. miR-126 Is a Negative Regulator for VCAM-1 and SDF-1/CXCL12 and a Positive Regulator for RGS16, CXCR4, and SDC-4 in HUVECs. Upregulation of miR-126 (transfection with pre-miR-126) increased RGS16 (1 versus 3.23 ± 1.48), SDC-4 (1 versus 6.39 ± 0.64), and CXCR4 expression (1 versus 6.39 ± 0.91) and decreased SDF-1/CXCL12 (1 versus 0.19 ± 0.03) and VCAM-1 expression (1 versus 0.65 ± 0.14) in HUVECs. However, no significant difference was observed for SDC-1 expression (1 versus 1.43 ± 0.61) after pre-miR-126 transfection (1 versus 0.87 ± 0.79) (Figure 4(c)).

In addition, miR-126 knockdown (transfection with anti-miR-126) decreased RGS16 (1 versus 0.74 ± 0.20), SDC-4 (1 versus 0.48 ± 0.08), and CXCR4 expressions (1 versus 0.56 ± 0.23). A significant increase of SDF-1/CXCL12 (1 versus 4.8 ± 0.38) and VCAM-1 (1 versus 28.05 ± 3.7) expression was observed under the same conditions. No significant difference was observed for SDC-1 expression after anti-miR-126 transfection (Figure 4(c)).

3.7. miR-126 and SDC-4 Expression Increased in Apo-E KO/CKD Mice. To confirm our findings in an *in vivo* model, we decided to analyze SDC-4 expression in vessels from a rodent model where miR-126 was shown to be increased [16]. We thus measured concomitantly miR-126 and SDC-4 in aortas from mice models with CKD, atherosclerosis, and vascular calcification [16]. Wild-type (WT) C57/BL6 as well as Apo-E KO mice were submitted to partial nephrectomy to induce CKD. Apo-E KO mice characteristically develop large atheromatous plaques and low-grade vascular calcification and the combination of Apo-E KO/CKD leads to atherosclerosis and aortic calcification [16]. Experiments were performed 10 weeks after induction of uremia, when CKD mice presented severe uremia and Apo-E KO mice presented marked atherosclerosis (data not shown). Under these conditions, miR-126 and SDC-4 expression were also correlated *in vivo*, as miR-126 expression was increased in the aorta of Apo-E KO/CKD mice (1 versus 3.77±1.29), compared to WT SHAM mice (Figure 5). A significant increase of

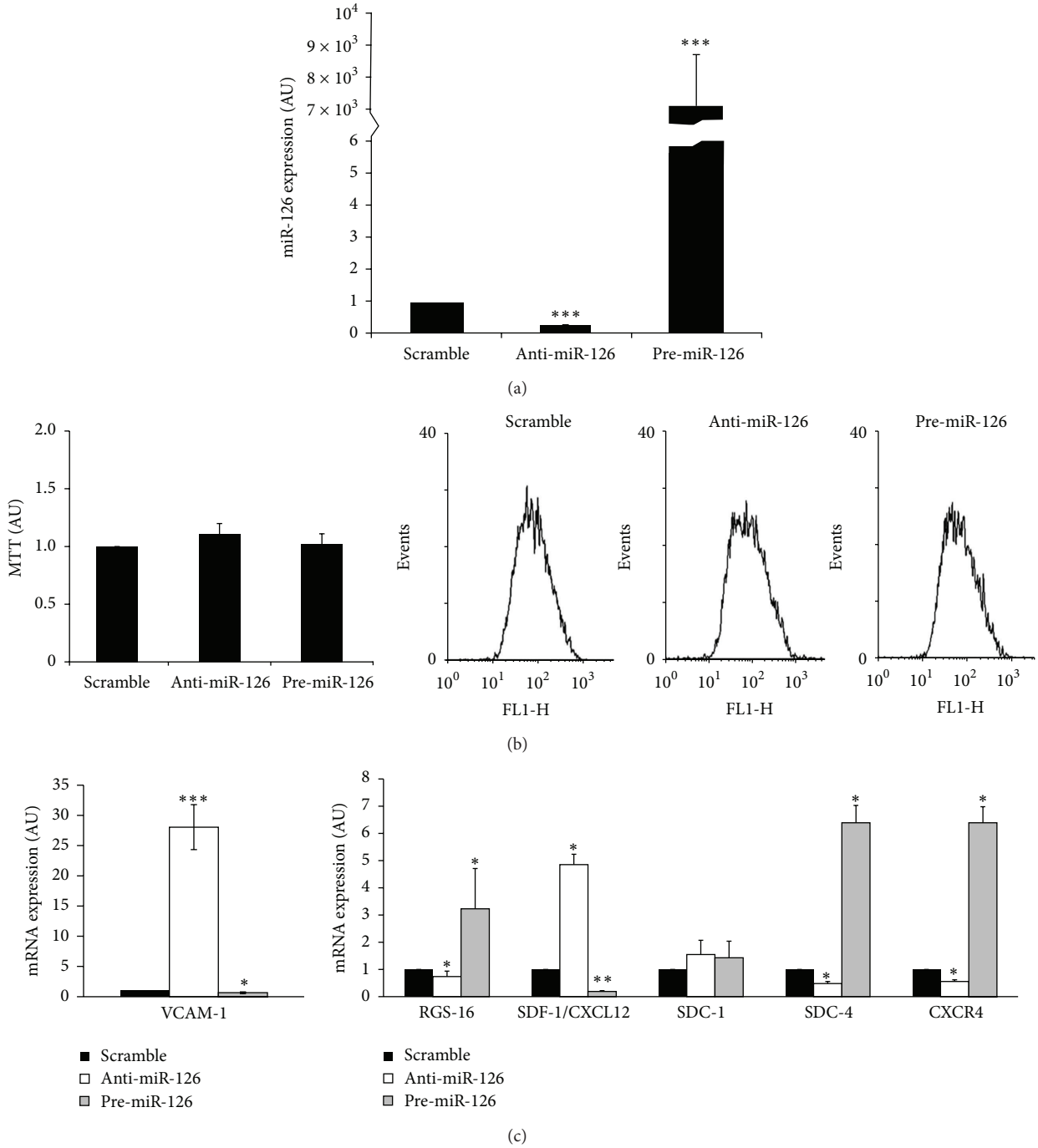


Figure 4: Study of HUVEC transfection after 48 h with scramble, anti-miR-126, and pre-miR-126 under static conditions. (a) Confirmation of miR-126 overexpression and knockdown under the respective conditions. Values are expressed as mean ± SD of 3 independent experiments (AU: arbitrary units). Student's t-test, for miR-126: $^{***}P < 0.0001$ (scramble versus anti-miR-126), $^{***}P < 0.0001$ (scramble versus pre-miR-126). (b) HUVEC metabolic activity was measured using MTT test after HUVEC transfection. Representative flow cytometry histogram study of apoptosis from HUVECs. (c) Expression of various mRNA targets in HUVECs after 48 h of transfection with anti-miR-126 and pre-miR-126. Values are expressed as mean ± SD of 3 independent experiments (AU: arbitrary units). Student's t-test, for VCAM-1: $^{***}P < 0.0001$ (scramble versus anti-miR-126), $^{*}P < 0.05$ (scramble versus pre-miR-126); for RGS-16, SDC-4, CXCR4: $^{*}P < 0.05$ (scramble versus anti-miR-126), $^{*}P < 0.05$ (scramble versus pre-miR-126); for CXCL12: $^{*}P < 0.05$ (scramble versus anti-miR-126), $^{**}P < 0.001$ (scramble versus pre-miR-126).

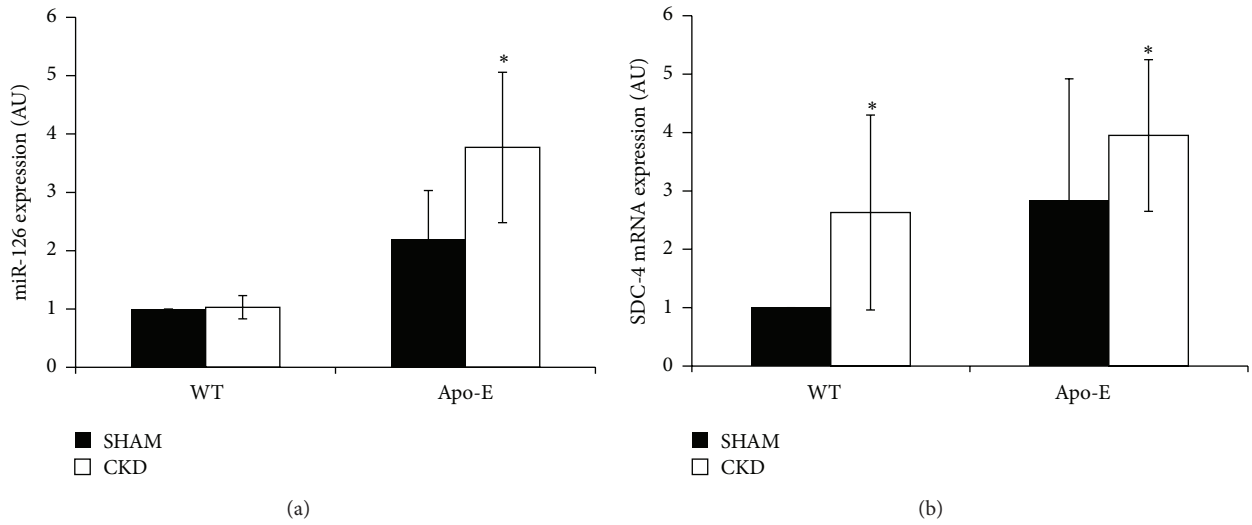


Figure 5: miR-126, miR-126, and SDC-4 expression in control and atherosclerotic mice. Twenty-week-old mice aortas were isolated from wild-type (WT) and Apo-E KO mice for miRNA and mRNA studies at the indicated times. CKD mice were subjected to 10 weeks of uremia. (a) miR-126 expression expressed as RQ normalized to U6. (b) SDC-4 expression normalized to GAPDH. Values are expressed as mean ± SD of 3 independent experiments (AU: arbitrary units). Student's t-test, for miR-126: $^{*}P < 0.05$ (WT sham versus Apo-E CKD); for SDC-4: $^{*}P < 0.05$ (WT sham versus WT CKD), $^{*}P < 0.05$ (WT sham versus Apo-E CKD).

SDC-4 expression was also observed in WT CKD (1 versus 2.63 ± 1.67) and Apo-E KO/CKD mice (1 versus 3.95 ± 1.3), compared to WT SHAM mice (Figure 5).

4. Discussion

LSS has been associated with a vasoprotective endothelial phenotype [5]. The present study demonstrates that LSS is associated with human endothelial cell (HUVEC) elongation after an intermediate exposure time to flow, but when submitted to longer exposure times to LSS, HUVECs changed their phenotype to a cobblestone shape with rearrangement of the cytoskeleton distribution in the cell, especially near to membrane, and increased proteoglycan expression. Increased shear stress induces tension which is accommodated by the endothelial cytoskeleton *via* rearrangement of actin and intermediate filament proteins at sites of cell-matrix contact [23]. When HUVECs were submitted to prolonged LSS, downregulation of adhesion molecule VCAM-1 was observed concomitantly with mechanical reorganization of the cytoskeleton. These changes were reflected by significant upregulation of miR-126 during long-term exposure to flow. These changes suggest that flow could confer an anti-inflammatory and atheroprotective phenotype to vascular endothelial cells.

It has been previously reported that mechanical and haemodynamic phenomena applied the forces to arterial walls and especially shear stresses to the vascular endothelium [24]. There are many values of shear in various vessels, <10 dyne/cm^2 (for vena cava, large veins, descending aorta), <20 dyne/cm^2 (for venules and ascending aorta), and <60 dyne/cm^2 (for capillaries and arterioles) [25]. In our study, we used shear stress at 0.8 dyne/cm^2, since this condition is optimal to study the mechanical properties of shear flow on adhesion molecule VCAM-1 and syndecans expression and to understand their role in blood-cell adhesion on endothelium [20].

VCAM-1 is expressed by vascular endothelial cells and plays an important role in monocyte or lymphocyte rolling and adhesion in early-onset atherosclerosis [26] and in the inflammatory process [27]. The decreased VCAM-1 expression observed in this study suggests that long-term LSS confers anti-inflammatory properties to endothelial cells, as already reported [22].

We therefore decided to analyze genes critical in conferring atheroprotective properties to the endothelial cell surface and investigated the expression of transcription factor KLF-2.

KLF-2is a zinc-finger transcription factor that is exclusively present in the adult vasculature and has distinct functions in vasomotor regulation, inflammation, homeostasis, and angiogenesis. In cardiovascular biology, KLF-2 is described to be an important molecular transducer that converts hemodynamic forces into the regulation of gene expression [28, 29]. Some studies have already reported results in favour of increased KLF-2 transcription when cells were subjected to laminar flow [28, 30, 31]. Shear stress induces KLF-2 by raising mRNA and protein levels by transcriptional activation at the KLF-2 promoter via the MEK5/ERK5 Mitogen-activated protein kinases (MAPK) cascade which leads to activation of myocyte enhancer binding factor 2 (MEF2) at the KLF2 promoter [21, 32]. In addition, van Thienen et al. [29] showed that shear stress induces KLF-2 mRNA stabilization.

In the present study, we observed an acute increase of KLF-2 mRNA after 10 min of exposure to LSS, followed by a decrease after 24 h. We therefore conclude that in our experimental condition LSS does not continuously sustain KLF-2 mRNA transcription and/or stabilization.

miRNAs have been described as important modulators of angiogenesis and atherosclerosis [33]. We measured miR-126 expression, as it is the most abundant miRNA found during endothelial cell (EC) differentiation and in adult ECs [12] and it is deregulated in various cardiovascular disorders [14]. miR-126 was diversely regulated when exposed to LSS. After a brief exposure to LSS (10 min), a slight decrease of miR-126 expression was observed concomitant with an increase of KLF-2 mRNA and, after a long exposure time to LSS (24 h), a marked increase of miR-126 expression was observed. This increase could possibly be induced by the increased KLF-2 expression observed after 10 min of exposure, as KLF-2 has been shown to increase miR-126 expression in zebrafish [31]. But Harris et al. [34] found a potential binding site for KLF-2 in the Egfl7/miR-126 5′ flanking region. In comparison, Hergenreider et al. used on HUVECs a higher shear at 20 dyne/cm^2 for a longer time (3 days) and found no effect on miR-126 expression level and no significant regulation of miR-126 by KLF-2 overexpression [30]. On the other hand, Schober et al., 2014, showed that shear stress on HUVECs induces KLF-2-dependent expression of pri-miR-126 but not miR-126-3p [35].

miR-126 is known to regulate many targets either directly (SDF-1/CXCL12, CXCR-4, VCAM-1 and RGS16) and/or indirectly (SDF-1/CXCL12, CXCR-4) [12, 13, 17, 36]. We did not find any evidence that SDC-4 is a direct target of miR-126 by looking at all known dedicated databases. Our results show that the increase in miR-126 after 24 h of LSS was concomitant with downregulation of its well-known target VCAM-1 at the protein level compared to 10 min LSS and miR-126 overexpression experiments resulted also in a decrease of VCAM-1 mRNA. In the same way, Harris et al. [12] observed that overexpression of miR-126 decreases VCAM-1 protein expression and consequently reduces leukocyte adhesion to endothelial cells. The changes we observed after 10 minutes of flux are probably not consequent to a change in gene expression but are likely due to other mechanisms such as the last steps of protein biosynthesis or phosphorylation of kinases.

As expected, overexpression of miR-126 under our experimental conditions provided results comparable to those published by van Solingen et al. [14], that is, a decrease of SDF-1/CXCL12 and VCAM-1, which are described targets of miR-126 [12]. This effect occurred in the absence of apoptosis. In contrast, Zernecke et al. showed that the increase in miR-126 expression was concomitant with enhancement of SDF-1/CXCL12 in an indirect pathway mediated by apoptosis [17]. Knowing that miR-126 decreases SDF-1/CXCL12 and VCAM-1 expression, we can hypothesize that overexpression of miR-126 is atheroprotective and may lead to a decrease of leukocyte homing from the blood circulation through the endothelium *in vivo* [29, 37]. On the other hand, miR-126 modulation (under- or overexpression) did not change in our hands cell proliferation or apoptosis. Van Solingen et al. showed in a mouse hindlimb ischemia model that modulating miR-126 does not affect shear-stress-induced arteriogenesis [38]. The same authors found also that short-term antagomir-126 treatment did not alter HUVEC cell migration or proliferation which is concordant with our present results.

It has been clearly documented that the remodelling of endothelial cells due to LSS is associated with an increase of proteoglycan distribution and marked modification of gene expression, resulting in protection of the endothelial layer from atherosclerosis [8, 9]. We therefore decided to investigate the link between the SDF-1/CXCL12/SDC-4 complex and miR-126, as SDF-1/CXCL12, another target of miR-126, is known to form a stable complex by binding SDC-4, which in turn binds CXCR4 [14, 17, 39]. We have previously shown that SDC-4 behaves like a SDF-1/CXCL12 coreceptor. In this study we explored the effect of upregulation of miR-126 on SDF-1/CXCL12, CXCR4, and SDC-4 expression and their impact on actin cytoskeleton remodeling. Interestingly, a new finding of our study is that miR-126 is a positive regulator of SDC-4, as miR-126 overexpression increased the level of SDC-4 mRNA. miR-126 overexpression was a positive regulator of CXCR4 in our model. This result is discordant with those of a study in colon cancer cells, which demonstrated that miR-126 is a negative regulator of CXCR4 expression [40]. We suggest that our model comprises a more complex system because transfection of miR-126 significantly decreased the SDF-1/CXCL12 level and increased its receptor CXCR4.

We decided to study whether our *in vitro* findings were mirrored in an *in vivo* model where miR-126 is increased. As expected, we confirm here that miR-126 expression is significantly increased in aorta from Apo-E KO mice with CKD as previously described [16]. We show here that, in mice expressing a high level of miR-126 in their aortas (Apo-E KO/CKD mice), the SDC-4 was also significantly increased. Apo-E KO/CKD mice present higher calcification and atherosclerosis levels than WT CKD mice; this could explain why we did not find an increase in WT CKD mice for miR-126 expression. Taken together, our *in vivo* findings therefore mirror our in vitro results, suggesting that miR-126 and SDC-4 levels are concomitantly upregulated in vivo in ApoE/CKD mice aortas and in vitro in endothelial cells [16].

In summary, our data demonstrate that LSS has an important impact on miR-126 expression in HUVECs (Figure 6), and its protein targets SDF-1/CXCL12. The results of our *in vitro* model confirm the previously described impacts of miR-126 on normal and inflammatory events. This study also highlights the potential role of miR-126 on regulation of syndecan-4 expression. Our finding emphasizes the importance of identifying different mechanisms to explain this regulation.

Conflict of Interests

The authors have no conflict of interests to declare.

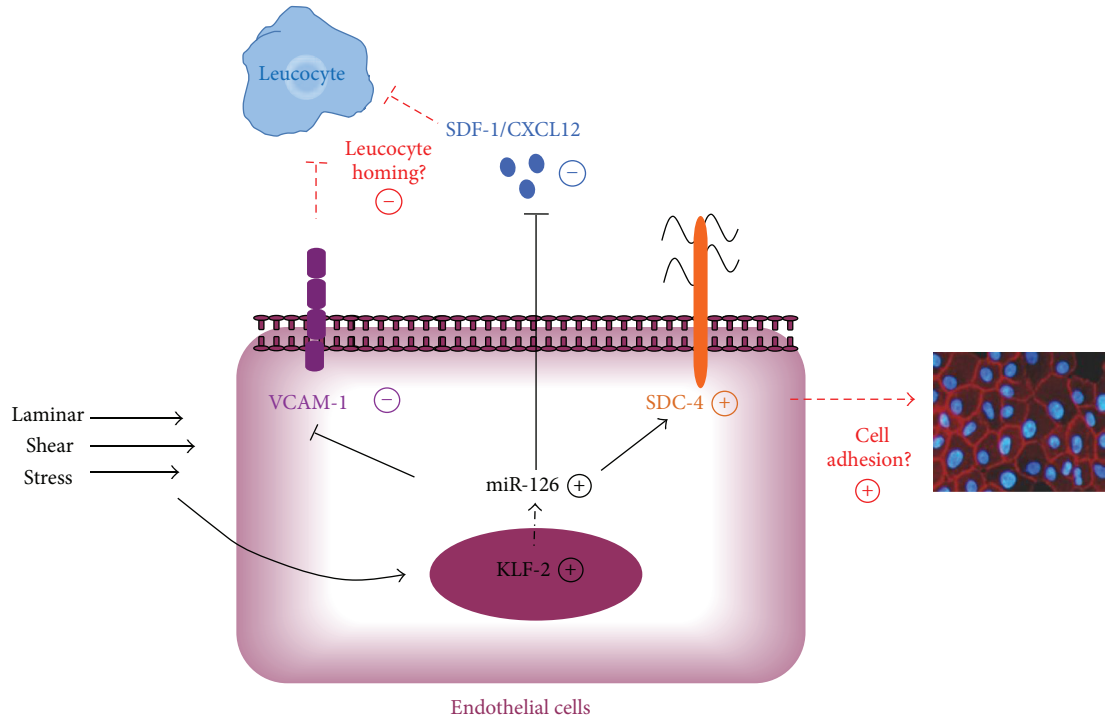


Figure 6: Representative network of miR-126 in HUVECs submitted to LSS. LSS induces at short-term the expression of transcription factor, KLF-2, and at long-term miR-126 expression. miR-126 downregulates the endothelial adhesion molecule VCAM-1 and chemokine SDF-1/CXCL12. Loss of VCAM-1 and SDF-1/CXCL12 can be associated with decrease of leukocyte homing over the endothelium. miR-126 overexpression enhanced the SDC-4 which can induce the transduction pathway leading to remodeling of F-actin cytoskeleton and favors cell adhesion and spreading. VCAM-1: vascular cell adhesion molecule-1; miR-126: small noncoding micro-RNA-126; SDF-1/CXCL12: stromal cell-derived factor-1; KLF-2: Krüppel-like factor-2; SDC-4: syndecan-4.

Authors' Contribution

Hanna Hlawaty and Valérie Metzinger-Le Meuth contributed equally to this work.

Acknowledgment

This work was funded by grants from the Picardie Regional Council (MARNO-MPCC and MIRNA), including a Ph.D. fellowship for Fatiha Taïbi and a postdoctoral fellowship for Eléonore M'baya-Moutoula.

References

[1] L. A. Harker, S. M. Schwartz, and R. Ross, "Endothelium and arteriosclerosis.," *Clinics in Haematology*, vol. 10, no. 2, pp. 283–296, 1981.

[2] A. Zlotnik and O. Yoshie, "Chemokines: a new classification system and their role in immunity," *Immunity*, vol. 12, no. 2, pp. 121–127, 2000.

[3] J. A. Burger and T. J. Kipps, "CXCR4: a key receptor in the cross-talk between tumor cells and their microenvironment," *Blood*, vol. 107, no. 5, pp. 1761–1767, 2006.

[4] M. Salmi and S. Jalkanen, "Cell-surface enzymes in control of leukocyte trafficking," *Nature Reviews Immunology*, vol. 5, no. 10, pp. 760–771, 2005.

[5] N. Davids, "Finite element methods of studying mechanical factors in blood flow," *Neurological Research*, vol. 3, no. 1, pp. 83–105, 1981.

[6] M. Gouverneur, B. Van Den Berg, M. Nieuwdorp, E. Stroes, and H. Vink, "Vasculoprotective properties of the endothelial glycocalyx: effects of fluid shear stress," *Journal of Internal Medicine*, vol. 259, no. 4, pp. 393–400, 2006.

[7] P. A. VanderLaan, C. A. Reardon, and G. S. Getz, "Site specificity of atherosclerosis: site-selective responses to atherosclerotic modulators," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 24, no. 1, pp. 12–22, 2004.

[8] A. Koo, C. F. Dewey Jr., and G. García-Cardeña, "Hemodynamic shear stress characteristic of atherosclerosis-resistant regions promotes glycocalyx formation in cultured endothelial cells," *American Journal of Physiology: Cell Physiology*, vol. 304, no. 2, pp. C137–C146, 2013.

[9] P. Zimmermann and G. David, "The syndecans, tuners of transmembrane signaling," *FASEB Journal*, vol. 13, supplement, pp. S91–S100, 1999.

[10] D. P. Bartel, "MicroRNAs: genomics, biogenesis, mechanism, and function," *Cell*, vol. 116, no. 2, pp. 281–297, 2004.

[11] M. Fabbri, "MicroRNAs and cancer: towards a personalized medicine," *Current Molecular Medicine*, vol. 13, no. 5, pp. 751–756, 2013.

[12] T. A. Harris, M. Yamakuchi, M. Ferlito, J. T. Mendell, and C. J. Lowenstein, "MicroRNA-126 regulates endothelial expression of vascular cell adhesion molecule 1," *Proceedings of the National*

Academy of Sciences of the United States of America, vol. 105, no. 5, pp. 1516–1521, 2008.

[13] S. A. Asgeirsdóttir, C. van Solingen, N. F. Kurniati et al., "MicroRNA-126 contributes to renal microvascular heterogeneity of VCAM-1 protein expression in acute inflammation," *The American Journal of Physiology—Renal Physiology*, vol. 302, no. 12, pp. F1630–F1639, 2012.

[14] C. van Solingen, H. C. de Boer, R. Bijkerk et al., "MicroRNA-126 modulates endothelial SDF-1 expression and mobilization of Sca-1(+)/Lin(−) progenitor cells in ischaemia," *Cardiovascular Research*, vol. 92, pp. 449–455, 2011.

[15] S. Wang, A. B. Aurora, B. A. Johnson et al., "The endothelial-specific microRNA miR-126 governs vascular integrity and angiogenesis," *Developmental Cell*, vol. 15, no. 2, pp. 261–271, 2008.

[16] F. Taïbi, V. Metzinger-Le Meuth, E. M'Baya-Moutoula et al., "Possible involvement of microRNAs in vascular damage in experimental chronic kidney disease," *Biochimica et Biophysica Acta—Molecular Basis of Disease*, vol. 1842, no. 1, pp. 88–98, 2014.

[17] A. Zernecke, K. Bidzhekov, H. Noels et al., "Delivery of microRNA-126 by apoptotic bodies induces CXCL12-dependent vascular protection," *Science Signaling*, vol. 2, article ra81, 2009.

[18] A. Y. Rangrez, E. M'Baya-Moutoula, V. Metzinger-Le Meuth et al., "Inorganic phosphate accelerates the migration of vascular smooth muscle cells: evidence for the involvement of miR-223," *PLoS ONE*, vol. 7, no. 10, Article ID e47807, 2012.

[19] P. F. Davies, A. Remuzzi, E. J. Gordon, C. F. Dewey Jr., and M. A. Gimbrone Jr., "Turbulent fluid shear stress induces vascular endothelial cell turnover in vitro," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 83, no. 7, pp. 2114–2117, 1986.

[20] K. D. Puri, S. Chen, and T. A. Springer, "Modifying the mechanical property and shear threshold of L-selectin adhesion independently of equilibrium properties," *Nature*, vol. 392, no. 6679, pp. 930–933, 1998.

[21] R. J. Dekker, S. van Soest, R. D. Fontijn et al., "Prolonged fluid shear stress induces a distinct set of endothelial cell genes, most specifically lung Krüppel-like factor (KLF2)," *Blood*, vol. 100, no. 5, pp. 1689–1698, 2002.

[22] R. A. Boon and A. J. G. Horrevoers, "Key transcriptional regulators of the vasoprotective effects of shear stress," *Hamostaseologie*, vol. 29, no. 1, pp. 39–43, 2009.

[23] P. F. Davies, A. Robotewskyj, and M. L. Griem, "Quantitative studies of endothelial cell adhesion. Directional remodeling of focal adhesion sites in response to flow forces," *The Journal of Clinical Investigation*, vol. 93, no. 5, pp. 2031–2038, 1994.

[24] H. H. Lipowsky, S. Kovalcheck, and B. W. Zweifach, "The distribution of blood rheological parameters in the microvasculature of cat mesentery," *Circulation Research*, vol. 43, no. 5, pp. 738–749, 1978.

[25] T. G. Papaioannou and C. Stefanadis, "Vascular wall shear stress: basic principles and methods," *Hellenic Journal of Cardiology*, vol. 46, no. 1, pp. 9–15, 2005.

[26] M. I. Cybulsky, K. Iiyama, H. Li et al., "A major role for VCAM-1, but not ICAM-1, in early atherosclerosis," *Journal of Clinical Investigation*, vol. 107, no. 10, pp. 1255–1262, 2001.

[27] P. Libby, "Inflammation in atherosclerosis," *Nature*, vol. 420, no. 6917, pp. 868–874, 2002.

[28] K. Kobus, J. Kopycinska, A. Kozlowska-Wiechowska et al., "Angiogenesis within the duodenum of patients with cirrhosis is modulated by mechanosensitive Kruppel-like factor 2 and microRNA-126," *Liver International*, vol. 32, no. 8, pp. 1222–1232, 2012.

[29] J. V. van Thienen, J. O. Fledderus, R. J. Dekker et al., "Shear stress sustains atheroprotective endothelial KLF2 expression more potently than statins through mRNA stabilization," *Cardiovascular Research*, vol. 72, no. 2, pp. 231–240, 2006.

[30] E. Hergenreider, S. Heydt, K. Treguer et al., "Atheroprotective communication between endothelial cells and smooth muscle cells through miRNAs," *Nature Cell Biology*, vol. 14, no. 3, pp. 249–256, 2012.

[31] S. Nicoli, C. Standley, P. Walker, A. Hurlstone, K. E. Fogarty, and N. D. Lawson, "MicroRNA-mediated integration of haemodynamics and Vegf signalling during angiogenesis," *Nature*, vol. 464, no. 7292, pp. 1196–1200, 2010.

[32] K. M. Parmar, H. B. Larman, G. Dai et al., "Integration of flow-dependent endothelial phenotypes by Kruppel-like factor 2," *Journal of Clinical Investigation*, vol. 116, no. 1, pp. 49–58, 2006.

[33] S. Anand and D. A. Cheresh, "Emerging role of micro-RNAs in the regulation of angiogenesis," *Genes & Cancer*, vol. 2, no. 12, pp. 1134–1138, 2011.

[34] T. A. Harris, M. Yamakuchi, M. Kondo, P. Oettgen, and C. J. Lowenstein, "Ets-1 and Ets-2 regulate the expression of MicroRNA-126 in endothelial cells," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 30, no. 10, pp. 1990–1997, 2010.

[35] A. Schober, M. Nazari-Jahantigh, Y. Wei et al., "MicroRNA-126-5p promotes endothelial proliferation and limits atherosclerosis by suppressing Dlk1," *Nature Medicine*, vol. 20, no. 4, pp. 368–376, 2014.

[36] Y. Wei, A. Schober, and C. Weber, "Pathogenic arterial remodeling: The good and bad of microRNAs," *The American Journal of Physiology—Heart and Circulatory Physiology*, vol. 304, no. 8, pp. H1050–H1059, 2013.

[37] J.-G. Wang, J.-S. Mu, H.-S. Zhu, and J.-G. Geng, "N-desulfated non-anticoagulant heparin inhibits leukocyte adhesion and transmigration in vitro and attenuates acute peritonitis and ischemia and reperfusion injury in vivo," *Inflammation Research*, vol. 51, no. 9, pp. 435–443, 2002.

[38] C. Van Solingen, L. Seghers, R. Bijkerk et al., "Antagomir-mediated silencing of endothelial cell specific microRNA-126 impairs ischemia-induced angiogenesis," *Journal of Cellular and Molecular Medicine*, vol. 13, no. 8, pp. 1577–1585, 2009.

[39] S. Brule, N. Charnaux, A. Sutton et al., "The shedding of syndecan-4 and syndecan-1 from HeLa cells and human primary macrophages is accelerated by SDF-1/CXCL12 and mediated by the matrix metalloproteinase-9," *Glycobiology*, vol. 16, no. 6, pp. 488–501, 2006.

[40] Z. Li, N. Li, M. Wu, X. Li, Z. Luo, and X. Wang, "Expression of miR-126 suppresses migration and invasion of colon cancer cells by targeting CXCR4," *Molecular and Cellular Biochemistry*, vol. 381, no. 1-2, pp. 233–242, 2013.

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 831641, 9 pages
http://dx.doi.org/10.1155/2015/831641

Review Article

MicroRNAs: Novel Players in Aortic Aneurysm

Xian-ming Fu,[1] Yang-zhao Zhou,[1] Zhao Cheng,[2] Xiao-bo Liao,[1] and Xin-min Zhou[1]

[1]*Department of Cardiothoracic Surgery, The Second Xiangya Hospital, Central South University, Changsha, Hunan 410011, China*
[2]*Department of Hematology, The Second Xiangya Hospital, Central South University, Changsha, Hunan 410011, China*

Correspondence should be addressed to Xiao-bo Liao; xiaoboliaoxiangya@gmail.com
and Xin-min Zhou; xinminzhouxiangya@hotmail.com

Received 14 August 2014; Revised 18 December 2014; Accepted 25 December 2014

Academic Editor: Ahmed Abdel-Latif

An aortic aneurysm (AA) is a common disease with potentially life-threatening complications. Despite significant improvements in the diagnosis and treatment of AA, the associated morbidity and mortality remain high. MicroRNAs (miRNAs, miR) are small noncoding ribonucleic acids that negatively regulate gene expression at the posttranscriptional level by inhibiting mRNA translation or promoting mRNA degradation. miRNAs are recently reported to be critical modulators for vascular cell functions such as cell migration, contraction, differentiation, proliferation, and apoptosis. Increasing evidences suggest crucial roles of miRNAs in the pathogenesis and progression of cardiovascular diseases such as coronary artery disease, heart failure, arterial hypertension, and cardiac arrhythmias. Recently, some miRNAs, such as miR-24, miR-155, miR-205, miR-712, miR-21, miR-26a, miR-143/145, miR-29, and miR-195, have been demonstrated to be differentially expressed in the diseased aortic tissues and strongly associated with the development of AA. In the present paper, we reviewed the recent available literature regarding the role of miRNAs in the pathogenesis of AA. Moreover, we discuss the potential use of miRNAs as diagnostic and prognostic biomarkers and novel targets for development of effective therapeutic strategies for AA.

1. Introduction

An aortic aneurysm (AA) is defined as a localized or diffuse dilation of aorta with a diameter at least 1.5 times greater than the expected normal size [1]. Risk factors for AA development include aging, cigarette smoking, hypertension, family history, male gender, aging, and atherosclerosis (AS) [2, 3]. Despite improvements over the years in the diagnostic and therapeutic techniques for AA, the associated morbidity and mortality remain high. The most fatal clinical consequence of AA is acute rupture, which leads to a mortality as high as 90% in 2009 [4]. Current available treatments, such as prosthetic replacement (open surgery) or strengthening (endoprosthesis) of the aorta, are associated with a high mortality rate and limited durability, respectively [5]. Until now, no nonsurgical (medical) treatments have been approved for prevention or limitation of AA in humans. Not only is a better understanding of the molecular mechanisms of AA formation essential for understanding the physiological processes of this disease, but it is also important for identifying new biomarkers and therapeutic targets.

The mechanisms underlying AA are incompletely understood. AA formation is thought to be a multifactorial and predominantly degenerative process that results from a complex interplay between biological processes in the arterial wall and the hemodynamic stimuli on the wall [5–9]. The pathology of AA is characterized by endothelial dysfunction, chronic inflammation, vascular smooth muscle cell (VSMC) phenotype switch (earlier) and apoptosis (later), and extracellular matrix (ECM) degradation. Some unknown inciting events result in aortic wall injury, whereby inflammatory cells are recruited into the aortic wall. The infiltrated inflammatory cells such as macrophages and lymphocytes secrete various inflammatory cytokines and chemokines such as interleukin- (IL-) 1β, IL-6, tumor necrosis factor- (TNF-) α, and monocyte chemoattractant protein-1 (MCP-1). These cytokines and chemokines induce activation of matrix metalloproteinases (MMPs), particularly MMP-2 and MMP-9, and apoptosis of VSMC, which contribute prominently to AAs development [5]. Moreover, the catalytic activities of MMPs are partly controlled by specific inhibitors named tissue inhibitors of

Table 1: MicroRNAs involved in vascular remodeling and aortic aneurysm (AA).

miRNA(s)	Cellular origin	Targets	Effects	References
miR-21	VSMC and EC	PTEN, SPRY1, PDCD4, and BCL2	Induces proliferation and decreases apoptosis of VSMC in AAA	[47, 49]
miR-24	VSMC and macrophage	CHI3L1	Inhibits vascular inflammation	[15]
miR-26a	VSMC	SMAD-1, SMAD-4	Promotes proliferation and inhibits differentiation, apoptosis of VSMC in AAA	[50]
miR-29	Fibroblast	COL1A1, COL1A2, COL3A1, FBN1, ELN	Downregulates ECM in AAA	[56–58, 69]
miR-126	EC	EGFL7	Inhibits vascular inflammation	[16, 18, 19]
miR-143/145	VSMC	Klf4, myocardin, Elk-1, and SRF	Promotes differentiation and represses proliferation of VSMC in AS and TAD	[41, 51, 52, 70]
miR-155	EC, BLC, TLC, macrophage, and DC	MAP3K10 CTLA4, SMAD2	Inhibits vascular inflammation in AS	[22, 23, 63]
miR-181b	EC	IPOA3	Inhibits vascular inflammation in AS	[24–26]
miR-195	VSMC	COL1A1, COL1A2, COL3A1, FBN1, ELN	Regulates MMPs in AAA	[59]
miR-223	Myeloid cell	CXCL2, CCL3, IL-6	Inhibits vascular inflammation in AAA	[21, 27–30]
miR-663	EC, VSMC	JunB, MLC9	Promotes differentiation and inhibits proliferation and migration of VSMC	[53, 71]
miR-712/miR-205	EC	TIMP3, RECK	Induces inflammation in AS and AAA	[31, 32, 72]

VSMC: vascular smooth muscle cell, EC: endothelial cell, BC: B lymphocytes, TC: T lymphocytes, DC: dendritic cells, AAA: abdominal aortic aneurysm, AS: atherosclerosis, TAD: thoracic aortic dissection, and ECM: extracellular matrix.

matrix metalloproteinases (TIMPs). TIMP1 binds to pro-MMP-9 forming a complex, while TIMP2 binds to pro-MMP-2. TIMPs were also found to play important roles in AA development [7].

MicroRNAs (miRNAs, miRs) are one class of small, conserved, single-stranded, noncoding RNA. They are approximately 18~25 nucleotides in length and can bind to complementary target sites in mRNA molecules, causing translation repression or the cleavage of the targets [10]. miRNAs have been reported to play important roles in a variety of pathophysiological processes [11–13]. Some recent studies have revealed that several miRNAs may be involved in vascular remodeling and pathogenesis of AA formation (Table 1). These findings clearly demonstrate the roles of miRNAs in the development of AA.

In the present paper, we reviewed the recent published literature about the role of miRNAs in the pathogenesis of AA. Moreover, we discussed their potential use as diagnostic and prognostic biomarkers and as novel targets for development of effective therapeutic strategies.

2. miRNAs in the Pathogenesis of AAs

2.1. miRNAs and Vascular Inflammation. Vascular inflammation contributes to the formation and development of AA [9]. Inflammatory reaction is characterized by infiltration of neutrophils, macrophages, T cells, and dendritic cells into the pathological aortic wall. Infiltrated inflammatory cells, particularly macrophages and lymphocytes, not only destruct directly ECM by releasing MMPs but also mediate activation of mesenchymal cells and apoptosis of VSMC, thus leading to progressive and pathological remodeling of aorta [14]. Recent studies have shown that miRNAs are involved in regulation of vascular inflammation and play crucial roles in AA development.

2.1.1. miR-24. The highly conserved miR-23b-24-27b cluster is involved in postinfarct cardiac angiogenesis, cardiomyocyte survival, and cancer. AA progression is associated with downregulation of the miR-23b-24-27b cluster in murine abdominal AA models, with miR-24 displaying the most

significant inverse regulation of its predicted targets in array profiling studies. Human abdominal AA also displays miR-24 downregulation, correlating inversely with aneurysm size. Maegdefessel group proved the function of miR-24 as a key regulator of vascular inflammation and abdominal AA pathology [15]. They revealed that chitinase 3-like 1 (CHI3L1) was a major target effector under the control of miR-24, regulating cytokine synthesis in macrophages as well as their survival, promoting aortic smooth muscle cell migration and cytokine production and stimulating adhesion molecule expression in vascular endothelial cells. Further, they showed that modulation of miR-24 alters abdominal AA progression in animal models and that miR-24 and CHI3L1 represent novel plasma biomarkers of abdominal AA disease progression in human.

2.1.2. miR-126. miR-126 is a human microRNA that is highly expressed in endothelial cells (ECs) [16]. It is located within the 7th intron of the EGF-like domain 7 (EGFL7) gene, involved in cell migration and blood vessel formation. miR-126 is regulated by the binding of two transcription factors: ETS-1 and ETS-2 [17]. Recent studies showed that miR-126 is involved in the vascular integrity and angiogenesis. Knockout of miR-126 in mice and zebrafish has decreased vascular integrity and impaired proliferation, migration, and angiogenic activity of ECs [18]. It also has been reported to be involved in vascular inflammation by modulating vascular cell adhesion molecule- (VCAM-) 1 expression, which hence inhibits leukocyte adhesion and inflammation [19]. Additionally, miR-126 in ECs regulates vascular remodeling by modulating the expression of stromal cell-derived factor-1 (SDF-1) [20]. Furthermore, downregulation of miR-126 in plasma and upregulation in abdominal aortic aneurysmal tissues have been observed and indicate the potential role of miR-126 in AA formation [21]. These evidences showed that miR-126 regulates vascular inflammation and is involved in vascular remodeling and aortic diseases.

2.1.3. miR-155. miR-155 is a typical multifunctional miRNA. It is expressed in a number of tissues and cell types and has been found to play a critical role in a wide variety of pathophysiological processes, including vascular inflammation. Chen and colleagues have reported that miR-155 was upregulated in primary murine macrophages and oxidized low density lipoprotein- (oxLDL-) stimulated monocytes, thus being involved in vascular inflammation [22]. In another study, Zhu and coworkers investigated the role of miR-155 in AS and found that miR-155 has increased in the aortic tissues of AS mice and in the plasma from patients with the coronary artery diseases [23]. They further showed that the miR-155 mimics have decreased secretion of IL-6 and TNF-α from oxLDL induced macrophages. On the contrary, the miR-155 inhibitor has promoted their secretions. Moreover, miR-155 has been shown to inhibit vascular inflammation and AS development by targeting mitogen-activated protein kinase 10 (MAP3K10). Taken together, all these evidences suggested that miR-155 represents an important modulator of vascular inflammation and may show important roles in inflammation associated vascular diseases, such as AS and AA.

2.1.4. miR-181b. miR-181b is one member of the miR-181 family and has recently been shown to play an important role in vascular inflammation. It modulates vascular inflammation by targeting importin-α3 (IPOA3), a protein required for nuclear translocation of NF-κB [24]. Sun and coworkers have showed that overexpression of miR-181b inhibited IPOA3 expression and downregulated NF-κB-responsive gene (VCAM-1 and E-selectin) expression in ECs [25]. In an endotoxemic mice model, miR-181b expression decreased with proinflammatory stimuli, while it was rescued with miR-181b mimics administration. Moreover, decreased lung injury and mortality of mice were observed with miR-181b mimics treatment, which has been reported to be associated with reduced NF-κB signaling and leukocyte influx in vascular endothelium. Furthermore, miR-181b expression in plasma was reduced in critically ill patients with sepsis. These findings indicated that miR-181b inhibits NF-κB mediated inflammation through reducing the expression of IPOA3. In a sequent study [26], the same research group further investigated the role of miR-181b in the development of AS. miR-181b expression was reduced in the aortic intima and plasma from ApoE$^{-/-}$ mice, as well as in the plasma from patients with coronary artery diseases. Systemic delivery of miR-181b resulted in overexpression of miR-181b and suppressed NF-κB signaling and AS lesion formation. Collectively, these results demonstrated that miR-181b could inhibit vascular inflammation and AS development through suppressing NF-κB activation. To date, there is no direct evidence about a role of miR-181b in the pathogenesis of AA; we believe that it may play a critical role because vascular inflammation is the central step of AA development.

2.1.5. miR-223. Hemodynamic stress triggers vascular remodeling and infiltration of inflammatory cells, especially macrophages, into the intracranial aneurysmal walls. miR-223 is a hematopoietic specific microRNA with crucial functions in myeloid lineage development. It has also been reported to be a novel regulator of inflammation [27–29], which suppresses proinflammatory pathways and enhances the anti-inflammatory response. Overexpression of miR-223 shows a protective role for vascular homeostasis and inflammation [30]. Moreover, miR-223 was shown to be unregulated in abdominal AA tissues and negatively correlated with MCP-1, TNF-α, and TGF-β expression in diseased aortic tissues [21].

2.1.6. miR-712 and miR-205. miR-712 is a murine specific atypical miRNA which derived from preribosomal RNA. miR-205 is thought to be potential human homolog of miR-712, which shares the same "seed sequence" and is highly conserved in most mammalian species including murine and human [31]. Recent studies have reported that microRNA-712 not only induces endothelial inflammation and AS but is also involved in AA formation. Son and colleagues have firstly identified that, by targeting 2 MMP inhibitors, tissue inhibitor of metalloproteinase 3 (TIMP3) and reversion-inducing cysteine-rich protein with Kazal motifs (RECK), miR-712 is an atypical mechanosensitive miRNA upregulated in ECs and

suppresses endothelial inflammation and AS [31]. Furthermore, treatment with specific antagonist of miR-712 inhibited endothelial inflammation and AS in a TIMP3-dependent manner. They also found that human miR-205, homolog of miR-712, targets TIMP3 in a flow-dependent manner. In a sequent study from the same institute, they investigated the role of miR-712 and miR-205 in AA development [32]. miR-712 and miR-205 were shown to be underregulated in the abdominal aortic endothelium during angiotensionII (AngII) -induced AA in ApoE$^{-/-}$ mice, associated with ECM degradation and AA development. Silencing of miR-712 and miR-205 by using anti-miR-712 and anti-miR-205 prevented AA development. Reduced aortic MMPs activity and vascular inflammation were observed. Moreover, upregulation of miR-205 expression was also identified in the human abdominal AA samples compared with the control. In summary, these results show that miR-712 and miR-205 stimulate MMPs activity and promote vascular inflammation by inhibiting TIMP3 and RECK, resulting in AA development. miR-712 and miR-205 may be potential modulators of AA.

2.2. miRNAs and VSMC Homeostasis. VSMC are the predominant cells in the media of aorta and essential in maintaining its structure and function through controlling proliferation and secretion and turnover of ECM. VSMC are plastic and can undergo reversible changes in their phenotypes in response to changing environmental cues. Two common phenotypic states of VSMC have been described: differentiated (also termed contractile) state and dedifferentiated (also termed synthetic) state. Differentiated phenotype is characterized by high levels of contractile gene expression and low rates of proliferation, migration, and ECM synthesis. Conversely, dedifferentiated phenotype has increased rates of proliferation, migration, and production of ECM, as well as reduced expression of contractile genes. In healthy vessels, VSMC can switch between states, but regulation of this switch is disrupted in vascular diseases and thought to contribute to the progression of diseases [33, 34]. Deregulation of phenotype switching and apoptosis of VSMC contribute to the development and progression of vascular pathologies like AA [35–38], and miRNAs are found to be critical modulators of VSMC function (phenotype) such as cell differentiation, contraction, migration, proliferation, calcification, and apoptosis [39–43]. Therefore, miRNAs are thought to be involved in AA formation. In several independent studies, miRNAs including miR-21, miR-26a, miR-126, miR-143/145, and miR-663 have been found to play crucial roles in AA development.

2.2.1. miR-21. miR-21 is the first miRNA demonstrated to be involved in regulation of VSMC phenotype. It is highly expressed in VSMC and ECs and targets phosphatase and tensin homolog (PTEN) [44, 45], programmed cell death 4 (PDCD4) [46], sprouty-1 (SPRY1) [47], and B cell lymphoma 2 (BCL2) [45]. Ji group [45] found that miR-21 promotes VSMC proliferation and inhibits apoptosis by downregulating PTEN and upregulating BCL2. Davis group [48] has showed that miR-21 also promotes differentiation of VSMC in response to transforming growth factor-β (TGF-β) and bone morphogenetic protein (BMP) stimulation via a decrease in PDCD4 expression. These studies indicate that miR-21 is important in the maintenance of VSMC phenotype. More recently, Maegdefessel and colleagues have investigated the role of miR-21 in AA development [49]. They identified that miR-21 expression increased during AA formation. Overexpression of miR-21 inhibited AA expansion, which is associated with decreased apoptosis and downregulation of PTEN in the aortic wall. In contrast, systemic injection of a locked nucleic acid- (LNA-) modified antagomir targeting miR-21 led to a marked increase in the size of AA. Similar results were found in mice with AA augmented by nicotine and in patients with AA. Taken together, these data suggest that miR-21 is a key regulator of VSMC proliferation and apoptosis during AA development, and modulation of miR-21 expression may be a potential strategy to prevent AA formation.

2.2.2. miR-26a. miR-26a is an important regulator of VSMC phenotype. miR-26a has been proved to inhibit VSMC differentiation and apoptosis and promote proliferation and migration through a mechanism that targets the TGFβ/BMP pathway. Leeper and colleagues have performed a microarray-based study during the process of human aortic VSMC differentiation *in vitro* [50]. They identified that miR-26a is the highest-ranked significant differential expression of miRNA. VSMC differentiation was promoted by underexpression of miR-26a and inhibited by overexpression of miR-26a. In order to elucidate the mechanism that miR-26a modulates VSMC phenotype, the effects of miR-26a on the expression of the prodifferentiation TGF-β/BMP cascade molecules were assessed. Inhibition increased TGF-β superfamily signaling cascade gene expression including SMAD-1 and SMAD-4, while overexpression of miRNA-26a inhibited SMAD-1 expression. Furthermore, the expression of miR-26a in two murine AA models was evaluated and was found progressively downregulated during AA development. These results suggest that miR-26a may serve as an important regulator of VSMC biology and a potential therapeutic target in AA.

2.2.3. miR-143/145. miR-143/145 cluster which is highly expressed in VSMC is the most studied miRNA cluster. miR-143 and miR-145 encoding genes are highly conserved and lie in close proximity with each other on murine chromosome 18 and human chromosome 5 [41, 42]. They have been shown to play crucial roles in regulating VSMC phenotypic switching and pathogenesis of vascular diseases. They modulate VSMC function through targeting several transcription factors, including Klf4, myocardin, and Elk-1. Two pioneering studies by Cheng group and Cordes group, respectively, investigated the role of miR-143/145 in determining VSMC phenotype *in vitro*. Cheng and colleagues have reported that overexpression of miR-145 promotes VSMC differentiation while miR-145 inhibitor represses differentiation [51]. In addition to regulating VSMC differentiation, miR-145 alone was reported to be able to maintain the differentiated spindle-like shape of VSMC and inhibit proliferation. The Cordes group [41] have further verified the regulatory effects of miR-145 on VSMC phenotype. The roles of miR-143/145 on VSMC phenotype have also been verified and validated *in vivo*.

Recent studies have revealed the transition of VSMC from contractile phenotype to synthetic one in the media of thoracic aortic dissection (TAD) aorta [35]. Liao group [52] have reported that miR-143/145 were underexpressed in TAD, which may account for VSMC underdifferentiated in TAD and contributes to the aortic remodeling. In addition, a negative correlation between the expression of miR-143/145 and the dedifferentiation of VSMC has been observed. These results indicate that miR-143 and miR-145 are critical modulators of VSMC function and may play important roles in AA development.

2.2.4. miR-663. miR-663 is highly expressed in ECs and VSMC. It is recently recognized as an important regulator of VSMC function. Li group have investigated the role of miR-663 in human VSMC phenotypic switch and the development of neointima formation [53]. They found that the expression of miR-663 decreased in human aortic VSMC with platelet-derived growth factor treatment but increased during VSMC differentiation. Furthermore, overexpression of miR-663 promotes VSMC differentiation and potently inhibits VSMC proliferation and migration, which are associated with downregulation of JunB and its downstream molecules, such as myosin light chain 9 (MLC9) and MMP-9. In addition, adeno-miR-663 suppressed the neointimal lesion formation after vascular injury via decreased JunB expression. Collectively, miR-663 is an important modulator of human VSMC phenotypic switch by targeting JunB/MLC9 expression and may represent an attractive approach for the treatment of AA.

2.3. miRNAs and Extracellular Matrix Remodeling. Aortic wall is comprised of layers of VSMC and an arrangement of ECM structural proteins, primarily collagen and elastin. AA is characterized by degradation of ECM, but the mechanisms underlying this process are incompletely understood. It has been established that TGF-β signaling plays key role in ECM remodeling and is involved in AA formation. TGF-β1 regulates the expression of certain miRNAs. In particularly, miR-29 and miR15 family have been increasingly noted to be associated with TGF-β signaling and ECM remodeling and AA development.

2.3.1. miR-29. The miR-29 family including miR-29a, miR-29b, and miR-29c are enriched in fibroblasts and encoded by two separate loci. miR-29 family directly target at least 16 ECM genes such as collagen isoforms (COL1A1, COL1A2, and COL3A1), fibrillin-1 (FBN1), and elastin (ELN) and are involved in ECM remodeling in several organs [54, 55]. Moreover, it was recently indicated that miR-29 plays a pivotal role in the formation of aneurysm. Boon and colleagues [56] firstly discovered that the expression of miR-29 family increased in the aortic tissues of aged mice (18 months old) compared with young mice (6 weeks old), which is associated with a significant downregulation of ECM in aged mouse aortas. Furthermore, they found that systemic LNA-modified anti-miR-29 treatment decreased aortic dilatation in aged AngII treated mice, and this process is associated with increased expression of COL1A1, COL3A1, and ELN proteins. They also investigated miR-29 expression in human tissues from TAA patients and found that only miR-29b was upregulated among miR-29 family. In two murine AAA models (PPE and AngII infusion), Maegdefessel and colleagues [57] reported that miR-29b was the only member of miR-29 family decreased in aortic tissues during murine AAAs development. Anti-miR-29b treatment not only increased the expression of genes including Col1a1, Col2a1, Col3a1, Col5a1, and Eln, which encode type I, III, and V collagen and elastin, but also downregulated MMP-2 and MMP-9 expression. Accordingly, limited aneurysm expansion was observed with anti-miR-29b treatment. In contrast, overexpression of miR-29b led to rapid AAA expansion and increased aortic rupture rate. Merk and colleagues [58] further elucidated the role of miR-29b in early AAs development in murine model of Marfan syndrome (MFS). They found that the expression of miR-29b increased in ascending TAA of MFS mice, accompanied with increased apoptosis and MMP-2 activity as well as decreased expression of antiapoptotic proteins (Mcl-1 and Bcl-2) and elastin. Furthermore, an LNA-anti-miR-29b treatment limited AA development, aortic wall apoptosis, and ECM degradation. Taken together, these results provide important new insights into the mechanisms of AA formation and potentially allow for the development of new therapies.

2.3.2. miR-195. Besides miR-29, Zampetaki group reported the miR-15 family to be the regulator of the collagen remodeling and the characteristic postnatal silencing of elastin [59]. Among the miR-15 family, miR-195 was proved to be differentially expressed in aortas of $ApoE^{-/-}$ mice upon AngII infusion. Furthermore, the expression of miR-195 was altered in human aortic specimen with evidence of dissection. Direct binding of miR-195 to several ECM transcripts was detected in H4 cancer cells. Proteomic analysis of the secretome of murine aortic VSMC revealed that miR-195 targets a group of ECM proteins, including collagens, proteoglycans, elastin, and proteins associated with elastic microfibrils. In mice treated with antagomiR-195, higher aortic elastin expression was associated with an increase of MMP-2 and MMP-9. In human plasma, an inverse correlation of miR-195 was observed with the presence of abdominal AA and aortic diameter. Based on the evidences mentioned above, the miR-195 functioning as a potent regulator of the aortic ECM may contribute to the pathogenesis of AA disease. In addition, the plasma levels of miR-195 reduced in patients with AA suggested that it may serve as a noninvasive biomarker of AA.

3. Clinical Applications of MicroRNA in AA

3.1. The Role of miRNAs in the Diagnosis and Prognosis of AA. Not only are miRNAs tissue- and cell-specific but they also show different expression patterns. One miRNA may be highly expressed in one kind of cell or one tissue but has no or low expression in another kind of cells or tissues. Moreover, miRNAs are remarkably stable in the extracellular milieu, and they are detectable in blood and other body fluids. Circulating miRNAs have been demonstrated to share many of the essential characteristics of a good biomarker such as high degree of sensitivity and specificity, allowing

Table 2: Use of some miRNAs in the treatment of aortic aneurysm (AA).

miRNA	Model	Target	Anti-miR/mimics	Effects	Year/author
miR-21	Mice AngII-AAA PPE-AAA	PTEN	Mimics	Induces VSMC proliferation, decreases apoptosis via PTEN/PI3K/AKT, and prevents AA	2012 Maegdefessel et al. [49]
miR-24	Mice AngII-AAA PPE-AAA	CHI3L1	Mimics	Limits aortic vascular inflammation and AA formation	2014 Maegdefessel et al. [15]
miR-29	Aged mice AngII-AAA	Col1A1, Col3A1, elastin, Mcl-1	Antagomir, LNA-anti-miR	Induces ECM expression and inhibits AA formation	2011 Boon et al. [56]
miR-29b	Mice AngII-AAA PPE-AAA	Col1A1, Col3A1, elastin, Mcl-1	Antagomir, LNA-anti-miR	Increases collagen expression, leading to an early fibrotic response in the abdominal aortic wall, and prevents AAA progression	2012 Maegdefessel et al. [57]
miR-29b	Marfan mice $Fbn1^{C1039G/+}$ AA	Col1A1, Col3A1, elastin, Mcl-1	Antagomir, LNA-anti-miR	Reduces ECM deposition and VSMC apoptosis and prevents AA formation	2012 Merk et al. [58]
miR-195	Mice AngII-AAA	Col1A1, Col3A1, elastin, Mcl-1	Antagomir, LNA-anti-miR	Reduces ECM deposition and VSMC apoptosis and prevents AA formation	2014 Zampetaki et al. [59]
miR-712/miR-205	Mice AngII-AAA	TIMP3, RECK	Antagomir, LNA-anti-miR	Inhibits both endothelial and circulating leukocyte inflammation Decreases MMPs activity and inflammation Prevents AAA formation	2014 Kim et al. [32]

AngII: angiotensin II, PPE: porcine pancreatic elastase, AAA: abdominal aortic aneurysm, LNA: alpha-linolenic acid, VSMC: vascular smooth muscle cell, ECM: extracellular matrix, and MMPs: matrix metalloproteinases.

early detection of pathological states; time-related changes during the course of disease; and a long half-life within the sample, as well as rapid and cost-effective laboratory detection. Accordingly, they have been shown to have roles in the diagnosis and prognosis of cardiovascular disease as biomarker. For example, miR-1, miR-133a, miR-499, and miR-208a have been reported to be upregulated in plasma of patients with acute MI, as a result of cardiomyocyte necrosis and massive release into the bloodstream [60]. The time dependent release of acute MI-related miRNAs has also been investigated: miR-1, miR-133a, and miR-208a increased continuously during the first 4 hours after the induction of MI, before conventional biomarkers of acute MI could be detected [61]. Furthermore, an inverse correlation of miR-195 in human plasma was observed with the presence of abdominal AA and aortic diameter. The plasma levels of miR-195 reduced in patients with abdominal AA suggested that it may serve as a noninvasive biomarker of abdominal AA [59]. These results indicate that circulating miRNAs could be used as biomarkers for diagnosis of MI in humans. More recently, the prognostic value of circulating miRNAs has been evaluated. miR-223 and miR-197 have been shown to have negative association, while miR-126 has a positive association with subsequent acute MI in a prospective study on a total of 19 miRNAs [62]. However, the potential role of miRNAs as biomarkers for the diagnosis and prognosis of aortic diseases has not yet been thoroughly evaluated. Several recent studies have identified differentially expressed miRNAs in aortic tissues as well as in plasma in aortic diseases [21, 63–65]. These miRNAs may not only contribute to the pathogenesis of aortic aneurysm or dissection but also provide biomarker potential. Further studies are necessary to explore the diagnostic and prognostic value of miRs as biomarkers in AA disease.

3.2. The Role of miRNAs in the Treatment of AA. As mentioned above, tissue- and cell-specific expression is one important characteristic of miRNA expression. Recent works have identified that changes in miRNA expression may contribute to the pathogenesis of AA. It is well established that multiple miRNAs are aberrantly expressed in diseased tissues. Several miRNAs-based treatments for AA have been proposed and reported in animal experiments according to the miRNA-based therapeutic strategies: restoring the expression of miRNAs reduced in diseases by miR-mimics or inhibiting overexpressed miRNAs by antagomirs (Table 2). The antagomirs (or anti-miRs) are small single-stranded antagonistic nucleotide sequences artificially synthesized to be perfectly complementary to a specific mature miRNA. When injected systemically or locally, antagomirs interact with miRNAs in the cytoplasm and hybridize specifically with the mature miRNA target hindering the binding of miRNA

with their corresponding mRNA. Thus, antagomirs act as competitive inhibitors of miRNA and lead to a decrease in the effect caused by the excessive increase in the expression of certain miRNAs [66]. On the other hand, the miR-mimics are artificial small nucleotide sequences, double-stranded, similar to miRNA precursors (pre-miRNA). When introduced into the cells, the miR-mimics are recognized by the miRNA biogenesis machinery and processed by the enzyme dicer and subsequently incorporated into the RISC enzyme complex. Thus, the miR-mimics will function as a replacement of some miRNAs downregulated by setting the mRNA-target as endogenous miRNAs [67]. Additionally, there exist some other methods which can also inhibit miRNA expression and consequentially therapeutically interfere with the disease process such as masking, sponges, and erasers [68]. It should be noted that there are some challenges and risks for miRNA-based therapy like safety, delivery, and selectivity [68].

4. Summary

Our understanding of the molecular pathophysiology of AA is improving very rapidly but is still limited. miRNAs in AA have emerged as a new research area. The initial exciting results have demonstrated that multiple miRNAs are involved in the development of both human and animal AA diseases via regulating vascular cells differentiation, contraction, migration, proliferation, and apoptosis through their target genes. Additional studies with more patients and more animal models will be needed to determine the precise roles of miRNAs in AA. We believe that, on the basis of thorough understanding, miRNAs may represent novel promising biomarkers and new therapeutic targets for AA diseases.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgment

This work was supported by Grants 81370973, 81470507, 81272584, and 81400343 from the China National Natural Scientific Foundation.

References

[1] JCS Joint Working Group, "Guidelines for diagnosis and treatment of aortic aneurysm and aortic dissection (JCS 2011): digest version," *Circulation Journal*, vol. 77, no. 3, pp. 789–828, 2013.

[2] K. Singh, K. H. Bønaa, B. K. Jacobsen, L. Bjørk, and S. Solberg, "Prevalence of and risk factors for abdominal aortic aneurysms in a population-based study: the Tromsø study," *American Journal of Epidemiology*, vol. 154, no. 3, pp. 236–244, 2001.

[3] A. Wills, M. M. Thompson, M. Crowther, R. D. Sayers, and P. R. F. Bell, "Pathogenesis of abdominal aortic aneurysms—cellular and biochemical mechanisms," *European Journal of Vascular and Endovascular Surgery*, vol. 12, no. 4, pp. 391–400, 1996.

[4] K. D. Kochanek, J. Xu, S. L. Murphy, A. M. Minino, and H. C. Kung, "Deaths: preliminary data for 2009," *National Vital Statistics Reports*, vol. 59, no. 4, pp. 1–51, 2009.

[5] J. Golledge, J. Muller, A. Daugherty, and P. Norman, "Abdominal aortic aneurysm: pathogenesis and implications for management," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 26, no. 12, pp. 2605–2613, 2006.

[6] H. Lu, D. L. Rateri, D. Bruemmer, L. A. Cassis, and A. Daugherty, "Novel mechanisms of abdominal aortic aneurysms," *Current Atherosclerosis Reports*, vol. 14, no. 5, pp. 402–412, 2012.

[7] P. E. Norman, T. M. E. Davis, M. T. Q. Le, and J. Golledge, "Matrix biology of abdominal aortic aneurysms in diabetes: mechanisms underlying the negative association," *Connective Tissue Research*, vol. 48, no. 3, pp. 125–131, 2007.

[8] R. W. Thompson, P. J. Geraghty, and J. K. Lee, "Abdominal aortic aneurysms: basic mechanisms and clinical implications," *Current Problems in Surgery*, vol. 39, no. 2, pp. 110–230, 2002.

[9] D.-C. Guo, C. L. Papke, R. He, and D. M. Milewicz, "Pathogenesis of thoracic and abdominal aortic aneurysms," *Annals of the New York Academy of Sciences*, vol. 1085, pp. 339–352, 2006.

[10] T. Li and W. C. S. Cho, "MicroRNAs: mechanisms, functions and progress," *Genomics, Proteomics and Bioinformatics*, vol. 10, no. 5, pp. 237–238, 2012.

[11] A. Carè, D. Catalucci, F. Felicetti et al., "MicroRNA-133 controls cardiac hypertrophy," *Nature Medicine*, vol. 13, no. 5, pp. 613–618, 2007.

[12] T. Thum, P. Galuppo, C. Wolf et al., "MicroRNAs in the human heart: a clue to fetal gene reprogramming in heart failure," *Circulation*, vol. 116, no. 3, pp. 258–267, 2007.

[13] E. van Rooij, L. B. Sutherland, N. Liu et al., "A signature pattern of stress-responsive microRNAs that can evoke cardiac hypertrophy and heart failure," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 48, pp. 18255–18260, 2006.

[14] W. H. Pearce and A. E. Koch, "Cellular components and features of immune response in abdominal aortic aneurysms," *Annals of the New York Academy of Sciences*, vol. 800, pp. 175–185, 1996.

[15] L. Maegdefessel, J. M. Spin, U. Raaz et al., "miR-24 limits aortic vascular inflammation and murine abdominal aneurysm development," *Nature Communications*, vol. 5, article 5214, 2014.

[16] S. Wang, A. B. Aurora, B. A. Johnson et al., "The endothelial-specific microRNA miR-126 governs vascular integrity and angiogenesis," *Developmental Cell*, vol. 15, no. 2, pp. 261–271, 2008.

[17] T. A. Harris, M. Yamakuchi, M. Kondo, P. Oettgen, and C. J. Lowenstein, "Ets-1 and Ets-2 regulate the expression of MicroRNA-126 in endothelial cells," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 30, no. 10, pp. 1990–1997, 2010.

[18] J. E. Fish, M. M. Santoro, S. U. Morton et al., "miR-126 regulates angiogenic signaling and vascular integrity," *Developmental Cell*, vol. 15, no. 2, pp. 272–284, 2008.

[19] T. A. Harris, M. Yamakuchi, M. Ferlito, J. T. Mendell, and C. J. Lowenstein, "MicroRNA-126 regulates endothelial expression of vascular cell adhesion molecule 1," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 5, pp. 1516–1521, 2008.

[20] C. van Solingen, H. C. de Boer, R. Bijkerk et al., "MicroRNA-126 modulates endothelial SDF-1 expression and mobilization of Sca-1^+/Lin^- progenitor cells in ischaemia," *Cardiovascular Research*, vol. 92, no. 3, pp. 449–455, 2011.

[21] K. Kin, S. Miyagawa, S. Fukushima et al., "Tissue- and plasma-specific MicroRNA signatures for atherosclerotic abdominal aortic aneurysm," *Journal of the American Heart Association*, vol. 1, no. 5, Article ID e000745, 2012.

[22] T. Chen, Z. Huang, L. Wang et al., "MicroRNA-125a-5p partly regulates the inflammatory response, lipid uptake, and ORP9 expression in oxLDL-stimulated monocyte/macrophages," *Cardiovascular Research*, vol. 83, no. 1, pp. 131–139, 2009.

[23] J. Zhu, T. Chen, L. Yang et al., "Regulation of microRNA-155 in atherosclerotic inflammatory responses by targeting MAP3K10," *PLoS ONE*, vol. 7, no. 11, Article ID e46551, 2012.

[24] X. Sun, A. Sit, and M. W. Feinberg, "Role of miR-181 family in regulating vascular inflammation and immunity," *Trends in Cardiovascular Medicine*, vol. 24, no. 3, pp. 105–112, 2014.

[25] X. Sun, B. Icli, A. K. Wara et al., "MicroRNA-181b regulates NF-κB-mediated vascular inflammation," *The Journal of Clinical Investigation*, vol. 122, no. 6, pp. 1973–1990, 2012.

[26] X. Sun, S. He, A. K. M. Wara et al., "Systemic delivery of MicroRNA-181b inhibits nuclear factor-kappaB activation, vascular inflammation, and atherosclerosis in apolipoprotein E-deficient mice," *Circulation Research*, vol. 114, no. 1, pp. 32–40, 2014.

[27] J. B. Johnnidis, M. H. Harris, R. T. Wheeler et al., "Regulation of progenitor cell proliferation and granulocyte function by microRNA-223," *Nature*, vol. 451, no. 7182, pp. 1125–1129, 2008.

[28] G. Zhuang, C. Meng, X. Guo et al., "A novel regulator of macrophage activation: miR-223 in obesity-associated adipose tissue inflammation," *Circulation*, vol. 125, no. 23, pp. 2892–2903, 2012.

[29] H.-J. Lee, J.-S. Yi, H.-J. Lee, I.-W. Lee, K.-C. Park, and J.-H. Yang, "Dysregulated expression profiles of microRNAs of experimentally induced cerebral aneurysms in rats," *Journal of Korean Neurosurgical Society*, vol. 53, no. 2, pp. 72–76, 2013.

[30] Y. Kanematsu, M. Kanematsu, C. Kurihara et al., "Critical roles of macrophages in the formation of intracranial aneurysm," *Stroke*, vol. 42, no. 1, pp. 173–178, 2011.

[31] D. J. Son, S. Kumar, W. Takabe et al., "The atypical mechanosensitive microRNA-712 derived from pre-ribosomal RNA induces endothelial inflammation and atherosclerosis," *Nature Communications*, vol. 4, article 3000, 2013.

[32] C. W. Kim, S. Kumar, D. J. Son, I. H. Jang, K. K. Griendling, and H. Jo, "Prevention of abdominal aortic aneurysm by anti-microrna-712 or anti-microRNA-205 in angiotensin II-infused mice," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 34, no. 7, pp. 1412–1421, 2014.

[33] S. S. M. Rensen, P. A. F. M. Doevendans, and G. J. J. M. Van Eys, "Regulation and characteristics of vascular smooth muscle cell phenotypic diversity," *Netherlands Heart Journal*, vol. 15, no. 3, pp. 100–108, 2007.

[34] G. K. Owens, M. S. Kumar, and B. R. Wamhoff, "Molecular regulation of vascular smooth muscle cell differentiation in development and disease," *Physiological Reviews*, vol. 84, no. 3, pp. 767–801, 2004.

[35] V. Lesauskaite, P. Tanganelli, C. Sassi et al., "Smooth muscle cells of the media in the dilatative pathology of ascending thoracic aorta: morphology, immunoreactivity for osteopontin, matrix metalloproteinases, and their inhibitors," *Human Pathology*, vol. 32, no. 9, pp. 1003–1011, 2001.

[36] E. L. Henderson, Y.-J. Geng, G. K. Sukhova, A. D. Whittemore, J. Knox, and P. Libby, "Death of smooth muscle cells and expression of mediators of apoptosis by T lymphocytes in human abdominal aortic aneurysms," *Circulation*, vol. 99, no. 1, pp. 96–104, 1999.

[37] K. Riches, T. G. Angelini, G. S. Mudhar et al., "Exploring smooth muscle phenotype and function in a bioreactor model of abdominal aortic aneurysm," *Journal of Translational Medicine*, vol. 11, no. 1, article 208, 2013.

[38] G. Ailawadi, C. W. Moehle, H. Pei et al., "Smooth muscle phenotypic modulation is an early event in aortic aneurysms," *Journal of Thoracic and Cardiovascular Surgery*, vol. 138, no. 6, pp. 1392–1399, 2009.

[39] X.-B. Liao, Z.-Y. Zhang, K. Yuan et al., "MiR-133a modulates osteogenic differentiation of vascular smooth muscle cells," *Endocrinology*, vol. 154, no. 9, pp. 3344–3352, 2013.

[40] R.-R. Cui, S.-J. Li, L.-J. Liu et al., "MicroRNA-204 regulates vascular smooth muscle cell calcification in vitro and in vivo," *Cardiovascular Research*, vol. 96, no. 2, pp. 320–329, 2012.

[41] K. R. Cordes, N. T. Sheehy, M. P. White et al., "miR-145 and miR-143 regulate smooth muscle cell fate and plasticity," *Nature*, vol. 460, no. 7256, pp. 705–710, 2009.

[42] T. Boettger, N. Beetz, S. Kostin et al., "Acquisition of the contractile phenotype by murine arterial smooth muscle cells depends on the *Mir143/145* gene cluster," *The Journal of Clinical Investigation*, vol. 119, no. 9, pp. 2634–2647, 2009.

[43] B. N. Davis-Dusenbery, C. Wu, and A. Hata, "Micromanaging vascular smooth muscle cell differentiation and phenotypic modulation," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 31, no. 11, pp. 2370–2377, 2011.

[44] S. Roy, S. Khanna, S.-R. A. Hussain et al., "MicroRNA expression in response to murine myocardial infarction: MiR-21 regulates fibroblast metalloprotease-2 via phosphatase and tensin homologue," *Cardiovascular Research*, vol. 82, no. 1, pp. 21–29, 2009.

[45] R. Ji, Y. Cheng, J. Yue et al., "MicroRNA expression signature and antisense-mediated depletion reveal an essential role of MicroRNA in vascular neointimal lesion formation," *Circulation Research*, vol. 100, no. 11, pp. 1579–1588, 2007.

[46] X. Liu, Y. Cheng, J. Yang, T. J. Krall, Y. Huo, and C. Zhang, "An essential role of PDCD4 in vascular smooth muscle cell apoptosis and proliferation: implications for vascular disease," *American Journal of Physiology—Cell Physiology*, vol. 298, no. 6, pp. C1481–C1488, 2010.

[47] T. Thum, C. Gross, J. Fiedler et al., "MicroRNA-21 contributes to myocardial disease by stimulating MAP kinase signalling in fibroblasts," *Nature*, vol. 456, no. 7224, pp. 980–984, 2008.

[48] B. N. Davis, A. C. Hilyard, G. Lagna, and A. Hata, "SMAD proteins control DROSHA-mediated microRNA maturation," *Nature*, vol. 454, no. 7200, pp. 56–61, 2008.

[49] L. Maegdefessel, J. Azuma, R. Toh et al., "MicroRNA-21 blocks abdominal aortic aneurysm development and nicotine-augmented expansion," *Science Translational Medicine*, vol. 4, no. 122, Article ID 122ra22, 2012.

[50] N. J. Leeper, A. Raiesdana, Y. Kojima et al., "MicroRNA-26a is a novel regulator of vascular smooth muscle cell function," *Journal of Cellular Physiology*, vol. 226, no. 4, pp. 1035–1043, 2011.

[51] Y. Cheng, X. Liu, J. Yang et al., "MicroRNA-145, a novel smooth muscle cell phenotypic marker and modulator, controls vascular neointimal lesion formation," *Circulation Research*, vol. 105, no. 2, pp. 158–166, 2009.

[52] M. Liao, S. Zou, J. Weng et al., "A microRNA profile comparison between thoracic aortic dissection and normal thoracic aorta indicates the potential role of microRNAs in contributing to thoracic aortic dissection pathogenesis," *Journal of Vascular Surgery*, vol. 53, no. 5, pp. 1341.e3–1349.e3, 2011.

[53] P. Li, N. Zhu, B. Yi et al., "MicroRNA-663 regulates human vascular smooth muscle cell phenotypic switch and vascular neointimal formation," *Circulation Research*, vol. 113, no. 10, pp. 1117–1127, 2013.

[54] E. van Rooij, L. B. Sutherland, J. E. Thatcher et al., "Dysregulation of microRNAs after myocardial infarction reveals a role of miR-29 in cardiac fibrosis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 35, pp. 13027–13032, 2008.

[55] C. E. Ott, J. Grünhagen, M. Jäger et al., "MicroRNAs differentially expressed in postnatal aortic development downregulate elastin via 39′ UTR and coding-sequence binding sites," *PLoS ONE*, vol. 6, no. 1, Article ID e16250, 2011.

[56] R. A. Boon, T. Seeger, S. Heydt et al., "MicroRNA-29 in aortic dilation: implications for aneurysm formation," *Circulation Research*, vol. 109, no. 10, pp. 1115–1119, 2011.

[57] L. Maegdefessel, J. Azuma, R. Toh et al., "Inhibition of microRNA-29b reduces murine abdominal aortic aneurysm development," *The Journal of Clinical Investigation*, vol. 122, no. 2, pp. 497–506, 2012.

[58] D. R. Merk, J. T. Chin, B. A. Dake et al., "MiR-29b participates in early aneurysm development in Marfan syndrome," *Circulation Research*, vol. 110, no. 2, pp. 312–324, 2012.

[59] A. Zampetaki, R. Attia, U. Mayr et al., "Role of miR-195 in aortic aneurysmal disease," *Circulation Research*, vol. 115, no. 10, pp. 857–866, 2014.

[60] G. Condorelli, M. V. G. Latronico, and E. Cavarretta, "MicroRNAs in cardiovascular diseases: current knowledge and the road ahead," *Journal of the American College of Cardiology*, vol. 63, no. 21, pp. 2177–2187, 2014.

[61] C. Liebetrau, H. Möllmann, O. Dörr et al., "Release kinetics of circulating muscle-enriched microRNAs in patients undergoing transcoronary ablation of septal hypertrophy," *Journal of the American College of Cardiology*, vol. 62, no. 11, pp. 992–998, 2013.

[62] A. Zampetaki, P. Willeit, L. Tilling et al., "Prospective study on circulating microRNAs and risk of myocardial infarction," *Journal of the American College of Cardiology*, vol. 60, no. 4, pp. 290–299, 2012.

[63] E. Biros, C. S. Moran, Y. Wang, P. J. Walker, J. Cardinal, and J. Golledge, "microRNA profiling in patients with abdominal aortic aneurysms: the significance of miR-155," *Clinical Science*, vol. 126, no. 11, pp. 795–803, 2014.

[64] G. Liu, Y. Huang, X. Lu et al., "Identification and characteristics of microRNAs with altered expression patterns in a rat model of abdominal aortic aneurysms," *Tohoku Journal of Experimental Medicine*, vol. 222, no. 3, pp. 187–193, 2010.

[65] M. C. Pahl, K. Derr, G. Gäbel et al., "MicroRNA expression signature in human abdominal aortic aneurysms," *BMC Medical Genomics*, vol. 5, article 25, 2012.

[66] J. Krützfeldt, N. Rajewsky, R. Braich et al., "Silencing of microRNAs in vivo with 'antagomirs'," *Nature*, vol. 438, no. 7068, pp. 685–689, 2005.

[67] E. van Rooij, W. S. Marshall, and E. N. Olson, "Toward microRNA-based therapeutics for heart disease: the sense in antisense," *Circulation Research*, vol. 103, no. 9, pp. 919–928, 2008.

[68] A. Caroli, M. T. Cardillo, R. Galea, and L. M. Biasucci, "Potential therapeutic role of microRNAs in ischemic heart disease," *Journal of Cardiology*, vol. 61, no. 5, pp. 315–320, 2013.

[69] L. Maegdefessel, J. Azuma, and P. S. Tsao, "MicroRNA-29b regulation of abdominal aortic aneurysm development," *Trends in Cardiovascular Medicine*, vol. 24, no. 1, pp. 1–6, 2014.

[70] L. Elia, M. Quintavalle, J. Zhang et al., "The knockout of miR-143 and -145 alters smooth muscle cell maintenance and vascular homeostasis in mice: correlates with human disease," *Cell Death and Differentiation*, vol. 16, no. 12, pp. 1590–1598, 2009.

[71] C.-W. Ni, H. Qiu, and H. Jo, "MicroRNA-663 upregulated by oscillatory shear stress plays a role in inflammatory response of endothelial cells," *American Journal of Physiology: Heart and Circulatory Physiology*, vol. 300, no. 5, pp. H1762–H1769, 2011.

[72] L. Maegdefessel, J. M. Spin, and P. S. Tsao, "New ways to dismantle a ticking time bomb: microRNA 712/205 and abdominal aortic aneurysm development," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 34, no. 7, pp. 1339–1340, 2014.

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 981841, 8 pages
http://dx.doi.org/10.1155/2015/981841

Review Article

The Emerging Role of miR-223 in Platelet Reactivity: Implications in Antiplatelet Therapy

Rui Shi, Xin Zhou, Wen-Jie Ji, Ying-Ying Zhang, Yong-Qiang Ma, Jian-Qi Zhang, and Yu-Ming Li

Tianjin Key Laboratory of Cardiovascular Remodeling and Target Organ Injury, Institute of Cardiovascular Disease and Heart Center, Pingjin Hospital, Logistics University of the Chinese People's Armed Police Forces, 220 Cheng-Lin Road, Tianjin 300162, China

Correspondence should be addressed to Yu-Ming Li; cardiolab@live.com

Received 12 August 2014; Revised 26 November 2014; Accepted 1 December 2014

Academic Editor: Vinicio A. de Jesus Perez

Platelets are anuclear cells and are devoid of genomic DNA, but they are capable of *de novo* protein synthesis from mRNA derived from their progenitor cells, megakaryocytes. There is mounting evidence that microRNA (miRNA) plays an important role in regulating gene expression in platelets. miR-223 is the most abundant miRNAs in megakaryocytes and platelets. One of the miR-223-regulated genes is ADP P2Y12, a key target for current antiplatelet drug therapy. Recent studies showed that a blunted response to P2Y12 antagonist, that is, high on-treatment platelet reactivity (HTPR), is a strong predictor of major cardiovascular events (MACEs) in coronary heart disease (CHD) patients receiving antiplatelet treatment. Recent clinical cohort study showed that the level of circulating miR-223 is inversely associated with MACE in CHD patients. In addition, our recent data demonstrated that the level of both intraplatelet and circulating miR-223 is an independent predictor for HTPR, thus providing a link between miR-223 and MACE. These lines of evidence indicate that miR-223 may serve as a potential regulatory target for HTPR, as well as a diagnostic tool for identification of HTPR in clinical settings.

1. Introduction

MicroRNAs were first identified in *C. elegans* in 1993 by the laboratories of Victor Ambros and Gary Ruvkin [1]. They are small noncoding RNAs that regulate gene expression from viruses to humans [2]. Approximately 30% of miRNAs genes are located in intergenic regions, and approximately 70% are located within introns or exons of protein coding genes [3, 4]. It has been estimated that miRNAs regulate between one-third and two-thirds of the human genome and are involved in most of the cellular functions [5, 6]. So far, more than 500 human miRNAs have been identified, and roughly 1000 are estimated to exist [7, 8]. Some miRNAs are expressed ubiquitously, but many are tissue and/or developmental stage specific [9]. Many studies have demonstrated that miRNAs expression is associated with disease (for different types of cancer, inflammatory disease, autoimmune disease, and cardiovascular disease) [10–14]. miR-223 has a key role mainly in the development and homeostasis of the immune system and hematological system [3]. In this review, we will discuss current knowledge of miR-223, with focus on its emerging role in the platelet function regulation.

2. miRNA Biogenesis and Function

miRNA genes are transcribed from the genomes of nucleated cells into primary miRNAs (pri-miRNAs), which are trimmed into miRNA precursors (pre-miRNAs) by the nuclear RNase III Drosha [15, 16], acting together with the DiGeorge syndrome critical region 8 (DGCR8) protein within the microprocessor complex [17]. The pre-miRNAs are transported out of the nucleus via exportin 5. In the cytoplasm, the 3′ overhang of the pre-miRNA is recognized by the Dicer-TAR RNA-binding protein (TRBP) complex [18]. Assisted by TAR RNA-binding protein 2 (TRBP2), Dicer cleaves the stem of pre-miRNA substrates at the base of the loop to generate miRNA-miRNA* duplexes. Dicer is another RNase type III endonuclease, which generates the miRNA duplex for many miRNAs. The strands separate and the mature miRNA associates with a macromolecular complex

called the RNA-induced silencing complex (RISC), which guides the miRNA to its mRNA target. The mature miRNAs are subsequently incorporated into effector ribonucleoprotein (RNP) complexes containing Argonaute 2 (Ago2) and fragile X mental retardation protein (FMRP), guiding the miRNPs for the regulation of specific mRNAs [19, 20]. The process of miRNA generation is regulated at both transcriptional and posttranscriptional levels, occasionally composed of positive feedforward or negative feedback loop, in which the miRNA targets a transcriptional activator or repressor of itself [21].

A fundamental aspect of miRNA function relates to mRNA targeting: most miRNAs are predicted to target multiple mRNAs, and most mRNAs have predicted targets for many miRNAs [22]. Up to now, many algorithms are publicly available for predicting miRNA-binding sites, including miRanda, TargetScan, PicTar, and miRBase [23, 24]. miRNAs regulate mRNA translation through recognition of binding sites of imperfect complementarity, in which pairing of miRNA nucleotides 2 to 8, or the seed region, is crucial. The seed region of nucleotides 2 to 8 at the $5'$ end of the miRNA has perfect complementarity, and this sequence defines families of miRNAs. The miRNA sequence $3'$ to the seed sequence has variable degrees of complementarity with the mRNA. miRNAs have been aptly referred to as "rheostats" because their regulatory impact is generally to fine-tune but not abolish protein expression [25].

3. MiR-223 Biogenesis and Function

The gene encoding miR-223 is located within the q12 locus of the X chromosome [26, 27], and its expression is regulated by several transcription factors, including transcription factor PU.1, CCAAT-enhancer-binding proteins- (C/EBP-) α and β, and nuclear factor I-A (NFI-A) [28–31]. Within the body, miR-223 appears to be most highly expressed in the bone marrow [28, 32], where its expression was shown to be restricted to the myeloid compartment [28]. The fact that the sequence of miR-223 has been remarkably conserved during evolution suggests that this miRNA has an important role in physiological processes. To date, miR-223's involvement has been demonstrated in many types of cancer, inflammatory disease, autoimmune disease, and other pathological processes [3].

The most important role of miR-223 was discovered in the field of hematology, since it was shown to modulate the differentiation of hematopoietic lineages [27]. This function takes place in the hematopoietic bone marrow and affects hematopoietic stem cells and myeloid, erythroid, and lymphoid cells at different stages during their development [27]. In bone marrow, miR-223 expression is mainly confined to myeloid cells and is induced during the lineage differentiation of myeloid progenitor cells. These myeloid cells will give rise to monocyte/macrophage and granulocyte cells. The level of miR-223 is reduced when granulocyte-monocyte progenitors start to differentiate into monocytes [31], whereas its level is increased when granulocyte-monocyte progenitors enter the granulocyte differentiation phase [27, 31], indicating a lineage-specific pattern. By contrast, using miR-223$^{-/-}$ mice, the same group showed that miR-223 is not strictly essential for granulocyte differentiation but was required for normal maturation of granulocytes and regulation of the granulocyte compartment size [27]. In addition, miR-223 was shown to play a role in monocyte/macrophage differentiation, by targeting IκB kinase subunit alpha (IKK-α), a component of nuclear factor-kappa B (NF-κB) pathway. During macrophage differentiation, a fall in miR-223 expression induces an increase of IKK-α expression which induces the expression of p52 followed by the repression of NF-κB pathways [33, 34]. Moreover, miR-223-rich microvesicles were suggested to induce the differentiation of recipient monocytes, activate hematopoietic cell production in the bone marrow, and then induce the release of more microvesicles [35]. Recently, miR-223 was demonstrated to regulate human embryonic stem cell (hESC) differentiation by targeting the IGF-1R/Akt signaling pathway [36]. The inhibition of miR-223 expression maintained hESCs in the undifferentiated state, while addition of exogenous miR-223 induced their differentiation. These effects were dependent on the IGF-1R/Akt pathway, due to the fact that IGF-1R mRNA was demonstrated to be a target of miR-223 [36].

The second field that miR-223 is actively involved in is angiogenesis and vascular remodeling, which may have therapeutic potential for certain cancer and cardiovascular disease. miR-223 is typically associated with myeloid cells, but recent investigation revealed that miR-223 is expressed in native endothelial cells and is rapidly downregulated after isolation and culture of the endothelial cells [37]. Additionally, miR-223 could attenuate endothelial cell proliferation by targeting the expression of β1 integrin and reducing the vascular endothelial cell growth factor- (VEGF-) induced and basic fibroblast growth factor- (bFGF-) induced phosphorylation of their receptors, as well as downstream Akt phosphorylation [37]. Accordingly, downregulation or deletion of miR-223 markedly increased β1 integrin expression, as well as angiogenesis and vascular repair. Therefore, it seems that miR-223 may be required for the maintenance of endothelial cell quiescence. It is interesting to determine whether the endothelial dysfunction associated with cardiovascular disease is also associated with altered endothelial miR-223 levels. Another intriguing, yet unproved, regulatory pathway of miR-223 in angiogenesis might be derived from recent evidence that hypoxia could downregulate the transcription factor CCAAT/enhancer binding protein-α (C/EBP-α) [38], which is also an upstream regulator for miR-223 expression [39]. A variety of cellular stresses, including replication stress and oxidative stress, play an important role in angiogenesis and vascular remodeling, which lead to the activation of DNA damage signaling [40]. Mounting evidence suggests that miRNA expression is regulated in response to DNA damage, and vice versa [41]. A recent work reported that an increased sensitivity to chemotherapy was observed in cancer cells with enforced miR-223 expression and reduced poly(ADP-ribose) polymerase-1 (PARP-1) [42], an enzyme which catalyzes the NAD^+-dependent polymerization of long chains of poly-ADP ribose (PAR) onto itself in response to DNA damage, leading to DNA repair. Thus, miR-223 may

serve as an endogenous PARP-1 inhibitor for cancer treatment. As DNA damage/repair response has been implicated in the pathogenesis of vascular remodeling [43, 44], future work is warranted to determine whether miR-223 may also participate in this process by targeting PARP-1.

The third field in which miR-223 was intensively investigated is osteoclast formation and bone remodeling [45]. Indeed, this miRNA is expressed in osteoclast precursors (RAW 264.7 cells); both under- and overexpression of miR-223 diminish the osteoclast-like cell formation induced by RANKL [29]. This indicates that the expression of miR-223 must be fine-tuned for normal osteoclastogenesis [29, 30, 45]. The third important role of miR-223 was in regulation of carcinogenesis [13, 46]. For example, miR-223 is poorly expressed in acute myeloid leukemia (AML), as well as in gastric [47] and ovarian cancer [48]. Depending on the context, miR-223 acts as either an oncogene or a tumor suppressor gene [49].

In addition to its role in hematopoietic differentiation, osteoclastogenesis, and embryonic stem cells differentiation, as well as in carcinogenesis, miR-223 also has an instrumental role in inflammatory diseases, such as adipocyte inflammation associated with morbid obesity, insulin resistance, rheumatoid arthritis, vascular damage, and atherosclerosis [3, 32, 49–52]. In brief, many studies show a clear trend towards a regulatory role of miR-223 in various clinical disorders. Further work will be warranted to determine the molecular mechanisms of miR-223 implicated in the physiopathology of these disorders.

4. Intraplatelet miR-223 and Platelet Reactivity

Platelet adhesion and aggregation play a pivotal role in the maintenance of hemostasis, as well as in thrombosis and vessel occlusion, which underlies the pathogenesis of stroke and acute coronary syndrome (ACS) [53]. Dual antiplatelet therapy with clopidogrel (a P2Y12 receptor antagonist) and aspirin is currently the cornerstone of pharmacological prevention of ischemic events in patients with atherosclerosis [54]. But epidemiological and clinical studies have identified a fraction of patients with increased risk of ischemic cardiovascular events despite receiving a standard regimen of antiplatelet therapy, which is termed "high on-treatment platelet reactivity" (HTPR) [55, 56]. Recent studies showed that HTPR is a strong predictor of myocardial infarction (MI) and cardiovascular death in patients after percutaneous coronary intervention (PCI) [57, 58]. Although heritability strongly influences the interindividual variation in platelet reactivity [59], the precious molecular mechanisms of this phenomenon remain to be poorly understood.

Platelets are devoid of a nucleus and genomic DNA, but they were shown to contain subcellular organelles, such as rough endoplasmic reticulum and ribosomes [60], as well as a small amount of poly(A) + RNA from their megakaryocyte progenitor cells [61], sufficient to support *de novo* protein synthesis. In fact, between 15% and 32% of protein coding genes are represented in the form of mRNAs in platelets [62–64]. In addition, the functionality of these platelet transcripts was supported by a strong correlation between transcript abundance and protein expression [63, 64]. These evidences prompted basic researchers to speculate whether circulating platelets harbor a gene-regulatory pathway based on miRNAs. This hypothesis was first confirmed by a landmark study by Landry and coworkers in 2009, which demonstrated that human platelets harbor an abundant and diverse array of miRNAs, and the three most abundant miRNAs are miR-223, let-7c, and miR-19a [65]. Further analyses revealed that platelets contain the Dicer and Ago2 complexes, which function in the processing of exogenous miRNA precursors and in the control of specific reporter transcripts, respectively. Accordingly, the authors could not detect the nuclear microprocessor components Drosha and DGCR8 in platelets, consistent with the anucleate nature of human platelets. They also found that miRNA-associated Ago2 proteins, in a complex reminiscent of recombinant RISC, may form the endogenous miRNA effector complex in platelets. Moreover, the presence of P2Y12 mRNA in Ago2 immunoprecipitates was detected, suggesting a potential regulation of P2Y12 expression by miRNAs in human platelets. Serving as a receptor for ADP, P2Y12 is a seven-transmembrane domain receptor coupled to Gi2 protein that mediates a number of biological processes, such as platelet aggregation, granule secretion, and thrombus growth and stability [66]. The experimental validation of the predicted binding site for miR-223 in its natural 3′UTR context in P2Y12 mRNA supports the concept that P2Y12 expression could be regulated by miR-223 in human platelets [65].

Another study by Nagalla and coworkers focused on the potential role of platelet miRNAs as both a biomarker of platelet reactivity and a regulator of platelet mRNA variation [67]. Using a genome-wide profiling strategy in 19 healthy donors, they demonstrated the expression of 284 miRNAs in platelets, among which miR-223 is present in greatest abundance. They then showed that miRNA profiles are associated with and may predict the response of platelet aggregation to epinephrine. In addition, the authors used computational approach to theoretically generate a high-priority list of miRNA-mRNA pair candidates, among which the functionality of three pairs (miR-200b:*PRKAR2B* (encoding the regulatory chain of cAMP-dependent protein kinase type II); miR-495:*KLHL5* (encoding a Kelch-like protein that binds to actin); and miR-107:*CLOCK* (encoding a major regulator of the cell circadian rhythm)) was further verified in cell lines [67]. Interestingly, a prior study by the same group showed that platelet vesicle-associated membrane protein 8 (VAMP8) expression (a protein involved in platelet granule secretion) is associated with differential platelet aggregation response to epinephrine in healthy donors, which is regulated by miR-96 [68]. These lines of evidence support a regulatory role of platelet miRNAs in protein expression that associated with variation in platelet reactivity.

Enlightened by the above studies, we hypothesized that the expression of miR-223 and miR-96, which are associated with platelet aggregation and secretion properties in healthy subjects, may influence the responsiveness to clopidogrel in patients with coronary heart disease (CHD). We thus enrolled 33 nondiabetic CHD patients with non-ST elevation acute

coronary syndrome (NSTE-ACS) confirmed by diagnostic coronary angiography (CAG) [69]. All patients received a loading dose of 300 mg aspirin plus 300 mg clopidogrel for at least 24 h before CAG or 100 mg aspirin plus 75 mg clopidogrel for at least 5 days prior to CAG. Platelet responsiveness after antiplatelet therapy was determined by two methods (platelet reactivity index (PRI), measured by vasodilator-stimulated phosphoprotein (VASP) phosphorylation flow cytometry and ADP-induced platelet aggregation (PAG), measured by light transmission aggregometry). All patients were dichotomized according to the medians of their PRI and PAG values. We determined the expression of miR-223 and miR-96 in purified platelets from each patient and found that only miR-223 expression was statistically downregulated in PRI-determined low responders. In addition, neither miR-223 nor miR-96 was found to be significantly different between PAG-determined normal and low responders. Accordingly, miR-223 expression, but not miR-96, was statistically correlated with PRI. Using a binary logistic regression model, we showed that decreased miR-223 expression was the only variable that was associated with PRI-determined low responders, even after being adjusted for known risk factors for HTPR (*CYP2C19* 2* genetic polymorphisms [59, 70], calcium channel blockers [71], proton-pump inhibitors [72], age, obesity, and smoking [73–75]). To our knowledge, this work provides the first evidence that platelet miR-223 may serve as a potential marker in evaluating the degree of P2Y12 receptor inhibition in CHD patients.

5. Circulating miR-223 and Platelet Reactivity

Despite the above evidence that platelet specific miRNAs are associated with varied response to antiplatelet therapy, the detection of intraplatelet miRNAs requires a large amount of blood (15–20 mL), as well as a white blood cell depletion protocol using magnetic beads, which may substantially limit its clinical application. Circulating miRNAs are stable in serum and plasma and were shown to have prognostic values for cardiovascular disease [76]. Currently, three pools of plasma circulating miRNAs have been identified, that is, microparticle- (MP-) associated, high density lipoprotein- (HDL-) associated, and exosome-associated miRNAs [77]. Among these three pools, it has been reported that a prominent amount of plasma miRNAs is associated to MPs [78].

The prognostic value of circulating miRNAs for major cardiovascular events (MACEs) was first confirmed by Zampetaki and coworkers [79]. In a cohort study consisting of 820 participants, this group found that baseline levels of three circulating miRNAs, that is, miR-126 (hazard ratio (HR) 2.69, 95% CI: 1.45–5.01), miR-223 (HR 0.47, 95% CI: 0.29–0.75), and miR-197 (HR 0.56, 95% CI: 0.32–0.96), were independently correlated with incident MI during a 10-year follow-up [79]. Notably, the authors showed that these miRNAs are highly expressed in platelets and platelet-derived MPs [79]. This finding is consistent with a recent study which showed that activated platelet-derived MP is the major source of plasma circulating miR-223, whereas HDL- and exosome-derived miR-223 is almost negligible compared with MPs [80]. Based on these findings, Willeit and colleagues extended their findings by profiling 377 miRNAs in human platelets, platelet MPs, and platelet rich and poor plasma and found that miR-223 is the most differentially expressed miRNAs [81]. Interestingly, the authors reported that the level of circulating miR-223 tended to be decreased after 10 mg prasugrel plus 75/300 mg aspirin in healthy volunteers ($n = 3$) and in patients ($n = 15$) with symptomatic carotid atherosclerosis after 300 mg clopidogrel plus 75 mg aspirin [81]. Thus far, the VASP index measured by flow cytometry is the most specific assay to evaluate P2Y12 receptor blockade and is not influenced by other antiplatelet agents or anticoagulants [82]. As the authors did not perform platelet VASP analysis, the relationship between platelet responsiveness after P2Y12 inhibition and the magnitude of miR-223 change remains unclear. In addition, as pointed out by the accompanying editorial [83], if miR-223 is suppressed by P2Y12 inhibition, its inverse relationship to incident MI seems obscure.

We recently extended our prior work by focusing on the prognostic value of circulating miR-223 for diminished response to clopidogrel in NSTE-ACS CHD patients [84]. In multivariate analysis, the level of circulating miR-223 independently predicted the differential response to clopidogrel after adjustment of known clinical factors associated with HTPR [84]. Admittedly, our study was not designed to investigate the influence of P2Y12 inhibition on circulating miR-223 level. However, the results from Zampetaki and colleagues [79, 81], as well as our independent work, indicate that circulating miR-223, presumably derived from platelets, may serve as a novel marker for platelet reactivity.

6. Controversy and Perspective

Based on multiple profiling studies providing evidence that miR-223 is one of the most abundant miRNAs in megakaryocytes and platelets [85–89], a recent work evaluated the platelet functions in miR-223 deficient mice [90]. Surprisingly, this study reported that, in miR-223-deficient mice, platelet number, volume, and lifespan, as well as platelet surface receptors, were expressed at the same level compared with those from wild type mice [90]. Moreover, loss of miR-223 did not affect the platelet properties for ADP-induced aggregation. In view of this evidence, the authors concluded that miR-223 plays a remarkably modest role in thrombopoiesis and that platelet function does not depend upon miR-223. This study seems contradictory to previous published human studies. However, an important issue should be noted. The central hypothesis that miR-223 is a biomarker for platelet reactivity is based on the evidence that there is a putative binding site for miR-223 in the 3′untranslated region (3′UTR) of the mRNA encoding P2Y12 [65], which is the molecular basis for miR-223 regulation of P2Y12 expression, whereas, as addressed in Leierseder and collaborators' recent work [90], the bioinformatics analysis revealed that, in contrast to the situation in humans, the mouse P2Y12 UTR does not contain a binding site for miR-223. Accordingly, they found that platelet P2Y12 mRNA expression was unchanged in miR-223 deficient mice compared with wide type mice. This finding pointed out a puzzling issue that the role of miR-233 may have substantial species difference and provided an

explanation to the inconsistent results derived from human and mouse studies.

In addition to platelet's contribution to peripheral blood MPs, recent studies also demonstrated that mononuclear phagocytes, especially macrophages, are the second most abundant population contributing to MPs which lead to phenotype switch in target cells via miRNAs transfer, including miR-223 [35]. Moreover, miR-223 is highly expressed in cells of the granulocytic lineage and plays an important role in regulating granulopoiesis and neutrophil function [33]. Up to now, several miR-223 target genes that are closely associated with inflammation have been identified, such as NLRP3 [91], IKKa [34], Pknox1 [32], Pax6 [92], NFI-A [30], and insulin-like growth factor-1 receptor (IGF-1R) [93]. Therefore, it is possible that platelet-derived miR-223 may regulate other immune cells harboring miR-223 target genes via MP transfer and in turn regulate platelet function via platelet-leukocyte interaction (aggregate formation), a process now called immunothrombosis [94].

Appropriate regulation of platelet function, which is the basis to maintain the elegant balance between hemorrhage and thrombosis, is of paramount importance in patients on antiplatelet therapy. There is mounting evidence that platelet miRNAs mediated regulatory network serves as basic mechanisms for megakaryocyte/platelet gene expression. Given its abundance in platelet, miR-223 has begun to receive considerable attention. Recent studies provide an emerging role of miR-223 as both a regulator and biomarker for HTPR and MACEs. Bearing in mind that miR-223 mediated regulatory network may have species difference, future studies on miR-223 in different models of cardiovascular disease, as well as in clinical settings, will be warranted for developing new diagnostic and therapeutic strategies.

Conflict of Interests

The authors have no financial conflict of interests.

Authors' Contribution

Rui Shi and Xin Zhou contributed equally to this work.

Acknowledgments

This work was supported by National Natural Science Foundation of China (81102088 and 81170238) and Tianjin Municipal Science and Technology Committee (11JCYBJC12000 and 12JCYBJC16600) and Intramural Research Program from Pingjin Hospital and Logistics University of the Chinese People's Armed Police Forces (FYM201101, FYM201422, and TJC1408).

References

[1] R. C. Lee, R. L. Feinbaum, and V. Ambros, "The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14," *Cell*, vol. 75, no. 5, pp. 843–854, 1993.

[2] D. P. Bartel, "MicroRNAs: genomics, biogenesis, mechanism, and function," *Cell*, vol. 116, no. 2, pp. 281–297, 2004.

[3] F. Taïbi, V. Metzinger-Le Meuth, Z. A. Massy, and L. Metzinger, "MiR-223: an inflammatory oncomiR enters the cardiovascular field," *Biochimica et Biophysica Acta (BBA)—Molecular Basis of Disease*, vol. 1842, no. 7, pp. 1001–1009, 2014.

[4] R. C. Friedman, K. K.-H. Farh, C. B. Burge, and D. P. Bartel, "Most mammalian mRNAs are conserved targets of microRNAs," *Genome Research*, vol. 19, no. 1, pp. 92–105, 2009.

[5] B. P. Lewis, C. B. Burge, and D. P. Bartel, "Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets," *Cell*, vol. 120, no. 1, pp. 15–20, 2005.

[6] W. P. Kloosterman and R. H. A. Plasterk, "The diverse functions of microRNAs in animal development and disease," *Developmental Cell*, vol. 11, no. 4, pp. 441–450, 2006.

[7] G. C. Shukla, J. Singh, and S. Barik, "MicroRNAs: processing, maturation, target recognition and regulatory functions," *Molecular and Cellular Pharmacology*, vol. 3, no. 3, pp. 83–92, 2011.

[8] D. P. Bartel, "MicroRNAs: target recognition and regulatory functions," *Cell*, vol. 136, no. 2, pp. 215–233, 2009.

[9] E. Wienholds, W. P. Kloosterman, E. Miska et al., "MicroRNA expression in zebrafish embryonic development," *Science*, vol. 309, no. 5732, pp. 310–311, 2005.

[10] J. D. Port and C. Sucharov, "Role of microRNAs in cardiovascular disease: therapeutic challenges and potentials," *Journal of Cardiovascular Pharmacology*, vol. 56, no. 5, pp. 444–453, 2010.

[11] M. S. Jamaluddin, S. M. Weakley, L. Zhang et al., "MiRNAs: roles and clinical applications in vascular disease," *Expert Review of Molecular Diagnostics*, vol. 11, no. 1, pp. 79–89, 2011.

[12] C. Urbich, A. Kuehbacher, and S. Dimmeler, "Role of microRNAs in vascular diseases, inflammation, and angiogenesis," *Cardiovascular Research*, vol. 79, no. 4, pp. 581–588, 2008.

[13] N. Nouraee and G. A. Calin, "MicroRNAs as cancer biomarkers," *MicroRNA*, vol. 2, no. 2, pp. 102–117, 2013.

[14] L. Tutar, E. Tutar, and Y. Tutar, "MicroRNAs and cancer; an overview," *Current Pharmaceutical Biotechnology*, vol. 15, no. 5, pp. 430–437, 2014.

[15] Y. Lee, C. Ahn, J. Han et al., "The nuclear RNase III Drosha initiates microRNA processing," *Nature*, vol. 425, no. 6956, pp. 415–419, 2003.

[16] H. Zhang, F. A. Kolb, L. Jaskiewicz, E. Westhof, and W. Filipowicz, "Single processing center models for human Dicer and bacterial RNase III," *Cell*, vol. 118, no. 1, pp. 57–68, 2004.

[17] J. Han, Y. Lee, K.-H. Yeom, Y.-K. Kim, H. Jin, and V. N. Kim, "The Drosha-DGCR8 complex in primary microRNA processing," *Genes & Development*, vol. 18, no. 24, pp. 3016–3027, 2004.

[18] T. P. Chendrimada, R. I. Gregory, E. Kumaraswamy et al., "TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing," *Nature*, vol. 436, no. 7051, pp. 740–744, 2005.

[19] S. Cheloufi, C. O. Dos Santos, M. M. W. Chong, and G. J. Hannon, "A dicer-independent miRNA biogenesis pathway that requires Ago catalysis," *Nature*, vol. 465, no. 7298, pp. 584–589, 2010.

[20] D. Cifuentes, H. Xue, D. W. Taylor et al., "A novel miRNA processing pathway independent of dicer requires argonaute2 catalytic activity," *Science*, vol. 328, no. 5986, pp. 1694–1698, 2010.

[21] S. Muller and K. Nowak, "Exploring the miRNA-mRNA regulatory network in clear cell renal cell carcinomas by next-generation sequencing expression profiles ," *BioMed Research International*, vol. 2014, Article ID 948408, 11 pages, 2014.

[22] S.-Y. Ying, D. C. Chang, and S.-L. Lin, "The MicroRNA (miRNA): overview of the RNA genes that modulate gene function," *Molecular Biotechnology*, vol. 38, no. 3, pp. 257–268, 2008.

[23] A. Kozomara and S. Griffiths-Jones, "MiRBase: integrating microRNA annotation and deep-sequencing data," *Nucleic Acids Research*, vol. 39, no. 1, pp. D152–D157, 2011.

[24] S. Griffiths-Jones, "miRBase, microRNA sequences and annotation," in *Current Protocols in Bioinformatics*, A. D. Baxevanis et al., Ed., Chapter 12, 2010.

[25] M. R. Fabian, T. R. Sundermeier, and N. Sonenberg, "Understanding how miRNAs post-transcriptionally regulate gene expression," *Progress in Molecular and Subcellular Biology*, vol. 50, pp. 1–20, 2010.

[26] A. E. Rodriguez, J. A. Hernandez, R. Benito et al., "Molecular characterization of chronic lymphocytic leukemia patients with a high number of losses in 13q14," *PloS ONE*, vol. 17, no. 11, Article ID e48485, 2012.

[27] J. B. Johnnidis, M. H. Harris, R. T. Wheeler et al., "Regulation of progenitor cell proliferation and granulocyte function by microRNA-223," *Nature*, vol. 451, no. 7182, pp. 1125–1129, 2008.

[28] C.-Z. Chen, L. Li, H. F. Lodish, and D. P. Bartel, "MicroRNAs modulate hematopoietic lineage differentiation," *Science*, vol. 303, no. 5654, pp. 83–86, 2004.

[29] K. Kapinas and A. M. Delany, "MicroRNA biogenesis and regulation of bone remodeling," *Arthritis Research and Therapy*, vol. 13, no. 3, article 220, 2011.

[30] F. Fazi, A. Rosa, A. Fatica et al., "A minicircuitry comprised of microRNA-223 and transcription factors NFI-A and C/EBPα regulates human granulopoiesis," *Cell*, vol. 123, no. 5, pp. 819–831, 2005.

[31] G. Zardo, A. Ciolfi, L. Vian et al., "Polycombs and microRNA-223 regulate human granulopoiesis by transcriptional control of target gene expression," *Blood*, vol. 119, no. 17, pp. 4034–4046, 2012.

[32] G. Zhuang, C. Meng, X. Guo et al., "A novel regulator of macrophage activation: miR-223 in obesity-associated adipose tissue inflammation," *Circulation*, vol. 125, no. 23, pp. 2892–2903, 2012.

[33] R. M. O'Connell, J. L. Zhao, and D. S. Rao, "MicroRNA function in myeloid biology," *Blood*, vol. 118, no. 11, pp. 2960–2969, 2011.

[34] T. Li, M. J. Morgan, S. Choksi, Y. Zhang, Y.-S. Kim, and Z.-G. Liu, "MicroRNAs modulate the noncanonical transcription factor NF-κB pathway by regulating expression of the kinase IKKα during macrophage differentiation," *Nature Immunology*, vol. 11, no. 9, pp. 799–805, 2010.

[35] N. Ismail, Y. Wang, D. Dakhlallah et al., "Macrophage microvesicles induce macrophage differentiation and miR-223 transfer," *Blood*, vol. 121, no. 6, pp. 984–995, 2013.

[36] Y.-H. Yu, L. Zhang, D.-S. Wu et al., "MiR-223 regulates human embryonic stem cell differentiation by targeting the IGF-1R/Akt signaling pathway," *PLoS ONE*, vol. 8, no. 11, Article ID e78769, 2013.

[37] L. Shi, B. Fisslthaler, N. Zippel et al., "MicroRNA-223 antagonizes angiogenesis by targeting β1 integrin and preventing growth factor signaling in endothelial cells," *Circulation Research*, vol. 113, no. 12, pp. 1320–1330, 2013.

[38] R. Seifeddine, A. Dreiem, E. Blanc et al., "Hypoxia down-regulates CCAAT/enhancer binding protein-α expression in breast cancer cells," *Cancer Research*, vol. 68, no. 7, pp. 2158–2165, 2008.

[39] M. Eyholzer, S. Schmid, J. A. Schardt, S. Haefliger, B. U. Mueller, and T. Pabst, "Complexity of miR-223 regulation by CEBPA in human AML," *Leukemia Research*, vol. 34, no. 5, pp. 672–676, 2010.

[40] M. K. Zeman and K. A. Cimprich, "Causes and consequences of replication stress," *Nature Cell Biology*, vol. 16, no. 1, pp. 2–9, 2014.

[41] G. Wan, R. Mathur, X. Hu, X. Zhang, and X. Lu, "miRNA response to DNA damage," *Trends in Biochemical Sciences*, vol. 36, no. 9, pp. 478–484, 2011.

[42] M. M. Streppel, S. Pai, N. R. Campbell et al., "MicroRNA 223 is upregulated in the multistep progression of barrett's esophagus and modulates sensitivity to chemotherapy by targeting *PARP1*," *Clinical Cancer Research*, vol. 19, no. 15, pp. 4067–4078, 2013.

[43] M. Durik, M. Kavousi, I. Van Der Pluijm et al., "Nucleotide excision DNA repair is associated with age-related vascular dysfunction," *Circulation*, vol. 126, no. 4, pp. 468–478, 2012.

[44] I. Gorenne, S. Kumar, K. Gray et al., "Vascular smooth muscle cell sirtuin 1 protects against dna damage and inhibits atherosclerosis," *Circulation*, vol. 127, no. 3, pp. 386–396, 2013.

[45] T. Sugatani and K. A. Hruska, "MicroRNA-223 is a key factor in osteoclast differentiation," *Journal of Cellular Biochemistry*, vol. 101, no. 4, pp. 996–999, 2007.

[46] E. M. Pinatel, F. Orso, E. Penna et al., "miR-223 is a coordinator of breast cancer progression as revealed by bioinformatics predictions," *PLoS ONE*, vol. 9, no. 1, Article ID e84859, 2014.

[47] X. Li, Y. Zhang, H. Zhang et al., "miRNA-223 promotes gastric cancer invasion and metastasis by targeting tumor suppressor EPB41L3," *Molecular Cancer Research*, vol. 9, no. 7, pp. 824–833, 2011.

[48] A. Laios, S. O'Toole, R. Flavin et al., "Potential role of miR-9 and miR-223 in recurrent ovarian cancer," *Molecular Cancer*, vol. 7, article 35, 2008.

[49] M. Haneklaus, M. Gerlic, L. A. J. O'Neill, and S. L. Masters, "MiR-223: infection, inflammation and cancer," *Journal of Internal Medicine*, vol. 274, no. 3, pp. 215–226, 2013.

[50] J. M. Wentworth, G. Naselli, W. A. Brown et al., "Pro-inflammatory $CD11c^+CD206^+$ adipose tissue macrophages are associated with insulin resistance in human obesity," *Diabetes*, vol. 59, no. 7, pp. 1648–1656, 2010.

[51] Z.-X. Meng, G.-X. Wang, and J. D. Lin, "A MicroRNA circuitry links macrophage polarization to metabolic homeostasis," *Circulation*, vol. 125, no. 23, pp. 2815–2817, 2012.

[52] M. C. Lu, C. L. Yu, H. C. Chen et al., "Increased miR-223 expression in T cells from patients with rheumatoid arthritis leads to decreased insulin-like growth factor-1-mediated interleukin-10 production," *Clinical and Experimental Immunology*, vol. 177, no. 3, pp. 641–651, 2014.

[53] S. Gosavi and D. Mukherjee, "Review of newer anticoagulants and anti-platelet agents in acute coronary syndrome and cardiovascular diseases," *Cardiovascular & Hematological Agents in Medicinal Chemistry*, vol. 11, no. 3, pp. 194–202, 2013.

[54] H. Jneid, J. L. Anderson, R. S. Wright et al., "2012 ACCF/AHA focused update of the guideline for the management of patients with unstable angina/non-ST-elevation myocardial infarction (updating the 2007 guideline and replacing the 2011 focused update): a report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice

Guidelines," *Journal of the American College of Cardiology*, vol. 60, no. 7, pp. 645–681, 2012.

[55] D. Trenk, S. D. Kristensen, W. Hochholzer, and F.-J. Neumann, "High on-treatment platelet reactivity and P2Y12 antagonists in clinical trials," *Thrombosis and Haemostasis*, vol. 109, no. 5, pp. 834–845, 2013.

[56] U. S. Tantry, L. Bonello, D. Aradi et al., "Consensus and update on the definition of on-treatment platelet reactivity to adenosine diphosphate associated with ischemia and bleeding," *Journal of the American College of Cardiology*, vol. 62, no. 24, pp. 2261–2273, 2013.

[57] D. Aradi, A. Komócsi, A. Vorobcsuk et al., "Prognostic significance of high on-clopidogrel platelet reactivity after percutaneous coronary intervention: systematic review and meta-analysis," *American Heart Journal*, vol. 160, no. 3, pp. 543–551, 2010.

[58] N. J. Breet, J. W. Van Werkum, H. J. Bouman et al., "High on-treatment platelet reactivity to both aspirin and clopidogrel is associated with the highest risk of adverse events following percutaneous coronary intervention," *Heart*, vol. 97, no. 12, pp. 983–990, 2011.

[59] J. Miao, R. Liu, and Z. Li, "Cytochrome P-450 polymorphisms and response to clopidogrel," *The New England Journal of Medicine*, vol. 360, no. 21, pp. 2250–2251, 2009.

[60] C. H. Ts'ao, "Rough endoplasmic reticulum and ribosomes in blood platelets," *Scandinavian Journal of Haematology*, vol. 8, no. 2, pp. 134–140, 1971.

[61] G. J. Roth, M. J. Hickey, D. W. Chung, and D. D. Hickstein, "Circulating human blood platelets retain appreciable amounts of poly $(A)^+$ RNA," *Biochemical and Biophysical Research Communications*, vol. 160, no. 2, pp. 705–710, 1989.

[62] P. Bugert, A. Dugrillon, A. Günaydin, H. Eichler, and H. Klüter, "Messenger RNA profiling of human platelets by microarray hybridization," *Thrombosis and Haemostasis*, vol. 90, no. 4, pp. 738–748, 2003.

[63] D. V. Gnatenko, J. J. Dunn, J. Schwedes, and W. F. Bahou, "Transcript profiling of human platelets using microarray and serial analysis of gene expression (SAGE)," *Methods in Molecular Biology*, vol. 496, pp. 245–272, 2009.

[64] J. P. McRedmond, S. D. Park, D. F. Reilly et al., "Integration of proteomics and genomics in platelets: a profile of platelet proteins and platelet-specific genes," *Molecular & Cellular Proteomics*, vol. 3, no. 2, pp. 133–144, 2004.

[65] P. Landry, I. Plante, D. L. Ouellet, M. P. Perron, G. Rousseau, and P. Provost, "Existence of a microRNA pathway in anucleate platelets," *Nature Structural and Molecular Biology*, vol. 16, no. 9, pp. 961–966, 2009.

[66] T. Höchtl and K. Huber, "P2Y12-receptor-inhibiting antiplatelet strategies in acute coronary syndromes," *Hamostaseologie*, vol. 34, no. 1, pp. 20–28, 2014.

[67] S. Nagalla, C. Shaw, X. Kong et al., "Platelet microRNA-mRNA coexpression profiles correlate with platelet reactivity," *Blood*, vol. 117, no. 19, pp. 5189–5197, 2011.

[68] A. A. Kondkar, M. S. Bray, S. M. Leal et al., "VAMP8/endobrevin is overexpressed in hyperreactive human platelets: suggested role for platelet microRNA," *Journal of Thrombosis and Haemostasis*, vol. 8, no. 2, pp. 369–378, 2010.

[69] R. Shi, L. Ge, X. Zhou et al., "Decreased platelet miR-223 expression is associated with high on-clopidogrel platelet reactivity," *Thrombosis Research*, vol. 131, no. 6, pp. 508–513, 2013.

[70] A. R. Shuldiner, J. R. O'Connell, K. P. Bliden et al., "Association of cytochrome P450 2C19 genotype with the antiplatelet effect and clinical efficacy of clopidogrel therapy," *The Journal of the American Medical Association*, vol. 302, no. 8, pp. 849–858, 2009.

[71] J. M. Siller-Matula, I. Lang, G. Christ, and B. Jilma, "Calcium-channel blockers reduce the antiplatelet effect of clopidogrel," *Journal of the American College of Cardiology*, vol. 52, no. 19, pp. 1557–1563, 2008.

[72] U. S. Tantry, D. J. Kereiakes, and P. A. Gurbel, "Clopidogrel and proton pump inhibitors: influence of pharmacological interactions on clinical outcomes and mechanistic explanations," *JACC: Cardiovascular Interventions*, vol. 4, no. 4, pp. 365–380, 2011.

[73] J. S. Berger, D. L. Bhatt, S. R. Steinhubl et al., "Smoking, clopidogrel, and mortality in patients with established cardiovascular disease," *Circulation*, vol. 120, no. 23, pp. 2337–2344, 2009.

[74] L. Ang, V. Palakodeti, A. Khalid et al., "Elevated plasma fibrinogen and diabetes mellitus are associated with lower inhibition of platelet reactivity with clopidogrel," *Journal of the American College of Cardiology*, vol. 52, no. 13, pp. 1052–1059, 2008.

[75] J. Silvain, G. Cayla, J.-S. Hulot et al., "High on-thienopyridine platelet reactivity in elderly coronary patients: the SENIOR-PLATELET study," *European Heart Journal*, vol. 33, no. 10, pp. 1241–1250, 2012.

[76] S. Fichtlscherer, A. M. Zeiher, and S. Dimmeler, "Circulating MicroRNAs: biomarkers or mediators of cardiovascular diseases?" *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 31, no. 11, pp. 2383–2390, 2011.

[77] R. A. Boon and K. C. Vickers, "Intercellular transport of MicroRNAs," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 33, no. 2, pp. 186–192, 2013.

[78] P. Diehl, A. Fricke, L. Sander et al., "Microparticles: major transport vehicles for distinct microRNAs in circulation," *Cardiovascular Research*, vol. 93, no. 4, pp. 633–644, 2012.

[79] A. Zampetaki, P. Willeit, L. Tilling et al., "Prospective study on circulating microRNAs and risk of myocardial infarction," *Journal of the American College of Cardiology*, vol. 60, no. 4, pp. 290–299, 2012.

[80] B. Laffont, A. Corduan, H. Plé et al., "Activated platelets can deliver mRNA regulatory Ago2*microRNA complexes to endothelial cells via microparticles," *Blood*, vol. 122, no. 2, pp. 253–261, 2013.

[81] P. Willeit, A. Zampetaki, K. Dudek et al., "Circulating MicroRNAs as novel biomarkers for platelet activation," *Circulation Research*, vol. 112, no. 4, pp. 595–600, 2013.

[82] B. Aleil, C. Ravanat, J. P. Cazenave, G. Rochoux, A. Heitz, and C. Gachet, "Flow cytometric analysis of intraplatelet VASP phosphorylation for the detection of clopidogrel resistance in patients with ischemic cardiovascular diseases," *Journal of Thrombosis and Haemostasis*, vol. 3, no. 1, pp. 85–92, 2005.

[83] A. M. Curtis and G. A. Fitzgerald, "Platelet microRNAs: novy mir∗ or mired in human complexity?" *Circulation Research*, vol. 112, no. 4, pp. 576–578, 2013.

[84] Y.-Y. Zhang, X. Zhou, W.-J. Ji et al., "Decreased circulating microRNA-223 level predicts high on-treatment platelet reactivity in patients with troponin-negative non-ST elevation acute coronary syndrome," *Journal of Thrombosis and Thrombolysis*, vol. 38, no. 1, pp. 65–72, 2014.

[85] K. Hussein, K. Theophile, W. Dralle, B. Wiese, H. Kreipe, and O. Bock, "MicroRNA expression profiling of megakaryocytes

in primary myelofibrosis and essential thrombocythemia," *Platelets*, vol. 20, no. 6, pp. 391–400, 2009.

[86] O. I. Petriv, F. Kuchenbauer, A. D. Delaney et al., "Comprehensive microRNA expression profiling of the hematopoietic hierarchy," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 35, pp. 15443–15448, 2010.

[87] H. Plé, P. Landry, A. Benham, C. Coarfa, P. H. Gunaratne, and P. Provost, "The repertoire and features of human platelet microRNAs," *PLoS ONE*, vol. 7, no. 12, Article ID e50746, 2012.

[88] A. Osman and K. Fälker, "Characterization of human platelet microRNA by quantitative PCR coupled with an annotation network for predicted target genes," *Platelets*, vol. 22, no. 6, pp. 433–441, 2011.

[89] P. F. Bray, S. E. McKenzie, L. C. Edelstein et al., "The complex transcriptional landscape of the anucleate human platelet," *BMC Genomics*, vol. 14, article 1, 2013.

[90] S. Leierseder, T. Petzold, L. Zhang, X. Loyer, S. Massberg, and S. Engelhardt, "MiR-223 is dispensable for platelet production and function in mice," *Thrombosis and Haemostasis*, vol. 110, no. 6, pp. 1207–1214, 2013.

[91] F. Bauernfeind, A. Rieger, F. A. Schildberg, P. A. Knolle, J. L. Schmid-Burgk, and V. Hornung, "NLRP3 inflammasome activity is negatively controlled by miR-223," *Journal of Immunology*, vol. 189, no. 8, pp. 4175–4181, 2012.

[92] B.-S. Huang, Q.-Z. Luo, Y. Han, X.-B. Li, L.-J. Cao, and L.-X. Wu, "MicroRNA-223 promotes the growth and invasion of glioblastoma cells by targeting tumor suppressor PAX6," *Oncology Reports*, vol. 30, no. 5, pp. 2263–2269, 2013.

[93] C. Y. Jia, H. H. Li, X. C. Zhu et al., "MiR-223 suppresses cell proliferation by targeting IGF-1R," *PLoS ONE*, vol. 6, no. 11, Article ID e27008, 2011.

[94] B. Engelmann and S. Massberg, "Thrombosis as an intravascular effector of innate immunity," *Nature Reviews Immunology*, vol. 13, no. 1, pp. 34–45, 2013.

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 391306, 6 pages
http://dx.doi.org/10.1155/2015/391306

Research Article

miR-27 and miR-125 Distinctly Regulate Muscle-Enriched Transcription Factors in Cardiac and Skeletal Myocytes

Estefania Lozano-Velasco, Jennifer Galiano-Torres, Alvaro Jodar-Garcia, Amelia E. Aranega, and Diego Franco

Cardiovascular Research Group, Department of Experimental Biology, University of Jaén, 23071 Jaen, Spain

Correspondence should be addressed to Diego Franco; dfranco@ujaen.es

Received 15 August 2014; Revised 27 November 2014; Accepted 1 December 2014

Academic Editor: Magdalena Król

MicroRNAs are noncoding RNAs of approximately 22–24 nucleotides which are capable of interacting with the 3′ untranslated region of coding RNAs (mRNAs), leading to mRNA degradation and/or protein translation blockage. In recent years, differential microRNA expression in distinct cardiac development and disease contexts has been widely reported, yet the role of individual microRNAs in these settings remains largely unknown. We provide herein evidence of the role of miR-27 and miR-125 regulating distinct muscle-enriched transcription factors. Overexpression of miR-27 leads to impair expression of *Mstn* and *Myocd* in HL1 atrial cardiomyocytes but not in Sol8 skeletal muscle myoblasts, while overexpression of miR-125 resulted in selective upregulation of *Mef2d* in HL1 atrial cardiomyocytes and downregulation in Sol8 cells. Taken together our data demonstrate that a single microRNA, that is, miR-27 or miR-125, can selectively upregulate and downregulate discrete number of target mRNAs in a cell-type specific manner.

1. Introduction

MicroRNAs are noncoding RNAs of approximately 22–24 nucleotides which are capable of interacting with the 3′ untranslated region of coding RNAs (mRNAs), leading to mRNA degradation and/or protein translation blockage [1]. Understanding microRNA biogenesis has been greatly achieved; however, knowledge about the tissue distribution and functional consequences remains more elusive. In recent years, an increasing body of evidence has demonstrated a highly relevant role of microRNAs in multiple aspects of cardiac development and diseases [2, 3].

Functional evidence of the role of microRNAs in developing heart was demonstrated by selective inhibition of *Dicer* in tissue-restricted manner. Conditional ablation of *Dicer* using Nkx2.5Cre driver mice resulted in embryonic lethality with pericardial oedema and cardiac hypoplasia [4, 5]. Furthermore, *Dicer* inhibition using alpha-MHC-Cre mice also resulted in cardiac developmental impairment [6]. Thus, these studies highlight the importance of microRNA biogenesis for heart development. In addition, diverse studies have provided evidences of differential expression of microRNAs during heart development, both during embryogenesis [7–9] and at postnatal stages [10, 11] supporting a pivotal role of microRNAs during heart development. Moreover, recent studies reported microarray analyses which determine whether miRNAs are deregulated in common cardiovascular physiopathological conditions, such as hypertrophic and/or dilated cardiomyopathy, heart failure, or atrial fibrillation [12–17]. Taken together these data demonstrate a key role for microRNAs in cardiac development and disease. However, the role of individual microRNAs in these settings remains largely unknown.

We have previously reported a discrete number of differentially expressed microRNAs during cardiac development and we further elaborated on the functional role of miR-27 as regulator of the transcription factor *Mef2c* [7]. Furthermore, we recently reported that a large number of these microRNAs also display differential expression during iPS-derived cardiomyogenesis [18]. Among those, miR-125 displayed

Table 1: List of the oligonucleotides sequences used in the qPCR assays. Note that all primers were designed using the Primer3 (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) online tool, fixing the primer length to 100–200 nucleotides and an annealing temperature of 60°C. $MgCl_2$ concentration was always the same since SSOFast EvaGreen Master mix was used in all qPCR experiments.

Gapdh	Fw: 5′-TCTTGCTCAGTGTCCTTGCTGG-3′ Rv: 5′-TCCTGGTATGACAATGAATACGC-3′	180 pb
β-Actin	Fw: 5′-CCAGAGGCATACAGGGAC-3′ Rv: 5′-TGAGGAGCACCCTGTGCT-3′	144 pb
Myocd	Fw: 5′-TTTTCAATTCCATCCCCAAC-3′ Rv: 5′-CCCAGGGATCTTTGGAATTT-3′	210 pb
Mdfi	Fw: 5′-CAGGCTCTGAACAGCATTGA-3′ Rv: 5′-GGTTCTGAGAGGTGGTCGTG-3′	125 pb
Mstn	Fw: 5′-GGCTCTTTGGAAGATGACGA-3′ Rv: 5′-GGAGTCTTGACGGGTCTGAG-3′	188 pb
Runx1	Fw: 5′-TACCTGGGATCCATCACCTC-3′ Rv: 5′-GACGGCAGAGTAGGGAACTG-3′	164 pb
Mef2c	Fw: 5′-GGGGTGAGTGCATAAGAGGAC-3′ Rv: 5′-AGAAGAAACACGGGGACTATGGG-3′	288 pb
Mef2d	Fw: 5′-TCTCCCAGTCTACCCACTCG-3′ Rv: 5′-CAGGTGAACTGAAGGCTGGT-3′	162 pb

increased expression during both cardiac development and iPS-derived cardiomyogenesis, suggesting that it might play a pivotal role during muscle development. We therefore went on into this study dissecting the discrete role of miR-27 and miR-125, respectively, regulating muscle-enriched transcription factors.

2. Material and Methods

2.1. Cell Culture and MicroRNA Transfection Assays. HL1 cells ($6 * 10^5$ cells per well) [19] and Sol8 (ATCC, USA) cells were transfected with corresponding pre-miR (Ambion, USA), respectively, at 50 nM using lipofectamine 2000 (Invitrogen, USA) according to manufacturer's guidelines. Negative controls included nontransfected cells as well as FAM-labeled pre-miR negative control transfected cells, which also allowed transfection efficiency evaluation. In all cases, transfection efficiencies were greater than 50%, as revealed by observation of FAM-labeled pre-miR transfection. After 4 hours after transfection, HL1 cells were cultured in appropriate cell culture media and collected after 48 hours as previously reported [7, 20].

2.2. qRT-PCR Analyses. mRNA qRT-PCR was performed in Mx3005Tm QPCR System with an MxPro QPCR Software 3.00 (Stratagene, USA) and SSOFast EvaGreen detection system (BioRad, USA). Two internal controls, mouse *β-actin* and *Gapdh*, were used in parallel for each run. Each PCR reaction was performed at least three times to obtain representative averages. Primers sequences are provided in Table 1.

MicroRNA qRT-PCR was performed using Exiqon LNA microRNA qRT-PCR primers and detection kit according to manufacturer's guidelines. All reactions were always run in triplicate using 5S as normalizing control, as recommended by the manufacturer. The Livak & Schmittgen method was used to analyze the relative quantification RT-PCR data [21] and normalized in all cases taking as 100% the wild-type (control) value, as previously described [22].

3. Results

3.1. Search for Muscle-Enriched Transcription Factor Candidate Targets for miR-27 and miR-125. Using TargetScan search engine, mouse miR-27 is predicted to target over a thousand genes. Hand-curate literature search on PubMed indicating previous involvement of these predicted targets in the cardiovascular and/or skeletal muscle biology setting suggests that approximately only a third of these genes (324/1002; ~32%) might be targeted in this context. A subclassification of these genes demonstrates that approximately ~13% corresponded to transcription factors. Among them, *Runx1* and *Mef2c* have been already validated as direct miR-27 targets [7, 23]. We focus our attention on those transcription factors playing a role in either cardiac or skeletal muscle development, such as myostatin (*Mstn*), myocardin (*Myocd*), and MyoD family inhibitor (*Mdfi*). Using a similar approach, we also search for putative muscle-related transcription factors that might be putatively targeted by miR-125, which resulted in the identification of myocyte enhancer factor 2D (*Mef2d*).

3.2. Divergent Tissue-Specific miRNA Effects in Muscle Cells. In order to dissect the functional role of miR-27 and miR-125 in muscle cells, we overexpressed these microRNAs in two distinct muscle cell types, Sol8 skeletal muscle myoblasts and HL1 atrial cardiomyocytes, respectively. After 48 hours of transfection, expression levels of these microRNAs and distinct muscle-enriched transcription factors were measured by qPCR as compared to lipofectamine nontransfected control cells. Figure 1 demonstrates that similar levels of microRNA overexpression were achieved for miR-27 and

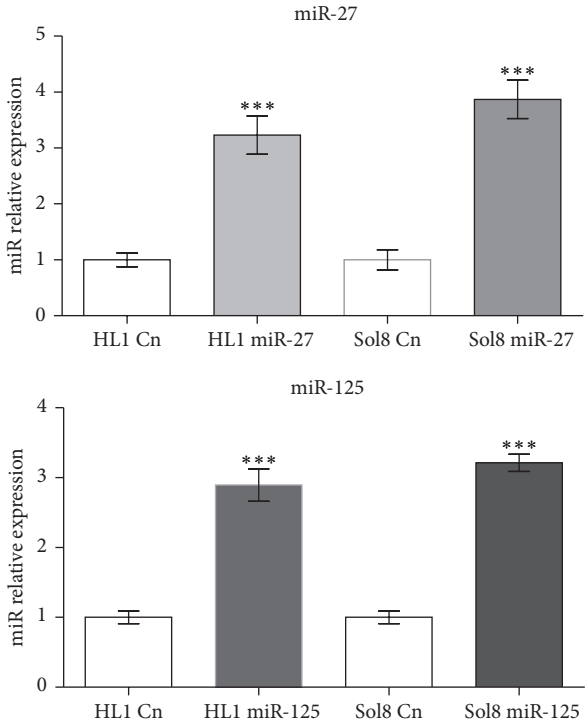


Figure 1: qPCR analyses of miR-27 and miR-125 expression levels in HL1 and Sol8 cells transfected with pre-miR-27 and pre-miR-125, respectively, as compared to nontransfected (lipofectamine only) control cells. Observe that a similar overexpression level is achieved for both miR-27 and miR-125 in HL1 and Sol8 cells, respectively ($n = 3$). $^{***}P < 0.001$, $^{****}P < 0.0001$.

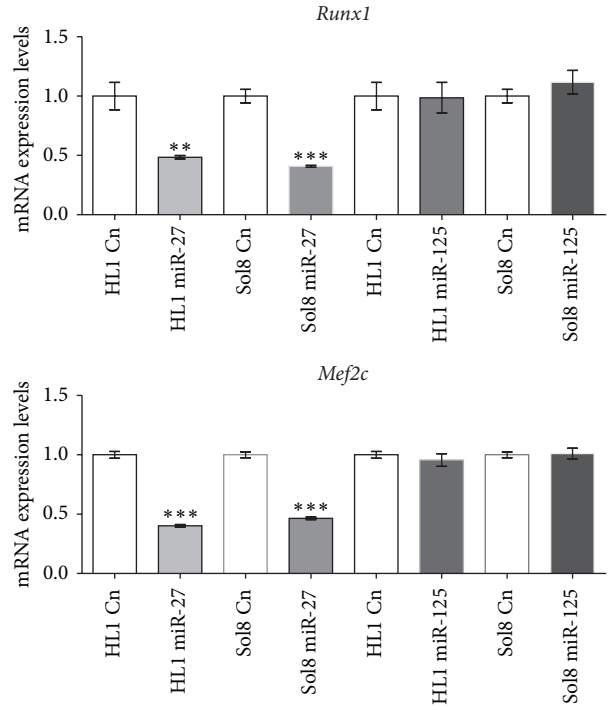


Figure 2: qPCR analyses of *Runx1* and *Mef2c* expression levels in HL1 and Sol8 cells transfected with pre-miR-27 and pre-miR-125, respectively, as compared to nontransfected (lipofectamine only) control cells. Note that *Runx1* and *Mef2c* expression levels are significantly downregulated in miR-27 but not in miR-125, overexpressing cells (HL1 and Sol8) ($n = 3$). $^{**}P < 0.01$, $^{***}P < 0.001$.

miR-125, respectively, in HL1 and Sol8 cells. Furthermore, functional assessment of microRNA overexpression was assayed by measuring *Mef2c* and *Runx1* expression levels, since these transcription factors were previously reported as direct targets of miR-27 [7, 23]. Figure 2 shows that overexpression of miR-27 selectively results in downregulation of both *Mef2c* and *Runx1* in cardiomyocytes (HL1) and skeletal myoblasts (Sol8), whereas no significant changes were observed upon miR-125 overexpression. Interestingly, selectively overexpression of miR-27 leads to downregulation of *Mstn* in HL1 atrial cardiomyocytes but not in Sol8 skeletal muscle myoblasts, as illustrated in Figure 3. On the other hand, miR-27 overexpression leads to significant upregulation of *Myocd* in HL1 atrial cardiomyocytes, whereas no changes are observed in Sol8 cells (Figure 3). Surprisingly, overexpression of miR-27 results in downregulation of *Mdfi* in HL1 cardiomyocytes and *Mdfi* upregulation in Sol8 cells. Importantly, expression of *Mstn*, *Myocd,* or *Mdfi* is not altered in Sol8 or HL1 cells after miR-125 expression, supporting the miR-27 specificity of these effects (Figure 3).

In line with the data obtained for miR-27, overexpression of miR-125 resulted in selective upregulation of *Mef2d* in HL1 atrial cardiomyocytes and *Mef2d* downregulation in Sol8 cells. Importantly, miR-27 overexpression led to no significant changes of *Mef2d* expression neither in Sol8 nor in HL1 cells.

4. Discussion

MicroRNAs have been demonstrated to play essential roles in multiple biological processes, such as embryonic development, cell tissue specification, and cell proliferation, as well as in distinct pathological conditions, such as cancer and cardiovascular diseases. miR-27 has been indeed implicated in several of these contexts, such as embryonic development [7], angiogenesis [24, 25], adipogenesis [26–28], and atherosclerosis [29]. In particular, miR-27 has been reported to selectively regulate *Pax3* [20, 30], *Runx1* [23], and *Mef2c* [7]. Similarly, miR-125 has been documented to play essential roles in stem cell differentiation [31, 32] and distinct cancer types [33–35], yet its role in muscle biology is more elusive [36].

In this study, we report that miR-27 overexpression leads to selective downregulation of *Mstn* and *Mdfi* in HL1 atrial cardiomyocytes, suggesting a direct role of miR-27 regulating these genes. Surprisingly, miR-27 overexpression leads to upregulation of *Myocd* in HL1 atrial cardiomyocytes. Selective microRNA-mediated downregulation of target genes is widely documented [37, 38], although some reports also demonstrate upregulation of target genes [39, 40] such as for miR-373 [Place et al., 2007]. Thus, our data suggest that miR-27 can equally act upregulating or downregulating genes in the cardiac muscle context. Intriguingly, in Sol8

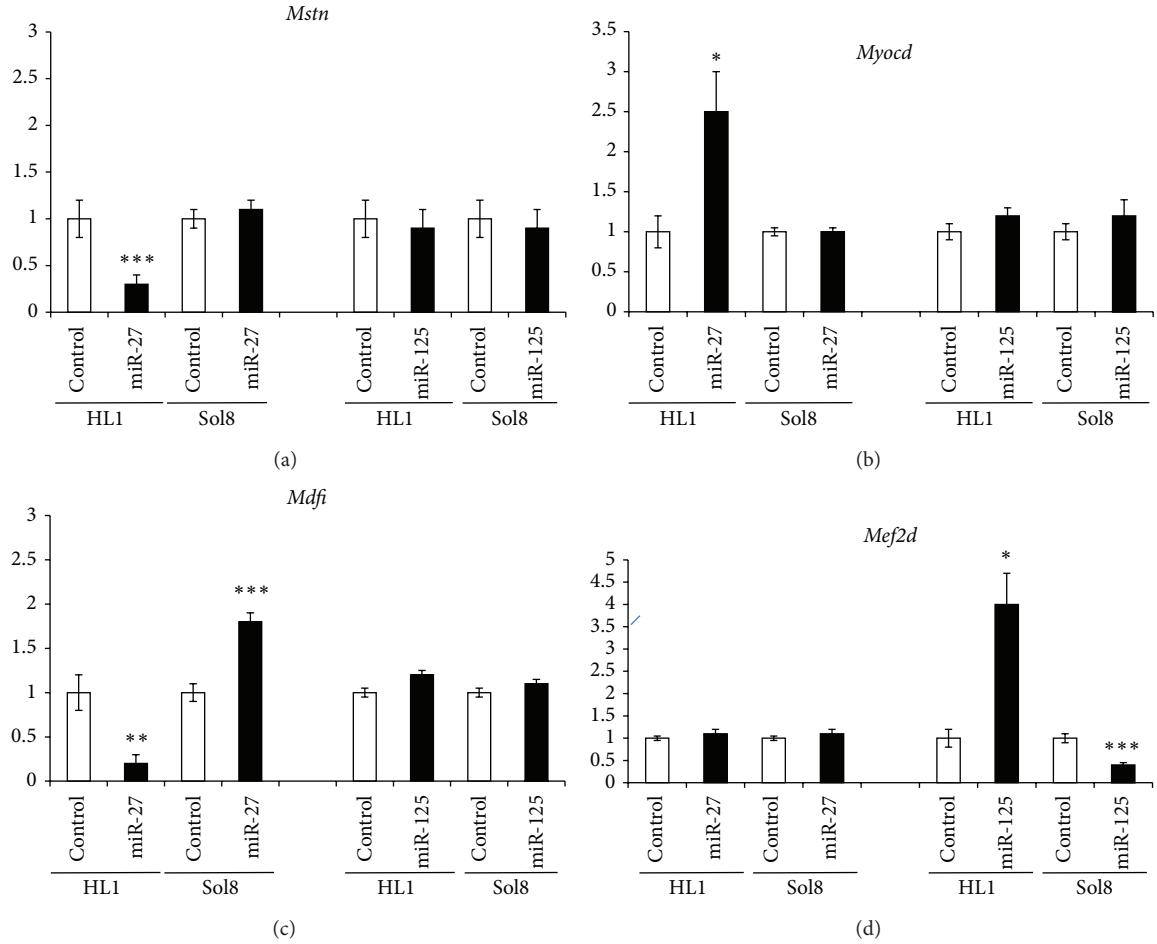


Figure 3: qPCR analyses of *Mstn* (a), *Myocd* (b), *Mdfi* (c), and *Mef2d* (d) expression in HL1 and Sol8 cells, respectively, transfected with miR-27 and miR-125, as stated in the corresponding panel. Observe that overexpression of miR-27 leads to downregulation of *Mstn* and upregulation of *Myocd* in HL1 cells, but not in Sol8 cells, while miR-125 overexpression does not alter any of these genes. Importantly, miR-27 overexpression downregulates *Mdfi* in HL1 cells, while it is upregulated in Sol8 cells. In the case of miR-125 overexpression, a similar effect is observed for *Mef2d* but in reverse mode; that is, miR-125 upregulates *Mef2d* in HL1 cells and downregulates it in Sol8 cells ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

cells, miR-27 overexpression does not alter *Mstn* or *Myocd*, yet it upregulates *Mdfi*. These data suggest that miR-27 does not regulate *Mstn* and *Myocd* in the skeletal muscle context, while it upregulates *Mdfi*. Taken together these data demonstrate a cell-type specific role of miR-27 for the same target genes. A similar finding is also documented for miR-125, since miR-125 overexpression in HL1 atrial cardiomyocytes upregulates *Mef2d*, while it is downregulated in Sol8 cells.

To our knowledge this is the first report that demonstrates distinct effects for a single microRNA for the same target gene in distinct cellular contexts. In a mechanistic way, this implies that miR-27 is capable of interacting with *Mstn* and *Mdfi* 3′UTR in cardiomyocytes but not in skeletal myoblasts, while miR-125 upregulates and downregulates *Mef2d* depending on the cell context. Importantly, overexpression of miR-125 does not modify expression of *Mstn*, *Myocd*, or *Mdfi* in any cell context, while miR-27 overexpression does not alter *Mef2d* expression in HL1 or Sol8 cells. These findings reinforce the notion of a specific regulatory role of miR-27 in *Mstn*, *Myocd*, or *Mdfi* and of miR-125 in *Mef2d*, in line with TargetScan predictions. Furthermore, they suggest that either a selective blocking mechanism is operative in one cell type, for example, skeletal myoblasts, or a coadjuvant facilitating interactive factor is exclusively expressed in the other cell type, for example, cardiomyocytes. Further research is required to sort out these hypotheses. We are aware that our biological assay does not provide direct biochemical evidence of microRNA-mRNA interaction, yet it reveals the overall biological output of miR-27/miR-125 overexpression, respectively. However, it is important to realize that 3′UTR luciferase report assays in heterologous systems, such as 3T3 fibroblasts or HeLa cells, will be rather inappropriate to give the cell-type specific effects revealed in our assays.

In summary, we provide evidence that miR-27 and miR-125, respectively, can selectively upregulate and downregulate discrete number of target mRNAs in a cell-type specific manner.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

References

[1] D. P. Bartel, "MicroRNAs: genomics, biogenesis, mechanism, and function," *Cell*, vol. 116, no. 2, pp. 281–297, 2004.

[2] R. A. Espinoza-Lewis and D.-Z. Wang, "MicroRNAs in heart development," *Current Topics in Developmental Biology*, vol. 100, pp. 279–317, 2012.

[3] W.-J. Chen, K. Yin, G.-J. Zhao, Y.-C. Fu, and C.-K. Tang, "The magic and mystery of MicroRNA-27 in atherosclerosis," *Atherosclerosis*, vol. 222, no. 2, pp. 314–323, 2012.

[4] Y. Zhao, J. F. Ransom, A. Li et al., "Dysregulation of cardiogenesis , cardiac conduction, and cell cycle in mice lacking miRNA-1-2," *Cell*, vol. 129, no. 2, pp. 303–317, 2007.

[5] A. Saxena and C. J. Tabin, "miRNA-processing enzyme Dicer is necessary for cardiac outflow tract alignment and chamber septation," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 1, pp. 87–91, 2010.

[6] J.-F. Chen, E. P. Murchison, R. Tang et al., "Targeted deletion of Dicer in the heart leads to dilated cardiomyopathy and heart failure," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 6, pp. 2111–2116, 2008.

[7] A. Chinchilla, E. Lozano, H. Daimi et al., "MicroRNA profiling during mouse ventricular maturation: a role for miR-27 modulating Mef2c expression," *Cardiovascular Research*, vol. 89, no. 1, pp. 98–108, 2011.

[8] J. E. Babiarz, M. Ravon, S. Sridhar et al., "Determination of the human cardiomyocyte mRNA and miRNA differentiation network by fine-scale profiling," *Stem Cells and Development*, vol. 21, no. 11, pp. 1956–1965, 2012.

[9] C. Vacchi-Suzzi, F. Hahne, P. Scheubel et al., "Heart structure-specific transcriptomic atlas reveals conserved microRNA-mRNA interactions," *PLoS ONE*, vol. 8, no. 1, Article ID e52442, 2013.

[10] J. Hsu, P. Hanna, D. R. Van Wagoner et al., "Whole genome expression differences in human left and right atria ascertained by RNA sequencing," *Circulation: Cardiovascular Genetics*, vol. 5, no. 3, pp. 327–335, 2012.

[11] E. R. Porrello, A. I. Mahmoud, E. Simpson et al., "Regulation of neonatal and adult mammalian heart regeneration by the miR-15 family," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 110, no. 1, pp. 187–192, 2013.

[12] E. Van Rooij, L. B. Sutherland, N. Liu et al., "A signature pattern of stress-responsive microRNAs that can evoke cardiac hypertrophy and heart failure," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 48, pp. 18255–18260, 2006.

[13] E. Van Rooij, L. B. Sutherland, J. E. Thatcher et al., "Dysregulation of microRNAs after myocardial infarction reveals a role of miR-29 in cardiac fibrosis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 35, pp. 13027–13032, 2008.

[14] S. Ikeda, S. W. Kong, J. Lu et al., "Altered microRNA expression in human heart disease," *Physiological Genomics*, vol. 31, no. 3, pp. 367–373, 2007.

[15] C. Sucharov, M. R. Bristow, and J. D. Port, "miRNA expression in the failing human heart: functional correlates," *Journal of Molecular and Cellular Cardiology*, vol. 45, no. 2, pp. 185–192, 2008.

[16] X. Luo, H. Zhang, J. Xiao, and Z. Wang, "Regulation of human cardiac Ion channel genes by MicroRNAs: theoretical perspective and pathophysiological implications," *Cellular Physiology and Biochemistry*, vol. 25, no. 6, pp. 571–586, 2010.

[17] N. Cooley, M. J. Cowley, R. C. Y. Lin et al., "Influence of atrial fibrillation on microRNA expression profiles in left and right atria from patients with valvular heart disease," *Physiological Genomics*, vol. 44, no. 3, pp. 211–219, 2012.

[18] F. Bonet, F. Hernandez-Torres, F. J. Esteban, A. Aranega, and D. Franco, "Comparative analyses of microrna microarrays during cardiogenesis: functional perspectives," *Microarrays*, vol. 2, pp. 81–96, 2013.

[19] W. C. Claycomb, N. A. Lanson Jr., B. S. Stallworth et al., "HL-1 cells: a cardiac muscle cell line that contracts and retains phenotypic characteristics of the adult cardiomyocyte," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 6, pp. 2979–2984, 1998.

[20] E. Lozano-Velasco, A. Contreras, C. Crist, F. Hernández-Torres, D. Franco, and A. E. Aránega, "Pitx2c modulates Pax3+/Pax7+ cell populations and regulates Pax3 expression by repressing miR27 expression during myogenesis," *Developmental Biology*, vol. 357, no. 1, pp. 165–178, 2011.

[21] K. J. Livak and T. D. Schmittgen, "Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method," *Methods*, vol. 25, no. 4, pp. 402–408, 2001.

[22] J. N. Domínguez, F. Navarro, D. Franco, R. P. Thompson, and A. E. Aránega, "Temporal and spatial expression pattern of β1 sodium channel subunit during heart development," *Cardiovascular Research*, vol. 65, no. 4, pp. 842–850, 2005.

[23] J. Feng, A. Iwama, M. Satake, and K. Kohu, "MicroRNA-27 enhances differentiation of myeloblasts into granulocytes by post-transcriptionally downregulating Runx1," *British Journal of Haematology*, vol. 145, no. 3, pp. 412–423, 2009.

[24] Q. Zhou, R. Gallagher, R. Ufret-Vincenty, X. Li, E. N. Olson, and S. Wang, "Regulation of angiogenesis and choroidal neovascularization by members of microRNA-23~27~24 clusters," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, no. 20, pp. 8287–8292, 2011.

[25] C. Urbich, D. Kaluza, T. Frömel et al., "MicroRNA-27a/b controls endothelial cell repulsion and angiogenesis by targeting semaphorin 6A," *Blood*, vol. 119, no. 6, pp. 1607–1616, 2012.

[26] T. Kang, W. Lu, W. Xu et al., "MicroRNA-27 (miR-27) targets prohibitin and impairs adipocyte differentiation and mitochondrial function in human adipose-derived stem cells," *Journal of Biological Chemistry*, vol. 288, no. 48, pp. 34394–34402, 2013.

[27] L. Sun and M. Trajkovski, "MiR-27 orchestrates the transcriptional regulation of brown adipogenesis," *Metabolism: Clinical and Experimental*, vol. 63, no. 2, pp. 272–282, 2014.

[28] Y. Zhu, X. Zhang, X. Ding et al., "miR-27 inhibits adipocyte differentiation via suppressing CREB expression," *Acta Biochimica et Biophysica Sinica*, vol. 46, no. 7, pp. 590–596, 2014.

[29] M. Zhang, J.-F. Wu, W.-J. Chen et al., "MicroRNA-27a/b regulates cellular cholesterol efflux, influx and esterification/hydrolysis in THP-1 macrophages," *Atherosclerosis*, vol. 234, no. 1, pp. 54–64, 2014.

[30] C. G. Crist, D. Montarras, G. Pallafacchina et al., "Muscle stem cell behavior is modified by microRNA-27 regulation of Pax3 expression," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 32, pp. 13383–13387, 2009.

[31] C. Boissart, X. Nissan, K. Giraud-Triboult, M. Peschanski, and A. Benchoua, "miR-125 potentiates early neural specification of human embryonic stem cells," *Development*, vol. 139, no. 7, pp. 1247–1257, 2012.

[32] S. Emmrich, M. Rasche, J. Schöning et al., "*miR-99a/100~125b* tricistrons regulate hematopoietic stem and progenitor cell homeostasis by shifting the balance between TGFβ and Wnt signaling," *Genes & Development*, vol. 28, no. 8, pp. 858–874, 2014.

[33] M. Zhou, Z. Liu, Y. Zhao et al., "MicroRNA-125b confers the resistance of breast cancer cells to paclitaxel through suppression of pro-apoptotic Bcl-2 antagonist killer 1 (Bak1) expression," *The Journal of Biological Chemistry*, vol. 285, no. 28, pp. 21496–21507, 2010.

[34] M. Leotta, L. Biamonte, L. Raimondi et al., "A p53-dependent tumor suppressor network is induced by selective miR-125a-5p inhibition in multiple myeloma cells in vitro," *Journal of Cellular Physiology*, vol. 229, no. 12, pp. 2106–2116, 2014.

[35] J. X. Jiang, S. Gao, Y. Z. Pan, C. Yu, and C. Y. Sun, "Overexpression of microRNA-125b sensitizes human hepatocellular carcinoma cells to 5-fluorouracil through inhibition of glycolysis by targeting hexokinase II," *Molecular Medicine Reports*, vol. 10, no. 2, pp. 995–1002, 2014.

[36] X. Wang, T. Ha, J. Zou et al., "MicroRNA-125b protects against myocardial ischaemia/reperfusion injury via targeting p53-mediated apoptotic signalling and TRAF6," *Cardiovascular Research*, vol. 102, no. 3, pp. 385–395, 2014.

[37] A. Heidersbach, C. Saxby, K. Carver-Moore et al., "MicroRNA-1 regulates sarcomere formation and suppresses smooth muscle gene expression in the mammalian heart," *eLife*, vol. 2, Article ID e01323, 2013.

[38] K. Wystub, J. Besser, A. Bachmann, T. Boettger, and T. Braun, "miR-1/133a clusters cooperatively specify the cardiomyogenic lineage by adjustment of myocardin levels during embryonic heart development," *PLoS Genetics*, vol. 9, no. 9, Article ID e1003793, 2013.

[39] S. Vasudevan, "Posttranscriptional upregulation by microRNAs," *Wiley Interdisciplinary Reviews: RNA*, vol. 3, no. 3, pp. 311–330, 2012.

[40] S. Lee and S. Vasudevan, "Post-transcriptional stimulation of gene expression by MicroRNAs," *Advances in Experimental Medicine and Biology*, vol. 768, pp. 97–126, 2013.

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 105620, 15 pages
http://dx.doi.org/10.1155/2015/105620

Review Article

Update on the Pathogenic Implications and Clinical Potential of microRNAs in Cardiac Disease

Mario Notari,[1] Julián Pulecio,[1,2] and Ángel Raya[1,2,3,4]

[1]*Center of Regenerative Medicine in Barcelona (CMRB), Barcelona Biomedical Research Park, Dr. Aiguader 88, 08003 Barcelona, Spain*
[2]*Center for Networked Biomedical Research on Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), Spain*
[3]*Control of Stem Cell Potency Group, Institute for Bioengineering of Catalonia (IBEC), Barcelona Science Park, Baldiri Reixac 15-21, 08028 Barcelona, Spain*
[4]*Institució Catalana de Recerca i Estudis Avançats (ICREA), Spain*

Correspondence should be addressed to Mario Notari; mnotari@cmrb.eu and Ángel Raya; araya@cmrb.eu

Received 18 September 2014; Accepted 19 December 2014

Academic Editor: Chi-Hsiao Yeh

miRNAs, a unique class of endogenous noncoding RNAs, are highly conserved across species, repress gene translation upon binding to mRNA, and thereby influence many biological processes. As such, they have been recently recognized as regulators of virtually all aspects of cardiac biology, from the development and cell lineage specification of different cell populations within the heart to the survival of cardiomyocytes under stress conditions. Various miRNAs have been recently established as powerful mediators of distinctive aspects in many cardiac disorders. For instance, acute myocardial infarction induces cardiac tissue necrosis and apoptosis but also initiates a pathological remodelling response of the left ventricle that includes hypertrophic growth of cardiomyocytes and fibrotic deposition of extracellular matrix components. In this regard, recent findings place various miRNAs as unquestionable contributing factors in the pathogenesis of cardiac disorders, thus begging the question of whether miRNA modulation could become a novel strategy for clinical intervention. In the present review, we aim to expose the latest mechanistic concepts regarding miRNA function within the context of CVD and analyse the reported roles of specific miRNAs in the different stages of left ventricular remodelling as well as their potential use as a new class of disease-modifying clinical options.

1. Introduction

Heart or cardiac disease is an overreaching term used to describe a large family of conditions that affect the myocardial muscle. Disorders that fall under this category include blood vessel illnesses such as coronary artery disease, heart rhythm conditions termed arrhythmias, and, in general, all types of cardiomyopathies. The term cardiac disease is often incorrectly used interchangeably with the term cardiovascular disease (CVD). However, CVDs comprise a broad class of disorders that involve narrowed or blocked blood vessel(s) that can lead to myocardial infarction (MI) and heart failure (HF). In both China and the United States, CVDs were the major cause of morbidity and mortality in 2012 according to the World Health Organization [1]. Moreover, the patients that survive heart injuries experience, at best, progressive deterioration of their heart function unequivocally leading to HF [2]. Altogether, this information underscores the need to develop new therapeutic strategies potentially appointed to regenerate the damaged cardiac tissue.

Notably, a MI episode can lead to the annihilation within a few hours of about 25% of the 2–4 billion cardiomyocytes present in the human left ventricle [3]. The response to MI as well as to other forms of cardiac insults is described as left ventricular remodeling which involves all the structural, molecular, and functional adaptations of the heart in response to injury [2]. It is usually associated with increased volume and altered chamber configuration of the heart driven by a combination of pathological changes that comprise cardiomyocyte hypertrophy, myocyte apoptosis, myofibroblast proliferation, and interstitial fibrosis [2]. In particular, a MI involves acute oxygen deprivation to

a portion of the ventricle leading to a confined area of ischemic cardiac cells, termed the infarct area. Given that adult cardiomyocytes have a very low intrinsic proliferation rate [4], vast replacement of damaged cardiomyocytes is precluded and a collagen-rich scar is formed instead. Hence, following an initial robust inflammatory response where dead cells are cleared by macrophage activity, cardiac fibroblasts infiltrate the damaged area to increase myocardial flexibility via deposition of extracellular matrix components [5], which marks the onset of the interstitial fibrosis process. While scarring culminates in a relatively quick preservation of myocardial integrity, it compromises the contractile activity of the heart permanently. Despite a wide range of available therapeutic options, the prevalence, mortality, and costs associated with CVDs continue to increase in the developed world as well as in the developing countries [6]. Current treatment options are limited to a surgical intervention to either restore patency or bypass affected coronary arteries, the application of passive cardiac support devices, and/or cardiac transplantation [6]. Although the ideal approach for HF patients would be to attempt myocyte replacement therapy with the objective of restoring cardiac muscle mass and function, current treatments and prospective research advances are geared towards the stimulation of the endogenous cardiac repair mechanisms [7–9]. Given the lack of a therapeutic approach to reverse the loss-of-functional myocardium, the development of efficient regenerative procedures is an urgent need in the field of modern cardiovascular research.

As an alternative, a promising cell-free strategy is based on the use of small molecules or paracrine factors to stimulate cardiomyocyte proliferation or differentiation of resident cardiac cells [10–12]. Recently, a new class of small noncoding RNAs or microRNAs (miRNAs) were identified as important posttranscriptional inhibitors of gene expression through their ability to block the translation of messenger RNA (mRNA) [13]. Hence, depending upon the presence and expression of a specific miRNA and/or its target as well as the physiological state of a cell, a miRNA can act as a "fine tuner" or even as an on/off switch of gene expression [13]. In this context, changes in miRNA levels as they relate to murine and human heart disorders and their implications in the development of different CVDs have been recently recognized; thus, certain miRNA expression signature patterns were shown to correlate with HF and cardiac hypertrophy [14]. To date, various studies have illustrated changes in miRNAs expression in a variety of human heart conditions such as MI [15], cardiac hypertrophy and fibrosis [16], and developmental heart disease [17]. They showed that the expression of numerous miRNAs is altered in such pathological processes and that different types of heart disease are associated with distinct changes in miRNA expression [18]. Therefore, understanding the regulation of molecules of mRNA biogenesis in both stem and cardiac progenitor cells will lead to a better understanding of the pathophysiological development of CVDs as well as contributing towards the development of novel therapeutic targets that promote endogenous cardiovascular regeneration. Not surprisingly, research on miRNAs in relation to the physiology and pathology of CVDs has become a rapidly evolving field. Recently, several excellent reviews have analyzed the progress made in understanding the role of miRNA therapeutics within the CVDs field [19–21] and circulating biomarkers within the blood stream for early detection of specific CVDs pathologies [22]. In this review, we examine the latest progress regarding the roles of miRNAs in the left ventricle remodeling process, as well as their potential for therapeutic intervention. We will focus our attention on the mechanistic concepts of miRNA function within the context of CVD progression and consider the specific roles of representative miRNAs in the different phases of left ventricular remodeling.

2. The Biology of miRNA: Overview

miRNAs are 21- to 23-nucleotide- (nt-) long noncoding RNAs able to modulate gene expression by targeting genes at the posttranscriptional level in a tissue-specific fashion. Thus, miRNAs bind mostly to the $3'$ untranslated region of target genes and inhibit gene expression translationally and/or by destabilizing the target mRNA [13]. miRNAs are transcribed as regular genes from DNA by RNA polymerase II into primary miRNAs, which are processed to pre-miRNAs (70-nt stem loop oligonucleotides) in the nucleus by the Drosha complex (a type III RNase complex), then exported to the cytoplasm, and further cleaved by the DICER1 complex into 22-nt double stranded fragments [23] (miRNA duplex, Figure 1). At this point, miRNAs can be discharged to the extracellular space within vesicles (i.e., exosomes) responsible for miRNA-mediated cell-to-cell communication. *In vitro* experimentations proved that endothelial cell line culture overexpressing the transcription factor Kruppel-like factor 2 (KLN2) was able to generate extracellular vesicles highly enriched in miRNAs. KLN2 is an important transcription factor involved in atheroprotective stimuli and able to enhance the expression of a particular cluster of miRNAs [24]. Similarly, the finding of circulating miRNAs in different human fluids indicates a conceivable role of miRNAs as paracrine signaling mediators *in vivo,* suggesting a crucial role of miRNAs in cell-cell communication and paracrine signaling during either physiological or pathological processes [24, 25]. Of note, involvement of miRNAs/vesicle-mediated communication has been discovered between different populations of myocardial cells such as endothelial and smooth muscle cells (SMC) [24] and also between fibroblasts and cardiomyocytes as well [26]. This latter study demonstrated a crosstalk between cardiac fibroblast and cardiomyocytes through extracellular vesicles highly enriched in miR-21*, a documented inducer of cardiac hypertrophy [26]. Alternatively, one of the strands of the miRNA can be selectively loaded into the RISC complex, which is responsible for binding to target mRNAs and repressing their expression through degradation or translational inhibition (Figure 1). In particular, a model for cytoplasmic mRNA repression/decay regulation has been proposed [27], in which argonaute (an essential protein of the RISC complex) directly interacts with the P-bodies (cellular foci involved in mRNA turnover containing most of the proteins related with miRNA gene silencing) [28] (Figure 1). Additionally, some miRNAs are

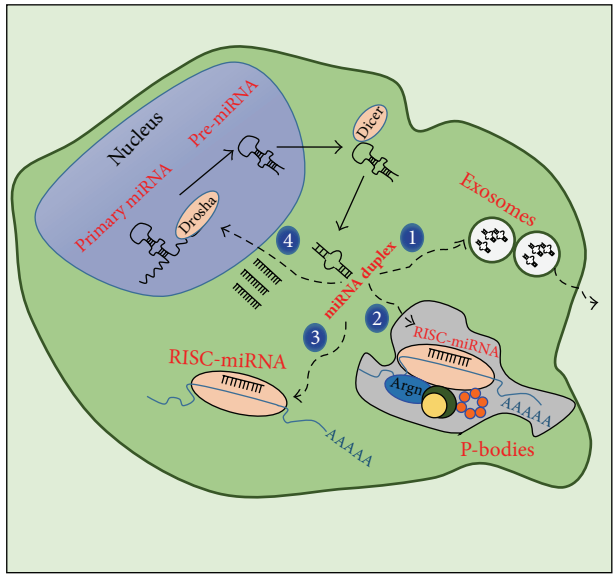


Figure 1: Representative scheme summarizing the biogenesis of the miRNA. The cartoon depicts the four main pathways followed by miRNA after extranuclear cleavage by DICER. The miRNA duplex can be processed to single mature miRNA and loaded into exosomes or microvesicles ready for extracellular release (1) or aggregates in P-bodies (2), a new model of mRNA regulation involving miRNA and other specific proteins [122]. However, miRNAs are preferentially loaded into the RISC complex in the cytoplasm, inducing silencing of target mRNA (3). Alternatively, miRNAs are able to enter back into the nucleus and directly bind the promoter region of target genes (4) and influence their expression. Argn = argonaute.

able to reenter the nucleus and directly bind to the promoter region of target genes to regulate their expression [29] (Figure 1).

Most miRNAs are predicted to target several mRNAs and most mRNAs have predicted binding sites for multiple miRNAs. In this manner, a single miRNA can affect an entire signaling pathway, converting them into appealing tools for cell-fate conversion approaches. Therefore, miRNAs perform diverse roles in many different cellular, developmental, and physiological processes [30, 31]. The relevance of the miRNAs as key players in phenotype specification mechanisms has been extensively confirmed by studies showing that the forced overexpression of miRNAs specifically upregulated in neuronal cells, embryonic stem cells, osteoblasts, blood progenitors, and cardiomyocyte stem cells, in specific culture conditions, was sufficient to induce the conversion of somatic cells towards each of these cell types [32, 33]. Hence, exogenous expression or inhibition of cardiac related miRNAs would presumably be a suitable tool for therapeutic applications in the context of CVD.

Relating to genomic organization, miRNAs are often clustered within miRNA families, comprising mature species similar enough to have evolved from a common ancestor and which are often regulated as a single cistron [34, 35]. This agrees with the notion that a family of miRNAs can affect a specific set of mRNAs in a redundant fashion, thereby significantly affecting their expression levels and hence the dynamics of any biological pathway.

A special mention in regards to the nomenclature of miRNAs, that is, the number assigned to a given primary miRNA, may not necessarily correspond to the same miRNA sequence across species. Moreover, the same miRNA sequence given or not the same nomenclature may not necessarily affect the same set of mRNAs in different species [35]. Hence, this lack of nomenclature consistency calls for caution when reading reports from research using different species' models.

3. miRNAs Implicated in Cardiac Development

The first evidence for an essential contribution of miRNAs in heart development came from analysis of transgenic animals with conditional knockout of the miRNA processing enzyme DICER1 in cardiac progenitor cells (CPCs), defined as cells expressing early cardiac-specific genes such as *Nkx2.5* [36]. Mouse embryos lacking DICER1 in CPCs died before birth with severe cardiac defects such as pericardial edema and a poorly developed ventricular myocardium [37]. Interestingly, conditional knockout DICER1 in the adult mouse myocardium induced rapid biventricular enlargement, characterized by myocyte hypertrophy, sarcomeric disarray, ventricular fibrosis, and strong induction of the fetal gene program [38]. Moreover, miR-1 and miR-133, two specific and widely conserved miRNAs derived from the same precursor transcript, have been well established as fundamental factors in cardiac development [39, 40]. However, while miR-1 and miR-133 cluster on the same chromosomal locus and are transcribed together in a tissue-specific manner, they have distinct roles in modulating skeletal muscle proliferation and differentiation [40]. For instance, mice lacking the miR-1-2 genomic locus developed a range of abnormalities such as ventricular septal defects, cardiac arrhythmia, and myocyte hyperplasia with nuclear division of cardiomyocytes persisting postnatally [37]. It was shown that miR-1 promoted myogenesis by targeting histone deacetylase 4 (HDAC4), a nuclear factor that represses the transcription of muscle specification genes [40]. In addition, increasing miR-1 levels in the developing heart lead to a decreased pool of proliferating cardiomyocytes via the targeting of Hand2 and thereby controlling cardiomyocyte differentiation and proliferation during cardiogenesis [39] (Figure 2). In regards to miR-133, it was shown to promote mesoderm formation in mouse embryonic stem cells [41]. However, mice lacking either the mir-133a-1 or miR-133a-2 variants were normal, while about half of double-mutant mice displayed lethal ventricular-septal defects already at 1 day postnatally (p1) and those that survived to adulthood developed signs of dilated cardiomyopathy and heart failure at around 4 months of age. These phenotypes were linked to deregulation of miR-133a-1 targets such as cyclin D2 and serum responsive factors (SRF) [42] (Figure 2). Interestingly, targeting EGFR expression via miR-133a-1 overexpression in human mesenchymal stem cells (MSCs) promoted cardiogenic differentiation [43]. Recently, another muscle-specific miRNA was shown to favor cardiac lineage differentiation of human CPC and rat MSCs. In

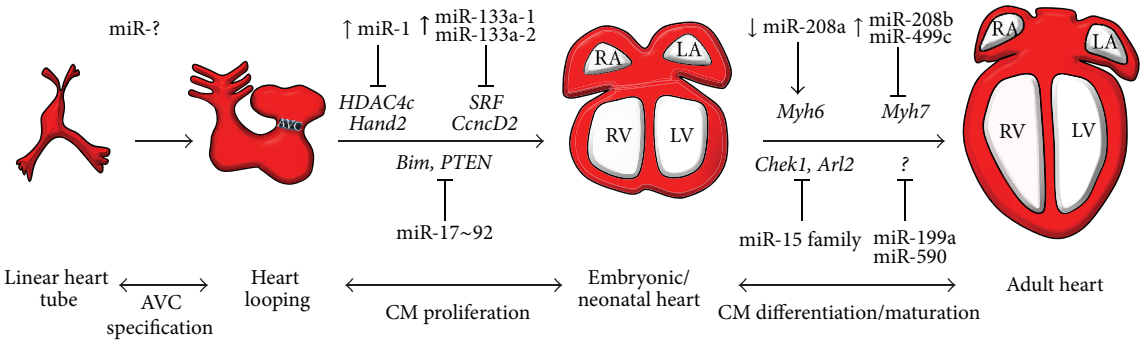


Figure 2: miRNAs control various stages of embryonic heart development. Schematic representation of the main stages of heart development. Murine cardiomyocyte progenitors become organized into a linear heart tube at day 7.5 post coitum (left). Subsequently, atrioventricular canal specification (AVC) as well as looping and swelling of the heart gives rise to the ventricular and atrial chambers. Between E13.5 and E15.5, the structure of the heart is complete and consists of the right atrium (RA), left atrium (LA), right ventricle (RV), and left ventricle (LV). miRNAs and their downstream targets are shown during embryonic heart development. Of note, compelling evidence for a critical role of miRNAs during AVC specification has been collected only in developing models such as zebrafish. It is however plausible that particular miRNAs also play an important role in AVC specification in mammals but has not yet been elucidated.

fact, miR-499 translocated from differentiated myocytes to CPCs via gap junctions promoting the differentiation of the latter into functionally differentiated cardiomyocytes [44], highlighting the role of miRNAs as paracrine factors that can influence gene expression and consequently cell behavior (Figure 2). This is of particular interest for the design of therapeutic strategies involving miRNAs as modulating agents considering that they are unusually stable in plasma and resistant to harsh conditions such as pH variation and high temperature [45]. Moreover, a 3′ mutation in the human miR-499 sequence altered the expression of different cardiac-specific mRNAs [46]. In a transgenic mouse model, it was similarly shown that increasing miR-499 expression resulted in myocardial hypertrophy and severe cardiac dysfunction by affecting the expression of early stress (i.e., *Erg1*, *Erg2*) and structural (i.e., *Myh7b*, *Acta1*) genes thereby magnifying the cardiac response to stress [47].

Finally, the miR-17~92 family, a well-known mRNAs cluster involved in cancer biology named OncomiR-1, consists of 6 different miRNAs all being described in different embryonic stages of heart development [48]. Data analysis from loss-of-function experimentations has suggested a specific role of the miR-17~92 cluster in ventricular septa specification as well as lung hypoplasia [48]. More importantly, the miR-17~92 family has emerged as important regulator of myocardial differentiation in particular in the specification of CPC in the second heart field which is required for normal outflow tract development [49] (Figure 2). Wang et al. demonstrated that the bone morphogenetic protein (BMP) signaling pathway triggers the transcription of several members of the miR-17~92 family which in turn repress the expression of cardiac progenitors genes such as *Isl-1* (ISL LIM homeobox 1)and *Tbx1* (T-box 1) [49].

At present, only scattered information is available on the expression of miRNAs in CPCs. In particular, a member of the miR-17~92 cluster, miR-17, was identified as the most differentially expressed miRNAs between neonatal and adult murine heart [50]. Preliminary attempts at manipulating CPCs behavior for stem cell therapy by altering miRNAs expression have met with limited success [44, 51]. However, an important role of miRNAs to improve homing, integration, and survival of CPCs engrafted in the infarcted myocardium has been described, and a mixture of miR-21, miR-24, and miR-221 is found to enhance the viability and survival of Sca-1$^+$ CPCs both *in vitro* and *in vivo* [52].

4. microRNAs and Myocardial Regeneration

Several reports support the notion that microRNAs play an important role in myocardial regeneration. Hence, an alternative option for restoring the number of loss myocytes in CVD patients may be through activation of endogenous mechanisms for cardiomyocyte proliferation, thereby increasing myocardial muscle mass. In order to achieve myocardial regeneration and push the development of efficient therapeutic options, it is essential to uncover molecular mechanisms that will support cardiac regeneration. By definition, regeneration is a complex biological process by which animals can restore the shape, structure, and function of body parts lost after injury or experimental amputation. For instance, a number of lower vertebrates such as the newt and axolotl are able to renew parts of their body and even entire organs during their lifetime [53]. In particular, adult zebrafish show a conspicuous capacity to regenerate large portions of their heart after partial resection [54–56]. Notably, zebrafish heart regeneration after partial amputation of the ventricular apex proceeds by *de novo* formation of myocardial tissue that is indistinguishable from the surrounding myocardium both functionally and histologically [57]. Moreover, in this model, cardiomyocytes produced during regeneration are unlikely to be derived from undifferentiated stem or progenitor cells. Rather, the source of regenerating tissue is preexisting cardiomyocytes that after dedifferentiation reenter the cell cycle in response to injury [58]. It was recently shown that many miRNAs display compelling changes and are involved in fundamental regenerative signaling during zebrafish fin

or organ regeneration [59]. Interestingly, a recent study purports that the neonatal mouse heart is also capable of complete regeneration following amputation of the apex or ischemic injury [60]. Hence, several groups have begun to investigate the involvement of microRNAs within the realms of the regeneration process using ventricular apex resection of the neonatal mouse heart, hoping this should be an ideal model to identify the miRNA profile regulating dedifferentiation and proliferation in the mammalian heart [61]. Although the mechanisms remain largely unknown, in zebrafish it was shown that miRNAs play a critical role in tissue regeneration [59]. For example, miR-133 was shown to promote cardiac regeneration in zebrafish [62], whereas miR-138 was associated with specification of the atrioventricular canal (AVC) which eventually gives rise to the cardiac valves indispensable for the unidirectional blood flow within the heart [63]. These observations prompted studies to ascertain whether similar mechanisms are present in the mammalian heart and, if so, whether experimental manipulation of miRNAs might trigger heart regeneration in mammals. More recently, Porrello and coworkers have established a model of ischemic MI in postnatal mice [64] and demonstrated that the neonatal heart can mount a regenerative response leading to cardiomyocyte proliferation of preexisting cardiomyocytes and a functional recovery in 21 days [60]. Interestingly, these authors also demonstrated that inhibition of the miR-15 family increased cardiomyocyte proliferation and improved ventricular systolic function after induction of MI in adult mice [64] (Figure 3). Members of the miR-15 family, including miR-15a, miR-15b, miR-16-1, miR-16-2, miR-195 and miR-497, have been implicated in cell cycle arrest and cell survival in different cell lineages via the regulation of many antiapoptotic genes and cell cycle inhibiting factors [65]. Members of this family are upregulated in the heart in response to stress conditions, MI, and cardiomyocyte death [16]. The multiplicity of miR-15 family members exemplifies one of the challenges associated with miRNA inhibition as a therapeutic strategy, as sequence divergence among different members of miRNA families prevents their collective inhibition by the delivery of a single antisense oligonucleotide inhibitor (antagomiR). Recent work performed using a model of ischemic and reperfusion in mice showed that inhibition of the miR-15 family reduced infarct size and improved cardiac function two weeks after delivering the injury [66]. Moreover, forced expression of miR-195 in heart was sufficient to cause cardiomyocyte loss leading to HF, and precocious activation of miR-195 in the heart suppressed the neonatal regeneration capacity of the murine heart [64]. The inhibitory influence of miR-195 on heart muscle regeneration appears to be attributable to the inhibition of a cohort of proliferative proteins and cell cycle inhibitors [64]. Hence, antagomiR-mediated inhibition of miR-15 family members represents an intriguing strategy to enhance cardiac repair following injury.

More recently, Senyo et al. found that a population of mononucleated proliferating cardiomyocytes appeared in the peri-infarct region in adult mice subjected to experimental MI, raising the rate of proliferating cardiomyocytes over the observed basal levels [67]. Interestingly, they also

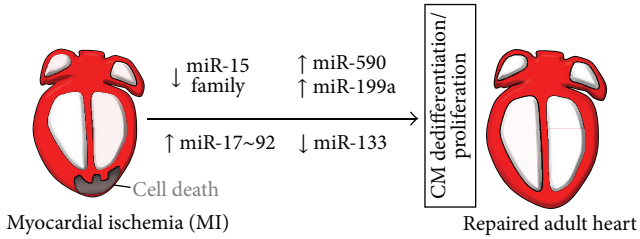


Figure 3: miRNAs can drive cardiac regeneration. Schematic representation of various miRNAs whose expression has been linked with the induction of cell cycle reentry mitosis and adult cardiomyocytes proliferation. Following myocardial infarction (MI), miRNAs that have been implicated in these processes are shown.

identified hypertrophic cardiomyocytes undergoing rounds of karyokinesis without mitosis through the measurement of DNA content, suggesting that at least two independent populations of cardiomyocytes with different proliferative capacities exist [67]. Although the response observed is insufficient to regenerate the damaged heart, the fact that the adult mammalian heart showed a cardiomyocyte-mediated regenerative response is a remarkable finding with exciting implications such as those related to miRNA manipulation to enhance this endogenous regenerative reaction. In fact, a report by Eulalio and colleagues had suggested that it is indeed possible to experimentally drive adult mammalian cardiomyocytes toward a proliferative state by manipulating miRNA expression [68]. The study focused on finding miRNAs that promoted the expression of proliferative markers. Using a library of 875 human miRNA mimics in a high-throughput screening approach, the authors found 204 miRNAs that induced greater than 2-fold induction of the proliferation rate of both rat and mouse cardiomyocytes and demonstrated that forced miR-199a and miR-590 expression with adenoassociated viruses increased the number of proliferating cardiomyocytes leading to a significant improvement of cardiac function after MI [68] (Figure 3). Other miRNAs that significantly increased postnatal cardiomyocyte proliferation in 7-day old mice were miR-590-3p, miR-199-3p, miR-33b, and miR-1825 [68]. miR-33b was previously shown to play a role in regulating cell proliferation and fatty acid metabolism [69, 70]. A recent study indicated that the miR-199a-204 cluster was involved in HF by facilitating a maladaptive metabolic shift to increased glucose metabolism rather than fatty acid utilization, as it would be normally found in the healthy myocardium [71]. Using a cardiac disease mouse model with transverse aortic constriction pressure overload, the authors found that mice treated with antagomirs of miR-199a and miR-214 displayed improved cardiac function as well as a normal arrangement of cardiomyocytes, with fibrosis and hypertrophy being significantly reduced compared to vehicle-treated control hearts [71]. Mechanistically both miR-199a and miR-214 directly repressed peroxisome proliferator-activated receptor delta (PPAR-δ), a critical regulator of mitochondrial fatty acid metabolism in the heart, but did not alter the expression of genes involved in glucose metabolism [71]. Another

group reported that loss of function of the miR-17~92 cluster in mice resulted in abnormal myocardial differentiation deriving from second heart field cardiac progenitors, through repression of the *lsl-1* gene during embryonic cardiac development [49], suggesting a specific role of this miRNA cluster in the differentiation towards cardiac lineages during embryogenesis. The miR-17~92 cluster encodes six polycistronic miRNAs such as miR-17, miR-18a, miR-19a, miR-19b, miR-20a, and miR-92b of which not all have the same seed site. A more recent study reported that the miR-17~92 cluster, particularly miR-19a and miR-19b, may induce proliferation of cardiomyocytes and help protect the heart from ischemic injury caused by MI [72]. Compared with controls, proliferation of cardiomyocytes was decreased in miR-17-92 loss-of-function hearts and increased in miR-17~92 gain-of-function hearts. Importantly, after MI, miR-17~92 gain-of-function hearts had improved cardiac function, reduced scar size, and increased numbers of proliferating cardiomyocytes at the injury border zone, suggesting a crucial role in cardiac regeneration. Further, *in vitro* studies indicated that the miR-17-92 cluster induced cardiomyocyte proliferation through direct repression of PTEN by miR-19a and miR-19b, making this cluster also an attractive therapeutic option [73] (Figure 2).

5. miRNAs and CVDs

In 2007, Ikeda and colleagues were among the first to analyze microRNA expression by genome-wide profiling of human hearts with several conditions. They measured the expression of 428 miRNAs in 67 human left ventricular postmortem samples belonging to ischemic cardiomyopathy, dilated cardiomyopathy, and aortic stenosis cases (Table 1). Recently, several studies have now compared genome-wide miRNA expression profiles between normal cardiac tissue and those derived from mice or human patients with an array of different CVDs [16, 18, 74–76]. Table 1 summarizes the data from these studies highlighting the miRNAs that were found to be most deregulated in CVD tissue as compared to the control samples tested. Interestingly, the 18 miRNAs listed in bold, alone, account for almost 90% of all miRNAs expressed within the heart [75]. It is also worth noting that the deregulation of these miRNAs in CVD hearts did not seem to have an effect on other tissues, despite being ubiquitously expressed and playing important roles in various organ systems. For instance, miR-125a and miR-125b, which typically display a dramatic alteration in their expression after cardiac stresses [14, 74], are also fundamental regulators of hematopoietic stem cell differentiation and maintenance [77]. Therefore, modulating the expression of these miRNAs could potentially result in unwanted side effects in additional off-target organs, which calls for extra caution when thinking about future treatment strategies. Collectively, these expression-profiling studies also revealed distinct miRNA signatures for heart diseases of different etiologies, supporting the clinical applicability of miRNAs not only as therapeutic targets but also as disease biomarkers.

Myocardial Infarction (MI). The first consequence of an event causing insufficient blood supply to the myocardium, that is, MI, is cardiac tissue necrosis and myocardial inflammation which leads, inexorably, to left ventricular pathological remodeling and dysfunction [78]. It has been well established that acute MI is associated with the deregulation of multiple genes and it is therefore reasonable for hypothesizing that miRNAs may be modulated and play a crucial role during an episode of MI. Many molecular targets investigated for their ability to limit ischemic cell death have been particularly focused on the mitochondrial permeability transition pore (MPTP) and oxidative stress pathways. In fact, MPTP opening is triggered by Ca^{2+} accumulation and also by various other stress conditions and plays a central role during myocardial necrosis [79]. A deeper understanding of miRNA involvement in these defensive mechanisms may provide new therapeutic targets to reduce cell death following MI.

It is well known that the Ca^{2+} ion is central for the cardiac contraction system and for the signaling networks that regulate pathological cardiac growth and remodeling. Intracellular Ca^{2+} overload can occur in cardiomyocytes as a consequence of ischemic injury or other stresses, leading to contractile dysfunction and ultimately cell death via apoptosis or necrosis through activation of proapoptotic Bcl-2 family members and opening of the MPTP [80]. Recently, Aurora and colleagues elegantly demonstrated that miR-214 plays a protective role against I/R damage by attenuating Ca^{2+} overload-induced cardiomyocyte death [81]. Thus, miR-214 transgenic mice were sensitized to I/R injury as evidenced by increased cardiac apoptosis following MI and I/R injury. From a mechanistic point of view, miR-214 deletion promoted the transcription of Ncx1 protein immediately following the ischemic events, the level of which continued rising 7 days after the injury; conversely, miR-214 downregulation of Ncx1 was sufficient to reestablish Ca^{2+} homeostasis in cardiomyocytes *in vitro* [81]. It has been proposed that the upregulation of miR-214 expression, in response to ischemic injury, protects myocytes from damage by attenuating Ncx1 levels in order to prevent excessive Ca^{2+} influx into the cytoplasm [81].

Another miRNA that has been associated with different types of CVDs and disease models generated from acute cardiomyocyte death is miR-126. Levels of miR-126 were increased in the noninfarcted zone at 6 h after coronary artery occlusion and at 24 h following a 30-minute ischemic insult [82]. miR-126 is encoded within one of the *EGFR7* gene introns and it was found to be highly expressed in both heart and lung tissues. Moreover, it has been implicated in the proangiogenic action of VEGF and FGF through the repression of Sprouty-related-protein-1 (Spred-1), an intracellular inhibitor of VEGF [83]. Interestingly, the detection of miR-126 within the apoptotic bodies of dying endothelial cells was shown to be involved in the paracrine signaling that repressed the function of regulator of G protein signaling 16 (RSG16), an inhibitor of G-proteins coupled with receptor signaling implicated in blocking apoptosis [84]. These studies suggest that pharmacological intervention on miR-126 expression may be a viable therapeutic strategy to enhance neoangiogenesis and cardiac repair in the ischemic myocardium.

Modulation of miR-499 levels attenuated cardiomyocyte death and the severity of MI induced by I/R [85]. This

Table 1: The literature review summary of miRNAs found to be deregulated in cardiovascular diseases. A number of studies have used high throughput miRNA profiling technologies to compare miRNA expression levels between control and affected tissues from cardiac disease inducing mice models or postmortem samples from failing human myocardium. The heading of the table identifies the specific study, followed by the species and diseases modeled, and the experimental design as well as the microarray technologies used to analyze the data. In bold, in rows below, the most deregulated miRNAs according to the different studies are displayed. The following miRNAs tabulated that are not in bold represent other cardiac specific miRNAs found to be deregulated in the five studies referenced but not directly mentioned in our review. Arrows illustrate the fold change of upregulation or downregulation of listed miRNAs as compared to control samples. miRNAs with the same seed region were combined into families. The family was labeled as regulated when at least 3 members of the family displayed a change in expression profile. Each family includes the following: let-7 family = let-7a, let-b, let-c, let-d, let-e, let-f, let-g, let-h, let-I, and let-j; miR-15 family = miR-15a, miR-15b, miR-16, miR-195, miR-424, and miR-497; miR-17 family = miR-17-5p, miR-20a, miR-20b, miR-93, miR-106a, and miR-106b; miR-29 family = miR-29a, miR-29b, and miR-29c; miR-30 family = miR-30a, miR-30b, miR-30c, miR-30d, and miR-30e. LAC = left coronary artery constriction; HF = heart failure; ICM = ischemic cardiomyopathy; DCM = dilated cardiomyopathy; d = days. ↑↑↑ ≥ 7.5; ↑↑ ≥ 2.5; ↑ ≥ 1 fold increase; —: not available; ↓ ≥ −1; ↓↓ ≥ −2.5; ↓↓↓ ≥ −7.5 fold decrease.

Study	van Rooij et al. [16]			Ikeda et al. [18]			Matkovich et al. [76]		Rao et al. [75]	Cheng et al. [74]		
Species	Mouse	Mouse	Human	Human	Human	Human	Human	Human	Mouse	Mouse		
Model of disease	LAC (3 days)	LAC (14 days)	HF	ICM	DCM	Aortic stenosis	DCM and ICM	HF	*Dgr8* conditional KO mouse	Thoracic aortic constriction		
Microarray platform	miRMAX microarray			Bead-based flow cytometric miRNA			Ncode microarray		Illumina microarray	mirVana microarray		
miR	LAC (3 d)	LAC (14 d)	HF	ICM	DCM	AS	DCM/ICM	HF	*Dgr8* KO mouse	TAC (7 d)	TAC (14 d)	TAC (21 d)
Let-7 family	—	↑	↑↑	↑	↑	↑	↑↑	↑↑	↑↑↑			
15 family	↑↑	↑↑	↑	↑	↑	↑	↑↑	↑↑		↑	↑	↑
29 family	↓↓	↓↓	↓↓				↑	↑	↑	↓	↓	↓
30 family	↓	↓↓	↓		↓	↓	↑↑	↑↑	↑	↓↓	↓↓	↓↓
1				↓	↓	↓	↑↑↑	↑↑	↑↑↑			
21	↑↑	↑↑↑	↑↑				↑↑	↑		↑↑↑	↑↑↑	↑↑
22	↓↓	↓↓	↓↓				↑↑↑	↑↑	↑↑			
23a, b				↑	↑	↑	↑↑	↑	↑			
24				↑	↑	↑	↑↑	↑	↑			
26a, b				↑	↑	↑	↑↑	↑	↑			
27a, b				↑	↑	↑	↑↑↑	↑	↑	↑	↑	↑
125a, b				↑	↑	↑	↑↑	↑↑	↑			
126	↓↓↓	↓↓↓	↓	↑	↑	↑	↑↑	↑↑	↑↑	↓	—	↑
133a, b							↑	↑	↑↑	↓	↓	↓
143	—	↓↓	↓↓↓				↑	↑	↑↑			
208									↑↑			
214	↑↑	↑↑	↑↑	↑	↑	↑				—	—	↑↑
379	↑	↑↑↑	↑									
486										↓↓	↓	↓
499	↓	↓↓	—	↑	↑	↑	↑↑	↑	↑			
638	—	↑↑↑	↑↑				↑	↑				
10a, b	—	↑↑	↑↑			↓						
146a, b	↑↑	↑↑↑	↑									
150	—	↓	↓	↑	↑	↑				↑↑	↑↑	↑↑
223	↑↑↑	↑↑	↑									
92	↑	↑	↑									

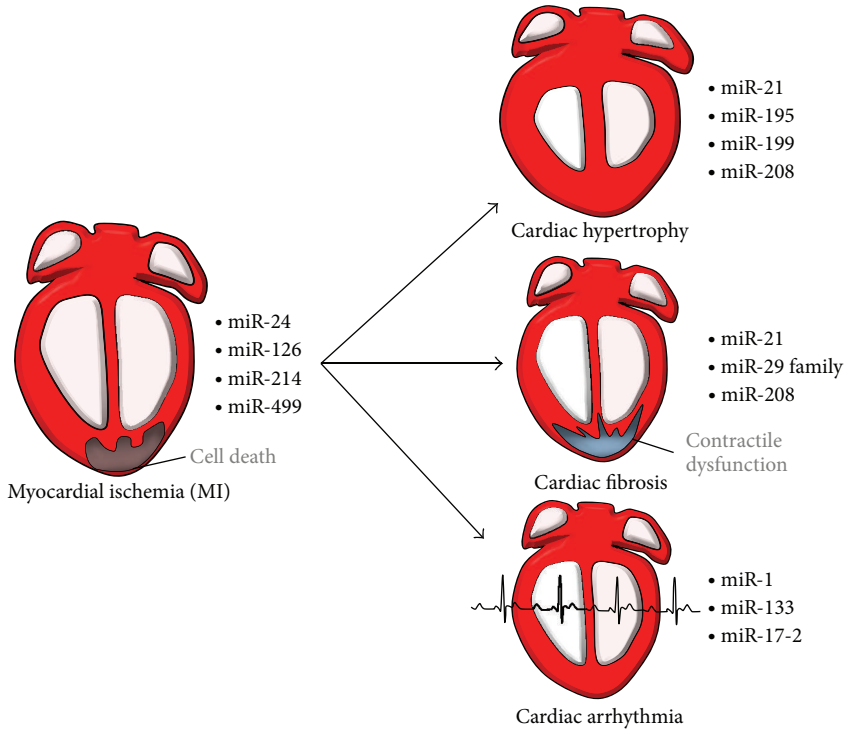


Figure 4: Schematic representation of the most relevant miRNAs reported during the different phases of left ventricular remodeling process.

protective mechanism was based upon the inhibition of the acute apoptotic response of the cardiomyocyte. Thus, Wang and colleagues showed that the α and β isoforms of the calcineurin catalytic subunit, an important enzyme for cardiomyocyte metabolism, are a specific target of miR-499 in mice [85]. Calcineurin plays an important role in inhibiting cardiomyocyte apoptosis through the dephosphorylation of dynamin-related protein-1 (Drp1), an effector of the activated apoptotic pathway; this decreases Drp1 accumulation on the mitochondrial membrane preventing oxidative stress-driven cell death. Furthermore, miR-24 expression was also shown to be downregulated in the ischemic border but not in the remote zone of the myocardium of the left ventricle a few hours after induced-MI in rat myocytes [86]. It was speculated that miR-24 suppressed cardiomyocyte apoptosis partially by repressing the expression of the proapoptotic protein Bim [86]. Accordingly, poor blood oxygenation during MI induced the expression of the HIF-1α protein that, in turn, increased endogenous levels of miR-24, protecting cardiomyocytes from oxygen deprivation in a rat MI model [85]. Moreover, *in vivo* miR-24 overexpression in a mouse MI model also inhibited cardiomyocyte apoptosis, decreased infarct size, and improved cardiac function, rendering this miRNA an excellent target to impede oxygen deprivation-driven apoptosis [86] (Figure 4).

Hypertrophy and Fibrosis. The initial post-MI phase of left ventricular remodeling involves fibrotic repair of the necrotic area associated with scar formation and elongation of resident cardiomyocytes associated thinning of the ventricular walls [2]. Consequently, there is a left ventricular volume increase related to stroke volume augmentation and maintenance of normal cardiac output [2]. However, the remodeling process is driven mainly by hypertrophic cardiomyocyte elongation with the resulting wall mass increase and chamber enlargement, as well as a shift from an elliptical to a more spherical ventricular chamber configuration [87].

By using experimental models of remodeling rather than that of acute MI cell death, several groups demonstrated that miR-21 is one of the most deregulated miRNAs following I/R injuries (Table 1), despite the fact of being weakly expressed in normal cardiac tissue. For instance, Roy and colleagues reported an upregulation of miR-21 expression 2 and 7 days after MI induction in mice. Moreover, miR-21 inhibition increased the expression of the metalloproteinase-2 (MMP-2) via suppression of the PTEN pathway in cardiac fibroblasts [88]. This is particularly interesting, given that MMP-2 is an enzyme involved in the breakdown of extracellular matrix during tissue remodeling [89] with a potential therapeutic use in controlling scar formation. However, the role of this miRNA in CVDs is still controversial. For example, in 2008 Thum and colleagues reported that altering miR-21 expression *in vivo* increased cardiomyocyte hypertrophy by indirectly affecting cardiac fibroblast behavior [90] and this was subsequently confirmed by miR-21 overexpression in various *in vitro* systems [74]. Conversely, other studies reported miR-21 to have an antihypertrophic effect on isolated cardiomyocytes *in vitro* [91], to reduce infarct size following I/R injury, and to support cellular outgrowths on cardiomyocytes [92] or even an antiapoptotic effect on isolated cardiomyocytes from transgenic mice [93]. The reasons for such inconsistencies among those studies are

unclear. It is possible that the biological consequences of miRNA-21 enrichment during CVDs are in part due to an increase in cardiac fibroblast cell number rather than from a direct effect of the injury itself, given that miR-21 is abundantly expressed in cardiac fibroblasts but not cardiomyocytes [17]. Moreover, miR-21 is expressed in many other tissues, including vasculature, hence precluding the discrimination between primary and secondary cardiac effects in models that use ubiquitous downregulation of miR-21 such as intravenous injections of antagomirs. This is supported by the fact that cardiomyocyte-specific overexpression of miR-21 *in vivo* does not induce a distinct cardiomyocyte phenotype [90]. Given that the effects of miR-21 modulation during MI *in vivo* and on isolated cardiomyocytes *in vitro* are not well understood, it is currently unclear whether miR-21 holds a value as a prospective therapeutic tool.

Van Rooij and colleagues described a deregulation of specific cardiac miRNAs during MI induced by a model based upon occlusion of the left coronary artery in mice. Among the miRNAs modulated in this system, all three members of the miR-29 family were downregulated. These results were validated by real-time PCR analysis and confirmed using human patient samples (Table 1) [16]. Interestingly, the miR-29 family targets a pool of mRNAs that encode proteins involved in fibrosis such as collagens, fibrillins, and elastin [92]. Thus, downregulation of miR-29 would predictively increase the expression of these mRNAs and enhance the fibrotic response. Indeed, downregulation of miR-29 with antagomirs induces the expression of collagens both *in vitro* and *in vivo*, whereas overexpression of miR-29 in fibroblasts reduces extra cellular matrix deposition [16]. These data were confirmed in bleomycin-induced fibrosis in the lung where the expression on miR-21 inversely correlated with the expression levels of profibrotic target genes and the severity of the fibrosis [94]. Thus, the miR-29 family acts as a regulator of cardiac fibrosis and represents a potential therapeutic target to control tissue fibrosis in general.

Also worth noting is the activity of miR-208 during heart fibrosis. This is a cardiac-specific miRNA encoded by an intron of the *MYH6* gene coding for α-myosin heavy chain (α-MHC), the major contractile protein expressed in rodent adult hearts [95]. In contrast, both miR-208b and miR-499 are encoded by introns of *MYH7* that codes the β isoform of myosin heavy chain (β-MHC), the isoform expressed during the fetal period. Thyroid hormone (T3) stimulates the expression of α-MHC and consequently of miR-208 after birth while repressing the expression of the embryonic isoform of the gene (Figure 2). A hallmark of pathologic hypertrophy subsequent to MI is the reactivation of a set of fetal genes, including those encoding for atrial natriuretic peptide (ANP), β-type natriuretic peptide (BNP), α-skeletal actin, and β-MHC [96]. Downregulation of α-MHC and upregulation of β-MHC are common responses to cardiac injury regardless of species; however, most relevant to cardiac disease is the finding that miR-208a knockout mice display reduced fibrosis and hypertrophy in response to thoracic aortic banding, which induces cardiac hypertrophy by increased afterload on the heart and is accompanied by downregulation of α-MHC and upregulation of β-MHC [95]. These transgenic animals fail to upregulate MYH7 expression, which is a common marker for pathological cardiac remodeling. These findings support the notion that therapeutic inhibition of miR-208a could induce benefits in the setting of cardiac remodeling. Indeed, Montgomery et al. showed that administration of the antagomirs against miR-208a reduced fibrosis and inhibited the activation of *MYH7* transcription in transgenic mice susceptible to hypertension and heart failure [97]. These findings provided the first proof of concept for the therapeutic use of antimir-208a agents in a setting of CVDs. Unexpectedly, mice treated with antimir-208a displayed resistance to obesity induced by a high-fat diet [98] indicating multiple effects of miR-208 on unknown targets that need to be accurately evaluated before any treatment strategy is devised (Figure 4).

Cardiac Arrhythmia. Heart rhythm problems or cardiac arrhythmias occur when the electrical impulses that coordinate heart contraction malfunction cause the heart to beat too fast or too slow. In fact, the terms arrhythmia refers to any changes from the normal sequence of electrical impulses of the heart. The electrical impulse of the heart is generated by specialized cells in the sinoatrial node and propagates to the atrial and ventricular myocardium through a specialized conduction system, whereas coordinated depolarization of adjacent cells in the myocardium is accomplished via gap junctions [99]. Membrane excitability depends on the activity of ion channels and, in particular, those for Na^+, Ca^{2+}, and K^+, which together with gap junction proteins such as connexin-43 are all critical regulators of cardiomyocyte polarization and depolarization during contraction and relaxation. It is important to note that fibrotic deposits and more in general scar tissue within the myocardium alter the normal electric conduction of the heart, generating profound changes in the normal electrical impulse. Several miRNAs, including miR-1 and miR-133, are predicted to target ion channel proteins and therefore play important roles in cardiac conduction and in the onset of arrhythmias during CVDs. Recently, using a combination of luciferase reporter assays and Western blotting, it was reported that gap junction protein a1 (GJA1) and the potassium inwardly rectifying channel member 2 (KCNJ2) are both specific targets of miR-1 in rats heart [100]. Moreover, miR-1 levels increased in individuals with coronary artery disease, and its overexpression in normal hearts resulted in slowed conduction velocity of the depolarization wave and prolonged repolarization, leading to the development of arrhythmias. Conversely, blocking miR-1 function with antisense oligo-miR-1 in rat infarcted hearts normalized the expression of connexin 43, thus reducing arrhythmias after MI [100]. In support of these findings, Zhao et al. described that mice knock-out for miR-1-2 has several ECG alterations such as a lower heart rate, shortened PR interval, and widened QRS and died as a result of cardiac arrhythmias [37], confirming the implication of miR-1 within the electric conduction system of the heart. Mechanistically, it has been suggested that at least part of the electrical alterations observed in knockout mice was due to the increased expression of Irx5 and Irx4, two transcription factors that

regulate endogenous levels of Kcnd2, a K^+ channel protein involved in cardiac repolarization [37] (Figure 4).

Nonischemic Cardiomyopathy. Literally, cardiomyopathy means "heart muscle disease" and it is defined as a progressive disorder of the myocardium associated with abnormalities in organ morphology and structure mainly due from MI events [101]. Cardiomyocyte death, cardiac fibrosis, and cardiomyocytes hypertrophy are all features of cardiomyopathies generated, usually, by ischemic insults. Nonischemic cardiomyopathies, instead, originate from congenital malformations of the heart's structure, such as alteration of the sarcomere complexes, cell-cell or cell-matrix junction dissolution, or electrical conduction abnormalities, but can also arise from various environmental factors such as hypertension, diabetes, or life style factors [99]. Among nonischemic cardiomyopathies, diabetes is an independent risk factor and a major cause of chronic cardiovascular complications [102]. About 80% of deaths associated with diabetes are due to heart complications in particular to diabetic cardiomyopathy [102]. Several recently published reviews outline important findings in the context of diabetic cardiomyopathy, regarding the putative mechanism of the disease progression in relation to the use of the miRNAs as potential diagnostic markers, as well as therapeutic targets [103–105]. Notably, Jaguszewski et al. identified four circulating miRNAs, namely, miR-1, miR-16, miR-26a, and miR-133a, as putative biomarkers for the differential diagnosis of two conditions that have until now been clinically indistinguishable [106].

6. miRNAs and Therapeutic Options

miRNAs may be of clinical value in the context of CVDs, both as therapeutic targets and as biomarkers to follow disease diagnosis and prognosis. The therapeutic potential of miRNAs in CVDs was first proposed in the light of results from animal studies that unveiled important roles of miRNAs in several contexts of cardiac development and disease. Since mice knockout for DICER1 shows phenotype in cardiomyocytes, smooth muscle cells, and endothelial cells leading to heart defects [107], it follows that reestablishing miRNA to normal levels could have a therapeutic effect on the context of cardiac disease. In principle, one of the advantages of the miRNAs as therapeutic tools in CVDs is their ability to target various factors in an established biological context. Because of the intrinsic nature of miRNAs, several mRNA targets may be downregulated by a single mature molecule, increasing the likelihood of affecting multiple components within a biological pathway. In addition, there are molecular tools that can affect simultaneously several miRNAs, thus further increasing the range of biological action. The design of specific inhibitory molecules, called miRNA sponges, may allow the inhibition of several miRNAs simultaneously, with a very high efficacy and the ability to specifically modify the dynamics of any pathway [108, 109]. In addition, inducing gain- or loss-of-function is technically more feasible, both *in vivo* and *in vitro*, using miRNAs rather than mRNA or DNAs, and miRNAs show higher stability [110]. Using a variety of animal models, it has been shown that inhibition of miRNAs upregulated upon cardiac malfunctioning by pharmaceutical or chemical molecules has reestablished normal cardiac function and, in some cases, even reduced the infarct size [65, 95, 96]. Considering this, therapeutic inhibition of miRNAs in CVDs is currently based on the fact that specific microRNAs have been investigated and found to be fundamental regulators in distinct phases of embryonic heart development and they were also described in the pathogenesis of different remodeling processes. Moreover, loss-of-function approaches could be designed using inhibitory tools such as miRNAs sponges to specifically block different miRNAs whose expression was related with different aspect of different CVDs in order to attenuate the response to cardiac injuries (Figure 5).

However, the therapeutic use of miRNAs faces a series of obstacles that should be taken into account and that hamper their current application on CVDs. The delivery of mature species mimicking miRNAs to induce gain of function or of chemically engineered molecules that specifically inhibit miRNA function should be accompanied by adjuvant strategies to address four outstanding issues: increasing tropism for the heart; blocking degradation by RNAse; reducing inflammatory response caused by naked nucleic acids; and achieving cytosolic delivery once it reaches the target organ. Delivery of small RNA molecules is often achieved by the means of liposomal or polymeric vehicles. Overall, these vehicles are able to protect the RNA species, increasing its stability and cellular uptake and strongly reducing any inflammatory response [111, 112]. However, delivery of these miRNA-containing vehicles to the heart is a random event, being the liver, spleen, and lungs the preferred organs that they reach [113, 114].

To circumvent the problem of targeting the heart in a specific-manner, different strategies have been devised ranging from direct intramyocardial injection which has been shown to reduce apoptosis and infarct size and to increase cardiomyocyte proliferation in infarcted rats [115] to the use of cardiac-specific antibodies that direct delivery vehicles to restricted cell populations. Nonetheless, in the case of CVDs, this last approach is still under debate because of the lack of reliable cardiac-specific antigens [116]. As an alternative, the use of delivery vectors traditionally used for long-term transgene expression can also be applied for miRNA gain or loss-of-function systems [68]. Primary miRNAs or sponge miRNAs can be engineered into adenoassociated viruses (AAV) to be expressed under strong promoters. The discovery of AAVs serotypes with increased cardiac tropism (AAV9), although in a nonspecific manner (it targets brain and liver as well), has created a window of opportunity for design clinically compatible strategies to inhibit or overexpress miRNAs in the future [117].

Regardless of these arguments, anti-miRNA therapeutics have recently turn into reality by the success of Phase I and Phase II clinical trials of Santaris Pharma antimir against miR-122, *miravisen*, which is currently in development as a cure for hepatitis C virus [118]. It is hoped that these promising findings will accelerate the development of

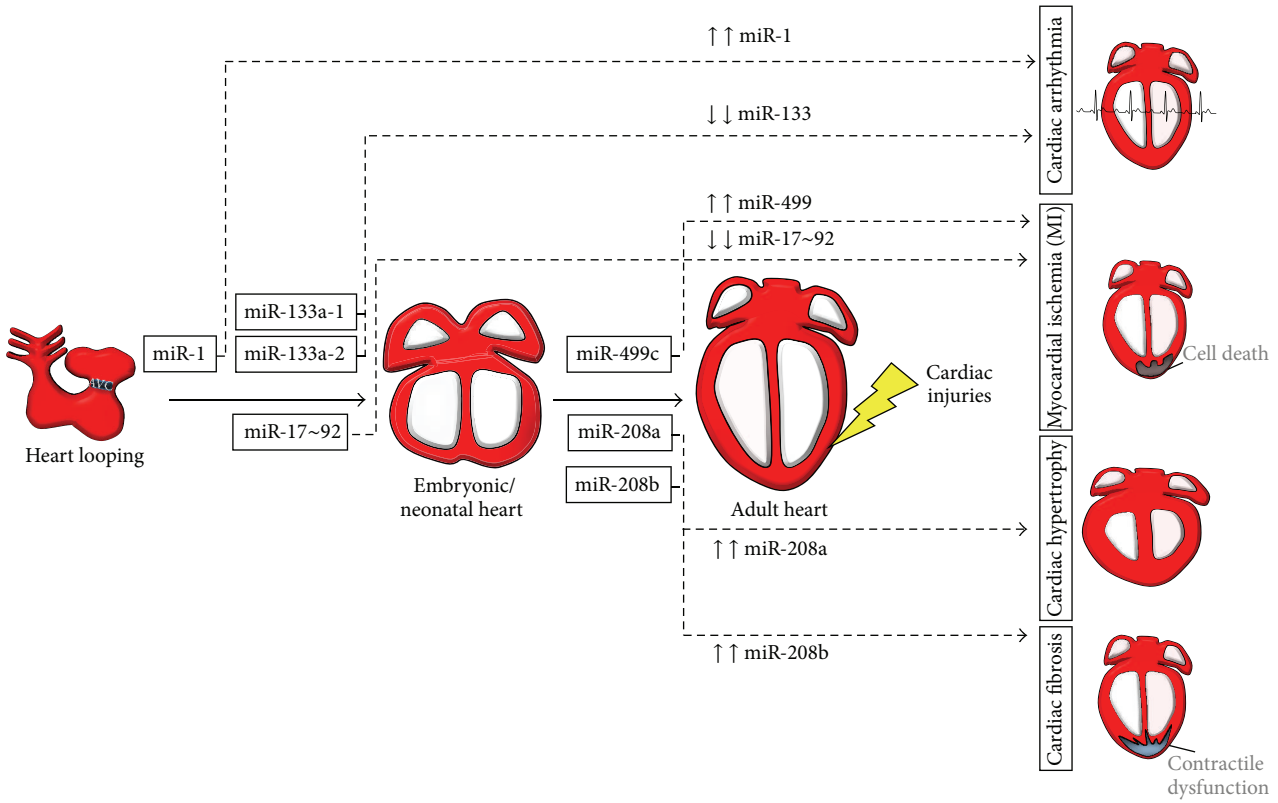


Figure 5: microRNA network in cardiac physiology and pathologies. The expression of miRNAs found of relevant importance in cardiac development (boxed miRNAs) is also involved in the remodeling process of the heart. Different modulation of miRNA expression enhances pathological outcomes at the myocardium and can be specifically targeted in a precise manner to improve the therapeutic applicability of miRNAs.

miRNA-targeting therapeutics into additional disease areas such as that of CVD.

Finally, miRNAs can be used as well as disease biomarkers for the diagnosis and/or prognosis of various types of CVDs. The existence of circulating miRNAs in the blood stream of CVDs patients can be harnessed in the clinical setting as promising prognosis or diagnostic tools. We direct interested readers to excellent review by Creemers and colleagues on the different issue that need to be taken into account for the use of miRNAs as diagnostic tool and a comprehensive list of circulating miRNAs described in the context of CVDs [117].

7. Conclusion

In the present era of genomic discoveries, miRNAs represent a unique class of endogenous noncoding RNAs that are highly conserved across species and that repress gene translation upon binding to mRNA, thereby having a versatile influence on various biological processes [13]. This inhibition can have profound effects on cardiac function, fueling the excitement for future exploration of miRNAs as therapeutic tools. To date, various studies have pointed to the importance and authority of miRNAs in controlling different mechanisms associated with the biogenesis and establishment of CVDs. This important role makes them fascinating targets for therapeutic interventions. Nonetheless, it is important to note that different genome-wide miRNAs expression analysis studies have reported controversial results regarding the deregulation of specific miRNA expression in the context of the same CVDs (Table 1). Hence, numerous challenges and hurdles remain in the path towards the development of miRNA-based therapeutics. Firstly, in contrast to the situation described for miRNAs in lower vertebrates, the effects of individual miRNAs on mRNA and protein processing in mammals are relatively modest, as judged by the results of various loss-of-function studies [19]. Secondly, unlike conventional drug molecules, miRNAs can target many mRNAs at the same time and therefore influence the translation of multiple genes that contribute to common cellular function mechanisms and the development of pathological conditions [19]. However, a single miRNA will probably target unrelated genes and possibly produce undesired changes in gene expression. For example, antagomirs directed against the cardiac-specific miR-208a were shown to prevent over-weight and metabolic disorder in mice maintained on a high-fat diet [98], a side effect not expected to be based on the restricted expression pattern of miR-208a. Conversely, inhibitions of miR-29 were proved to be effective against cardiac fibrosis in two different organs such as heart and lung using a mouse model [94]. Most of the animal studies performed to date have focused on the phenotypic effects of miRNA inhibition in target organs and have largely overlooked off-targets effects that

might be present in other tissues. Moreover, the doses used in most laboratory studies are unlikely to be feasible in a clinical setting. Follow-up preclinical studies will have to monitor appropriate dosing regimens in order to establish the lowest possible efficacious doses while attempting to prevent unwanted offside effects.

As an alternative to microRNA-based therapies, some investigators have turned their attention to direct lineage reprogramming conversion strategies, which involve switching one somatic cell type into another. The direct reprogramming of fibroblasts within the heart into functional cardiomyocytes using a combination of three or four factors (GATA4, Mef2C, Tbx5, and/or Hand2) is an exciting new therapeutic approach to treat CVDs and replace the damaged myocardium [119, 120]. Considering this, recent investigations suggested that miRNAs in collaboration with defined cardiac transcription factors might regulate directly a reprogramming process of a somatic cell type into another, adding a new exciting level for therapeutic intervention [121]. As biomarkers, instead, miRNAs have been convincingly shown to be an important tool to aid in early diagnosis of CVDs [117] and might have a brighter future as such clinical tools.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

The authors would like to thank Dr. Sylvia J. Bedford-Guaus for critical reading of the paper. The work on this topic in the authors' laboratories is partially supported by grants from MINECO (PLE2009/0147, PLE2009/0164, and SAF2012-33526), ISCIII-FEDER (RETICS-RD06/0010/0016, RD12/0019/0019, and RD12/0019/0034), and AGAUR (2014-SGR-1460, 2014-SGR-1570). Julián Pulecio was partially supported by a Juan de la Cierva Postdoctoral Fellowship (JCI-2012-15293).

References

[1] B. B. Kelly, J. Narula, and V. Fuster, "Recognizing global burden of cardiovascular disease and related chronic diseases," *The Mount Sinai Journal of Medicine*, vol. 79, no. 6, pp. 632–640, 2012.

[2] L. H. Opie, P. J. Commerford, B. J. Gersh, and M. A. Pfeffer, "Controversies in ventricular remodelling," *The Lancet*, vol. 367, no. 9507, pp. 356–367, 2006.

[3] R. S. Whelan, V. Kaplinskiy, and R. N. Kitsis, "Cell death in the pathogenesis of heart disease: mechanisms and significance," *Annual Review of Physiology*, vol. 72, pp. 19–44, 2010.

[4] A. P. Beltrami, K. Urbanek, J. Kajstura et al., "Evidence that human cardiac myocytes divide after myocardial infarction," *The New England Journal of Medicine*, vol. 344, no. 23, pp. 1750–1757, 2001.

[5] D. Fan, A. Takawale, J. Lee, and Z. Kassiri, "Cardiac fibroblasts, fibrosis and extracellular matrix remodeling in heart disease," *Fibrogenesis and Tissue Repair*, vol. 5, article 15, 2012.

[6] A. S. Go, D. Mozaffarian, V. L. Roger et al., "Heart disease and stroke statistics—2014 update: a report from the American Heart Association," *Circulation*, vol. 129, pp. e28–e292, 2014.

[7] J. C. Garbern and R. T. Lee, "Cardiac stem cell therapy and the promise of heart regeneration," *Cell Stem Cell*, vol. 12, no. 6, pp. 689–698, 2013.

[8] S. E. Senyo, R. T. Lee, and B. Kuhn, "Cardiac regeneration based on mechanisms of cardiomyocyte proliferation and differentiation," *Stem Cell Research*, vol. 13, no. 3, pp. 532–541, 2014.

[9] E. R. Porrello and E. N. Olson, "A neonatal blueprint for cardiac regeneration," *Stem Cell Research*, vol. 13, no. 3, pp. 556–570, 2014.

[10] F. B. Engel, M. Schebesta, M. T. Duong et al., "p38 MAP kinase inhibition enables proliferation of adult mammalian cardiomyocytes," *Genes & Development*, vol. 19, no. 10, pp. 1175–1187, 2005.

[11] K. Bersell, S. Arab, B. Haring, and B. Kühn, "Neuregulin1/ErbB4 signaling induces cardiomyocyte proliferation and repair of heart injury," *Cell*, vol. 138, no. 2, pp. 257–270, 2009.

[12] D. Srivastava and M. Ieda, "Critical factors for cardiac reprogramming," *Circulation research*, vol. 111, no. 1, pp. 5–8, 2012.

[13] D. P. Bartel, "MicroRNAs: target recognition and regulatory functions," *Cell*, vol. 136, no. 2, pp. 215–233, 2009.

[14] E. van Rooij, L. B. Sutherland, N. Liu et al., "A signature pattern of stress-responsive microRNAs that can evoke cardiac hypertrophy and heart failure," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 48, pp. 18255–18260, 2006.

[15] A. Bonauer, G. Carmona, M. Iwasaki et al., "MicroRNA-92a controls angiogenesis and functional recovery of ischemic tissues in Mice," *Science*, vol. 324, no. 5935, pp. 1710–1713, 2009.

[16] E. van Rooij, L. B. Sutherland, J. E. Thatcher et al., "Dysregulation of microRNAs after myocardial infarction reveals a role of miR-29 in cardiac fibrosis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 35, pp. 13027–13032, 2008.

[17] T. Thum, P. Galuppo, C. Wolf et al., "MicroRNAs in the human heart: a clue to fetal gene reprogramming in heart failure," *Circulation*, vol. 116, no. 3, pp. 258–267, 2007.

[18] S. Ikeda, S. W. Kong, J. Lu et al., "Altered microRNA expression in human heart disease," *Physiological Genomics*, vol. 31, no. 3, pp. 367–373, 2007.

[19] E. van Rooij and E. N. Olson, "MicroRNA therapeutics for cardiovascular disease: opportunities and obstacles," *Nature Reviews Drug Discovery*, vol. 11, no. 11, pp. 860–872, 2012.

[20] T. Thum, "MicroRNA therapeutics in cardiovascular medicine," *EMBO Molecular Medicine*, vol. 4, no. 1, pp. 3–14, 2012.

[21] E. M. Small, R. J. A. Frost, and E. N. Olson, "MicroRNAs add a new dimension to cardiovascular disease," *Circulation*, vol. 121, no. 8, pp. 1022–1032, 2010.

[22] A. J. Tijsen, Y. M. Pinto, and E. E. Creemers, "Circulating microRNAs as diagnostic biomarkers for cardiovascular diseases," *The American Journal of Physiology—Heart and Circulatory Physiology*, vol. 303, no. 9, pp. H1085–H1095, 2012.

[23] Y. Tomari and P. D. Zamore, "MicroRNA biogenesis: drosha can't cut it without a partner," *Current Biology*, vol. 15, no. 2, pp. R61–R64, 2005.

[24] E. Hergenreider, S. Heydt, K. Tréguer et al., "Atheroprotective communication between endothelial cells and smooth muscle

cells through miRNAs," *Nature Cell Biology*, vol. 14, pp. 249–256, 2012.

[25] L. Xu, B.-F. Yang, and J. Ai, "MicroRNA transport: a new way in cell communication," *Journal of Cellular Physiology*, vol. 228, no. 8, pp. 1713–1719, 2013.

[26] C. Bang, S. Batkai, S. Dangwal et al., "Cardiac fibroblast-derived microRNA passenger strand-enriched exosomes mediate cardiomyocyte hypertrophy," *The Journal of Clinical Investigation*, vol. 124, no. 5, pp. 2136–2146, 2014.

[27] J. Liu, M. A. Valencia-Sanchez, G. J. Hannon, and R. Parker, "MicroRNA-dependent localization of targeted mRNAs to mammalian P-bodies," *Nature Cell Biology*, vol. 7, no. 7, pp. 719–723, 2005.

[28] M. Kulkarni, S. Ozgur, and G. Stoecklin, "On track with P-bodies," *Biochemical Society Transactions*, vol. 38, no. 1, pp. 242–251, 2010.

[29] J. Winter, S. Jung, S. Keller, R. I. Gregory, and S. Diederichs, "Many roads to maturity: microRNA biogenesis pathways and their regulation," *Nature Cell Biology*, vol. 11, no. 3, pp. 228–234, 2009.

[30] D. Baek, J. Villén, C. Shin, F. D. Camargo, S. P. Gygi, and D. P. Bartel, "The impact of microRNAs on protein output," *Nature*, vol. 455, pp. 64–71, 2008.

[31] M. Selbach, B. Schwanhäusser, N. Thierfelder, Z. Fang, R. Khanin, and N. Rajewsky, "Widespread changes in protein synthesis induced by microRNAs," *Nature*, vol. 455, no. 7209, pp. 58–63, 2008.

[32] R. Yi and E. Fuchs, "MicroRNAs and their roles in mammalian stem cells," *Journal of Cell Science*, vol. 124, pp. 1775–1783, 2011.

[33] J. Pulecio, E. Nivet, I. Sancho-Martinez et al., "Conversion of human fibroblasts into monocyte-like progenitor cells," *Stem Cells*, vol. 32, no. 11, pp. 2923–2938, 2014.

[34] A. Kozomara and S. Griffiths-Jones, "miRBase: integrating microRNA annotation and deep-sequencing data," *Nucleic Acids Research*, vol. 39, no. 1, pp. D152–D157, 2011.

[35] A. Kozomara and S. Griffiths-Jones, "MiRBase: annotating high confidence microRNAs using deep sequencing data," *Nucleic Acids Research*, vol. 42, no. 1, pp. D68–D73, 2014.

[36] I. Lyons, L. M. Parsons, L. Hartley et al., "Myogenic and morphogenetic defects in the heart tubes of murine embryos lacking the homeo box gene Nkx2-5," *Genes and Development*, vol. 9, no. 13, pp. 1654–1666, 1995.

[37] Y. Zhao, J. F. Ransom, A. Li et al., "Dysregulation of cardiogenesis, cardiac conduction, and cell cycle in mice lacking miRNA-1-2," *Cell*, vol. 129, no. 2, pp. 303–317, 2007.

[38] P. A. da Costa Martins, M. Bourajjaj, M. Gladka et al., "Conditional Dicer gene deletion in the postnatal myocardium provokes spontaneous cardiac remodeling," *Circulation*, vol. 118, no. 15, pp. 1567–1576, 2008.

[39] Y. Zhao, E. Samal, and D. Srivastava, "Serum response factor regulates a muscle-specific microRNA that targets Hand2 during cardiogenesis," *Nature*, vol. 436, no. 7048, pp. 214–220, 2005.

[40] J.-F. Chen, E. M. Mandel, J. M. Thomson et al., "The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation," *Nature Genetics*, vol. 38, no. 2, pp. 228–233, 2006.

[41] K. N. Ivey, A. Muth, J. Arnold et al., "MicroRNA regulation of cell lineages in mouse and human embryonic stem cells," *Cell Stem Cell*, vol. 2, no. 3, pp. 219–229, 2008.

[42] N. Liu, S. Bezprozvannaya, A. H. Williams et al., "microRNA-133a regulates cardiomyocyte proliferation and suppresses smooth muscle gene expression in the heart," *Genes & Development*, vol. 22, no. 23, pp. 3242–3254, 2008.

[43] S.-Y. Lee, O. Ham, M.-J. Cha et al., "The promotion of cardiogenic differentiation of hMSCs by targeting epidermal growth factor receptor using microRNA-133a," *Biomaterials*, vol. 34, no. 1, pp. 92–99, 2013.

[44] J. P. G. Sluijter, A. van Mil, P. van Vliet et al., "MicroRNA-1 and-499 regulate differentiation and proliferation in human-derived cardiomyocyte progenitor cells," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 30, no. 4, pp. 859–868, 2010.

[45] P. S. Mitchell, R. K. Parkin, E. M. Kroh et al., "Circulating microRNAs as stable blood-based markers for cancer detection," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 30, pp. 10513–10518, 2008.

[46] G. W. Dorn, S. J. Matkovich, W. H. Eschenbacher, and Y. Zhang, "A human 3′ miR-499 mutation alters cardiac mRNA targeting and function," *Circulation Research*, vol. 110, no. 7, pp. 958–967, 2012.

[47] J. T. C. Shieh, Y. Huang, J. Gilmore, and D. Srivastava, "Elevated miR-499 levels blunt the cardiac stress response," *PLoS ONE*, vol. 6, no. 5, Article ID e19481, 2011.

[48] A. Ventura, A. G. Young, M. M. Winslow et al., "Targeted deletion reveals essential and overlapping functions of the *miR-17~92* family of miRNA clusters," *Cell*, vol. 132, no. 5, pp. 875–886, 2008.

[49] J. Wang, S. B. Greene, M. Bonilla-Claudio et al., "Bmp signaling regulates myocardial differentiation from cardiac progenitors through a MicroRNA-mediated mechanism," *Developmental Cell*, vol. 19, no. 6, pp. 903–912, 2010.

[50] P. Sirish, J. E. López, N. Li et al., "MicroRNA profiling predicts a variance in the proliferative potential of cardiac progenitor cells derived from neonatal and adult murine hearts," *Journal of Molecular and Cellular Cardiology*, vol. 52, no. 1, pp. 264–272, 2012.

[51] T. Hosoda, H. Zheng, M. Cabral-da-Silva et al., "Human cardiac stem cell differentiation is regulated by a mircrine mechanism," *Circulation*, vol. 123, pp. 1287–1296, 2011.

[52] S. Hu, M. Huang, P. K. Nguyen et al., "Novel microRNA prosurvival cocktail for improving engraftment and function of cardiac progenitor cell transplantation," *Circulation*, vol. 124, no. 11, pp. S27–S34, 2011.

[53] P. A. Tsonis, "Regeneration in vertebrates," *Developmental Biology*, vol. 221, no. 2, pp. 273–284, 2000.

[54] R. O. Becker, S. Chapin, and R. Sherry, "Regeneration of the ventricular myocardium in amphibians," *Nature*, vol. 248, no. 5444, pp. 145–147, 1974.

[55] J. P. Brockes, "Amphibian limb regeneration: rebuilding a complex structure," *Science*, vol. 276, no. 5309, pp. 81–87, 1997.

[56] J. P. Brockes and A. Kumar, "Comparative aspects of animal regeneration," *Annual Review of Cell and Developmental Biology*, vol. 24, pp. 525–549, 2008.

[57] Á. Raya, A. Consiglio, Y. Kawakami, C. Rodriguez-Esteban, and J. C. Izpisúa-Belmonte, "The zebrafish as a model of heart regeneration," *Cloning and Stem Cells*, vol. 6, no. 4, pp. 345–351, 2004.

[58] C. Jopling, E. Sleep, M. Raya, M. Martí, A. Raya, and J. C. I. Belmonte, "Zebrafish heart regeneration occurs by cardiomyocyte dedifferentiation and proliferation," *Nature*, vol. 464, no. 7288, pp. 606–609, 2010.

[59] E. J. Thatcher, I. Paydar, K. K. Anderson, and J. G. Patton, "Regulation of zebrafish fin regeneration by microRNAs," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 47, pp. 18384–18389, 2008.

[60] E. R. Porrello, A. I. Mahmoud, E. Simpson et al., "Transient regenerative potential of the neonatal mouse heart," *Science*, vol. 331, no. 6020, pp. 1078–1080, 2011.

[61] J. L. Whited and C. J. Tabin, "Regeneration review reprise," *Journal of Biology*, vol. 9, no. 2, article 15, 2010.

[62] V. P. Yin, J. M. Thomson, R. Thummel, D. R. Hyde, S. M. Hammond, and K. D. Poss, "Fgf-dependent depletion of microRNA-133 promotes appendage regeneration in zebrafish," *Genes & Development*, vol. 22, no. 6, pp. 728–733, 2008.

[63] S. U. Morton, P. J. Scherz, K. R. Cordes, K. N. Ivey, D. Y. R. Stainier, and D. Srivastava, "microRNA-138 modulates cardiac patterning during embryonic development," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 46, pp. 17830–17835, 2008.

[64] E. R. Porrello, B. A. Johnson, A. B. Aurora et al., "MiR-15 family regulates postnatal mitotic arrest of cardiomyocytes," *Circulation Research*, vol. 109, no. 6, pp. 670–679, 2011.

[65] M. J. Bueno and M. Malumbres, "MicroRNAs and the cell cycle," *Biochimica et Biophysica Acta—Molecular Basis of Disease*, vol. 1812, no. 5, pp. 592–601, 2011.

[66] T. G. Hullinger, R. L. Montgomery, A. G. Seto et al., "Inhibition of miR-15 protects against cardiac ischemic injury," *Circulation Research*, vol. 110, pp. 71–81, 2012.

[67] S. E. Senyo, M. L. Steinhauser, C. L. Pizzimenti et al., "Mammalian heart renewal by pre-existing cardiomyocytes," *Nature*, vol. 493, no. 7432, pp. 433–436, 2013.

[68] A. Eulalio, M. Mano, M. D. Ferro et al., "Functional screening identifies miRNAs inducing cardiac regeneration," *Nature*, vol. 492, no. 7429, pp. 376–381, 2012.

[69] D. Cirera-Salinas, M. Pauta, R. M. Allen et al., "Mir-33 regulates cell proliferation and cell cycle progression," *Cell Cycle*, vol. 11, no. 5, pp. 922–933, 2012.

[70] A. Dávalos, L. Goedeke, P. Smibert et al., "miR-33a/b contribute to the regulation of fatty acid metabolism and insulin signaling," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, no. 22, pp. 9232–9237, 2011.

[71] H. el Azzouzi, S. Leptidis, E. Dirkx et al., "The hypoxia-inducible microRNA cluster miR-199a~214 targets myocardial PPARδ and impairs mitochondrial fatty acid oxidation," *Cell metabolism*, vol. 18, pp. 341–354, 2013.

[72] J. Chen, Z.-P. Huang, H. Y. Seok et al., "Mir-17-92 cluster is required for and sufficient to induce cardiomyocyte proliferation in postnatal and adult hearts," *Circulation Research*, vol. 112, no. 12, pp. 1557–1566, 2013.

[73] V. Olive, M. J. Bennett, J. C. Walker et al., "miR-19 is a key oncogenic component of mir-17-92," *Genes and Development*, vol. 23, no. 24, pp. 2839–2849, 2009.

[74] Y. Cheng, R. Ji, J. Yue et al., "MicroRNAs are aberrantly expressed in hypertrophic heart: do they play a pole in cardiac hypertrophy?" *The American Journal of Pathology*, vol. 170, no. 6, pp. 1831–1840, 2007.

[75] P. K. Rao, Y. Toyama, R. Chiang et al., "Loss of cardiac microRNA-mediated regulation leads to dilated cardiomyopathy and heart failure," *Circulation Research*, vol. 105, pp. 585–594, 2009.

[76] S. J. Matkovich, D. J. van Booven, K. A. Youker et al., "Reciprocal regulation of myocardial microRNAs and messenger RNA in human cardiomyopathy and reversal of the microRNA signature by biomechanical support," *Circulation*, vol. 119, no. 9, pp. 1263–1271, 2009.

[77] M. Vitaloni, J. Pulecio, J. Bilic, B. Kuebler, L. Laricchia-Robbio, and J. C. I. Belmonte, "MicroRNAs contribute to induced pluripotent stem cell somatic donor memory," *The Journal of Biological Chemistry*, vol. 289, no. 4, pp. 2084–2098, 2014.

[78] C. E. Murry, R. B. Jennings, and K. A. Reimer, "Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium," *Circulation*, vol. 74, no. 5, pp. 1124–1136, 1986.

[79] M. Chiong, Z. V. Wang, Z. Pedrozo et al., "Cardiomyocyte death: mechanisms and translational implications," *Cell Death & Disease*, vol. 2, p. e244, 2011.

[80] G. W. Dorn II, "Apoptotic and non-apoptotic programmed cardiomyocyte death in ventricular remodelling," *Cardiovascular Research*, vol. 81, no. 3, pp. 465–473, 2009.

[81] A. B. Aurora, A. I. Mahmoud, X. Luo et al., "MicroRNA-214 protects the mouse heart from ischemic injury by controlling Ca^{2+} overload and cell death," *Journal of Clinical Investigation*, vol. 122, no. 4, pp. 1222–1232, 2012.

[82] S. Dong, Y. Cheng, J. Yang et al., "MicroRNA expression signature and the role of microRNA-21 in the early phase of acute myocardial infarction," *Journal of Biological Chemistry*, vol. 284, no. 43, pp. 29514–29525, 2009.

[83] T. A. Harris, M. Yamakuchi, M. Ferlito, J. T. Mendell, and C. J. Lowenstein, "MicroRNA-126 regulates endothelial expression of vascular cell adhesion molecule 1," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 5, pp. 1516–1521, 2008.

[84] A. Zernecke, K. Bidzhekov, H. Noels et al., "Delivery of microRNA-126 by apoptotic bodies induces CXCL12-dependent vascular protection," *Science Signaling*, vol. 2, no. 100, article ra81, 2009.

[85] J.-X. Wang, J.-Q. Jiao, Q. Li et al., "MiR-499 regulates mitochondrial dynamics by targeting calcineurin and dynamin-related protein-1," *Nature Medicine*, vol. 17, no. 1, pp. 71–78, 2011.

[86] L. Qian, L. W. Van Laake, Y. Huang, S. Liu, M. F. Wendland, and D. Srivastava, "miR-24 inhibits apoptosis and represses Bim in mouse cardiomyocytes," *The Journal of Experimental Medicine*, vol. 208, no. 3, pp. 549–560, 2011.

[87] M. A. Konstam, D. G. Kramer, A. R. Patel, M. S. Maron, and J. E. Udelson, "Left ventricular remodeling in heart failure: current concepts in clinical significance and assessment," *JACC: Cardiovascular Imaging*, vol. 4, no. 1, pp. 98–108, 2011.

[88] S. Roy, S. Khanna, S.-R. A. Hussain et al., "MicroRNA expression in response to murine myocardial infarction: miR-21 regulates fibroblast metalloprotease-2 via phosphatase and tensin homologue," *Cardiovascular Research*, vol. 82, no. 1, pp. 21–29, 2009.

[89] F. G. Spinale, "Matrix metalloproteinases: regulation and dysregulation in the failing heart," *Circulation Research*, vol. 90, no. 5, pp. 520–530, 2002.

[90] T. Thum, C. Gross, J. Fiedler et al., "MicroRNA-21 contributes to myocardial disease by stimulating MAP kinase signalling in fibroblasts," *Nature*, vol. 456, no. 7224, pp. 980–984, 2008.

[91] M. Tatsuguchi, H. Y. Seok, T. E. Callis et al., "Expression of microRNAs is dynamically regulated during cardiomyocyte hypertrophy," *Journal of Molecular and Cellular Cardiology*, vol. 42, no. 6, pp. 1137–1141, 2007.

[92] D. Sayed, S. Rane, J. Lypowy et al., "MicroRNA-21 targets Sprouty2 and promotes cellular outgrowths," *Molecular Biology of the Cell*, vol. 19, no. 8, pp. 3272–3282, 2008.

[93] Y. Cheng, X. Liu, S. Zhang, Y. Lin, J. Yang, and C. Zhang, "MicroRNA-21 protects against the H_2O_2-induced injury on cardiac myocytes via its target gene PDCD4," *Journal of Molecular and Cellular Cardiology*, vol. 47, no. 1, pp. 5–14, 2009.

[94] L. Cushing, P. P. Kuang, J. Qian et al., "miR-29 is a major regulator of genes associated with pulmonary fibrosis," *American Journal of Respiratory Cell and Molecular Biology*, vol. 45, no. 2, pp. 287–294, 2011.

[95] E. van Rooij, L. B. Sutherland, X. Qi, J. A. Richardson, J. Hill, and E. N. Olson, "Control of stress-dependent cardiac growth and gene expression by a microRNA," *Science*, vol. 316, no. 5824, pp. 575–579, 2007.

[96] M. Vanderheyden, W. Mullens, L. Delrue et al., "Myocardial gene expression in heart failure patients treated with cardiac resynchronization therapy: responders versus nonresponders," *Journal of the American College of Cardiology*, vol. 51, no. 2, pp. 129–136, 2008.

[97] R. L. Montgomery, T. G. Hullinger, H. M. Semus et al., "Therapeutic inhibition of miR-208a improves cardiac function and survival during heart failure," *Circulation*, vol. 124, no. 14, pp. 1537–1547, 2011.

[98] C. E. Grueter, E. van Rooij, B. A. Johnson et al., "A cardiac microRNA governs systemic energy homeostasis by regulation of MED13," *Cell*, vol. 149, no. 3, pp. 671–683, 2012.

[99] F. Ahmad, J. G. Seidman, and C. E. Seidman, "The genetic basis for cardiac remodeling," *Annual Review of Genomics and Human Genetics*, vol. 6, pp. 185–216, 2005.

[100] B. Yang, H. Lin, J. Xiao et al., "The muscle-specific microRNA miR-1 regulates cardiac arrhythmogenic potential by targeting GJA1 and KCNJ2," *Nature Medicine*, vol. 13, no. 4, pp. 486–491, 2007.

[101] P. Richardson, R. W. McKenna, M. Bristow et al., "Report of the 1995 world health organization/international society and federation of cardiology task force on the definition and classification of cardiomyopathies," *Circulation*, vol. 93, no. 5, pp. 841–842, 1996.

[102] B. H. Chew, S. S. Ghazali, M. Ismail, J. Haniff, and M. A. Bujang, "Age ≥ 60 years was an independent risk factor for diabetes-related complications despite good control of cardiovascular risk factors in patients with type 2 diabetes mellitus," *Experimental Gerontology*, vol. 48, no. 5, pp. 485–491, 2013.

[103] S. Rawal, P. Manning, and R. Katare, "Cardiovascular microRNAs: as modulators and diagnostic biomarkers of diabetic heart disease," *Cardiovascular Diabetology*, vol. 13, article 44, 2014.

[104] A. D. McClelland and P. Kantharidis, "microRNA in the development of diabetic complications," *Clinical Science*, vol. 126, no. 2, pp. 95–110, 2014.

[105] K. C. Vickers, K.-A. Rye, and F. Tabet, "MicroRNAs in the onset and development of cardiovascular disease," *Clinical Science*, vol. 126, no. 3, pp. 183–194, 2014.

[106] M. Jaguszewski, J. Osipova, J.-R. Ghadri et al., "A signature of circulating microRNAs differentiates takotsubo cardiomyopathy from acute myocardial infarction," *European Heart Journal*, vol. 35, no. 15, pp. 999–1006, 2014.

[107] J.-F. Chen, E. P. Murchison, R. Tang et al., "Targeted deletion of Dicer in the heart leads to dilated cardiomyopathy and heart failure," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 6, pp. 2111–2116, 2008.

[108] M. S. Ebert and P. A. Sharp, "MicroRNA sponges: progress and possibilities," *RNA*, vol. 16, no. 11, pp. 2043–2050, 2010.

[109] F. C. Tay, J. K. Lim, H. Zhu, L. C. Hin, and S. Wang, "Using artificial microRNA sponges to achieve microRNA loss-of-function in cancer cells," *Advanced Drug Delivery Reviews*, vol. 81, pp. 117–127, 2018.

[110] M. P. Gantier, C. E. McCoy, I. Rusinova et al., "Analysis of microRNA turnover in mammalian cells following Dicer1 ablation," *Nucleic Acids Research*, vol. 39, no. 13, pp. 5692–5703, 2011.

[111] K. Buyens, S. C. de Smedt, K. Braeckmans et al., "Liposome based systems for systemic siRNA delivery: stability in blood sets the requirements for optimal carrier design," *Journal of Controlled Release*, vol. 158, no. 3, pp. 362–370, 2012.

[112] K. A. Whitehead, R. Langer, and D. G. Anderson, "Knocking down barriers: advances in siRNA delivery," *Nature Reviews Drug Discovery*, vol. 8, pp. 129–138, 2009.

[113] N. Jiang, X. Zhang, X. Zheng et al., "A novel in vivo siRNA delivery system specifically targeting liver cells for protection of ConA-induced fulminant hepatitis," *PLoS ONE*, vol. 7, no. 9, Article ID e44138, 2012.

[114] R. M. Schiffelers, A. Ansari, J. Xu et al., "Cancer siRNA therapy by tumor selective delivery with ligand-targeted sterically stabilized nanoparticle," *Nucleic Acids Research*, vol. 32, no. 19, article e149, 2004.

[115] D. Kim, J. Hong, H.-H. Moon et al., "Anti-apoptotic cardioprotective effects of SHP-1 gene silencing against ischemia-reperfusion injury: use of deoxycholic acid-modified low molecular weight polyethyleneimine as a cardiac siRNA-carrier," *Journal of Controlled Release*, vol. 168, no. 2, pp. 125–134, 2013.

[116] A. Santel, M. Aleku, O. Keil et al., "A novel siRNA-lipoplex technology for RNA interference in the mouse vascular endothelium," *Gene Therapy*, vol. 13, no. 16, pp. 1222–1234, 2006.

[117] E. E. Creemers, A. J. Tijsen, and Y. M. Pinto, "Circulating microRNAs: novel biomarkers and extracellular communicators in cardiovascular disease?" *Circulation Research*, vol. 110, no. 3, pp. 483–495, 2012.

[118] H. L. Janssen, H. W. Reesink, E. J. Lawitz et al., "Treatment of HCV infection by targeting microRNA," *The New England Journal of Medicine*, vol. 368, pp. 1685–1694, 2013.

[119] L. Qian, Y. Huang, C. I. Spencer et al., "In vivo reprogramming of murine cardiac fibroblasts into induced cardiomyocytes," *Nature*, vol. 485, no. 7400, pp. 593–598, 2012.

[120] M. Ieda, "Heart regeneration using reprogramming technology," *Proceedings of the Japan Academy Series B: Physical and Biological Sciences*, vol. 89, no. 3, pp. 118–128, 2013.

[121] K. Song, Y. J. Nam, X. Luo et al., "Heart repair by reprogramming non-myocytes with cardiac transcription factors," *Nature*, vol. 485, no. 7400, pp. 599–604, 2012.

[122] R. Parker and U. Sheth, "P bodies and the control of mRNA translation and degradation," *Molecular Cell*, vol. 25, no. 5, pp. 635–646, 2007.

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 624037, 7 pages
http://dx.doi.org/10.1155/2015/624037

Research Article

Runx2/miR-3960/miR-2861 Positive Feedback Loop Is Responsible for Osteogenic Transdifferentiation of Vascular Smooth Muscle Cells

Zhu-Ying Xia,[1] Yin Hu,[1] Ping-Li Xie,[2] Si-Yuan Tang,[3] Xiang-Hang Luo,[1] Er-Yuan Liao,[1] Fei Chen,[4] and Hui Xie[1]

[1]*Institute of Endocrinology and Metabolism, Second Xiang-Ya Hospital of Central South University, Changsha, Hunan 410011, China*
[2]*Department of Forensic Medicine, School of Basic Medical Science of Central South University, Changsha, Hunan 410013, China*
[3]*School of Nursing of Central South University, Changsha, Hunan 410013, China*
[4]*Spine Surgery Department, Second Xiang-Ya Hospital of Central South University, Changsha, Hunan 410011, China*

Correspondence should be addressed to Fei Chen; chenfei91138@aliyun.com and Hui Xie; hxie5_csu@163.com

Received 15 August 2014; Accepted 2 January 2015

Academic Editor: Xiao-Bo Liao

We previously reported that Runx2/miR-3960/miR-2861 regulatory feedback loop stimulates osteoblast differentiation. However, the effect of this feedback loop on the osteogenic transdifferentiation of vascular smooth muscle cells (VSMCs) remains unclear. Our recent study showed that miR-2861 and miR-3960 expression increases significantly during β-glycerophosphate-induced osteogenic transdifferentiation of VSMCs. Overexpression of miR-2861 or miR-3960 in VSMCs enhances β-glycerophosphate-induced osteoblastogenesis, whereas inhibition of miR-2861 or miR-3960 expression attenuates it. MiR-2861 or miR-3960 promotes osteogenic transdifferentiation of VSMCs by targeting histone deacetylase 5 or Homeobox A2, respectively, resulting in increased runt-related transcription factor 2 (Runx2) protein production. Furthermore, overexpression of Runx2 induces miR-2861 and miR-3960 transcription, and knockdown of Runx2 attenuates β-glycerophosphate-induced miR-2861 and miR-3960 transcription in VSMCs. Thus, our data show that Runx2/miR-3960/miR-2861 positive feedback loop plays an important role in osteogenic transdifferentiation of VSMCs and contributes to vascular calcification.

1. Introduction

Medial artery calcification is a common and serious problem with ageing of the population. It is a nonocclusive process which leads to decreased vessel elasticity, increased blood pressure, and higher risk of cardiovascular mortality [1–3]. Medial artery calcification is now recognized as a highly regulated process which is similar in many ways to bone mineralization. Osteoblast-like phenotypes transformation of vascular smooth muscular cells (VSMCs), the predominant cells in the tunica media of arteries, is currently considered responsible for the formation of medial artery calcification [4–6]. However, the specific mechanism governing this process is still elusive.

MicroRNAs (miRNAs) are endogenous, small (16~25 nucleotides), single-stranded noncoding RNAs [7]. MicroRNAs mediate translational repression or degradation of target transcript by binding to sites of complementarity in the 3′-UTRs of target mRNAs. During the past decade, they have emerged as powerful posttranscriptional regulators of gene expression [8]. To date, more than 3% of the genes in the human genome have been found to encode for miRNAs, and over 30% of genes in the human genome are estimated to be regulated by miRNAs [9]. It has been documented that miRNAs participate in cellular proliferation, differentiation, migration, and apoptosis [10–12]. However, their roles in osteogenic transdifferentiation or calcification of VSMC have just begun to be understood.

Recently, we reported that Runx2/miR-3960/miR-2861 positive feedback loop is responsible for osteoblast differentiation [13]. Osteoblast differentiation signals lead to the activation of Runx2 transcription factor in stromal cells. In addition to induction of genes essential for osteoblast differentiation, Runx2 transactivates miR-3960/miR-2861. In turn, miR-3960 and miR-2861 maintain the levels of *Runx2* mRNA and protein via repressing Homeobox A2 (Hoxa2) and histone deacetylase 5 (HDAC5) and stabilizing the osteoblast differentiation. The objective of this study was to elucidate the effect of Runx2/miR-3960/miR-2861 feedback loop on regulation of the osteogenic transdifferentiation of VSMCs.

2. Materials and Methods

2.1. Cell Culture and Transfection. To isolate primary cells, eight-week-old C57BL/6 male wild type mice received a 150 mg/kg intraperitoneal dose of pentobarbital sodium prior to euthanasia which was confirmed by the absence of a heartbeat. Vascular smooth muscle cells (VSMCs) were isolated from aorta as previously described [14]. VSMCs were cultured in DMEM supplemented with 10% FBS. To induce osteogenic transdifferentiation, VSMCs between passages 3 and 7 were cultured in DMEM supplemented with 10 mM β-glycerophosphate. For transient transfection of miR-2861, miR-3960, anti-miR-2861, anti-miR-3960, and Runx2 siRNA, a mixture of each of them or their control with Lipofectamine 2000 (Invitrogen) was added to cells in 24-well plates at a density of 3×10^4 cells per well for 48 hours.

2.2. Northern Blot. Total RNA was extracted from cells by Trizol reagent (Invitrogen). Northern blotting was performed as described previously [15]. 20 μg of RNA was separated on a 15% urea polyacrylamide gel with 0.5x Tris borate-EDTA and transferred to a Hybond-N+ nylon membrane (Amersham Biosciences) using a semidry transfer cell (Bio-Rad). Hybridization was performed according to a standard protocol. ^{32}P-labeled oligonucleotide probes complementary to the mature miR-2861 and miR-3960 were used in the hybridization. The probes for miR-2861, miR-3960, and U6 were 5′-CCGCCCGCCGCCAGGCCCC-3′, 5′-GCCCCC-GCCTCCGCCGCCGCC-3′, and 5′-ATATGGAACGCT-TCACGAATT-3′, respectively.

2.3. Western Blot. To detect Runx2, HDAC5, and Hoxa2 protein expression, Western blot analysis was performed as previously described [16, 17]. Briefly, equal amounts of protein (40 μg/lane) were subjected to SDS gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Amersham Pharmacia Biotech, GE Healthcare Biosciences, Pittsburgh, PA, USA). The membranes were incubated with primary antibodies against Runx2 (Santa Cruz), HDAC5 (Santa Cruz), Hoxa2 (Santa Cruz), and β-actin (Abcam) overnight at 4°C and then incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (Santa Cruz) for 1 h at room temperature. The blots then were visualized with the chemiluminescent detection method using the SuperSignal West Pico Substrate (Pierce Biotechnology).

2.4. ALP Activity and Osteocalcin Secretion Assay. Cells were grown to confluence in 24-well plates. The cells were then washed with PBS and scraped into a solution containing 20 mM Tris-HCl, pH 8.0, and 150 mM NaCl and 1% Triton X-100, 0.02% NaN_3, and 1 μg/mL aprotinin. The lysates were homogenized; then alkaline phosphatase (ALP) activity was assayed by spectrophotometric measurement of p-nitrophenol release at 37°C. Osteocalcin released into the culture media was measured using a specific radioimmunoassay kit (DiaSorin). To normalize protein expression to total cellular protein, a fraction of the lysate solution was used in a Bradford protein assay.

2.5. qRT-PCR Analysis. Total RNA was isolated using the Trizol reagent (Invitrogen), and reverse transcription was performed using 1.0 mg of total RNA and the SuperScript II Kit (Invitrogen). qRT-PCR was performed using a Roche Molecular Light Cycler (Roche Molecular Biochemicals, Indianapolis, IN, USA). Amplification reactions were set up in 25 μL reaction volumes containing SYBR Green PCR Master Mix (PE Applied Biosystems, Waltham, MA, USA), template cDNA, and amplification primers. The following primers were used: 5′-TGTCACCGCCAGATGTTTTG-3′, 5′-TGAGCAGAGCCGAGACACAG-3′; Hoxa2 forward, 5′-GTCACTCTTTGAGCAAGCCC-3′; reverse, 5′-TAGGCC-AGCTCCACAGTTCT-3′; GAPDH sense, 5′-CACCAT-GGAGAAGGCCGGGG-3′; and antisense, 5′-GACGGA-CACATTGGGGGTAG-3′. PCR amplifications were performed, and the amplification data were analyzed using the sequence detector system software (PE Applied Biosystems). Relative quantification was calculated by normalizing the test crossing thresholds (Ct) with the GAPDH amplified control Ct.

2.6. Statistical Analyses. Data were presented as mean ± s.e.m. Comparisons were made using a one-way analysis of variance. All experiments were repeated at least three times, and representative experiments were shown. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Increased Expression of miR-2861 and miR-3960 during the Osteogenic Transdifferentiation of VSMCs. We performed Northern blot analysis to examine the expression of miR-2861 and miR-3960 in VSMCs during β-glycerophosphate-induced osteogenesis. Both miR-2861 and miR-3960 could be detected after treatment with β-glycerophosphate for 12 h and increased with time throughout osteoblastic differentiation of VSMCs (Figure 1).

3.2. miR-2861 or miR-3960 Promotes Osteoblast Differentiation of Vascular Smooth Muscle Cells. We next determined the role of miR-2861 or miR-3960 during osteogenic transdifferentiation of VSMCs by changing the functional levels

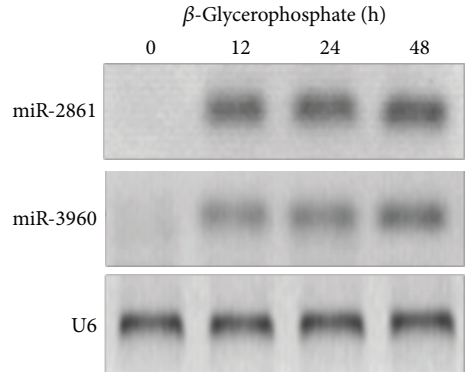


Figure 1: Increased expression of miR-2861 and miR-3960 during β-glycerophosphate-induced osteogenic transdifferentiation of VSMCs. Northern blot analysis of miR-2861 and miR-3960 expression in 10 mM β-glycerophosphate-treated VSMCs for the indicated times. U6 snRNA is used as a loading control.

of miR-2861 or miR-3960 in VSMCs. Northern blot data confirmed overexpression of miR-2861 and miR-3960 in VSMCs stably transfected with miR-2861 precursor and miR-3960 precursor, respectively (Figure 2(a)). The transfected VSMCs were treated with β-glycerophosphate for 48 h for inducing osteogenesis. Then, ALP activity, osteocalcin secretion, and Runx2 protein expression, as markers of osteoblast differentiation, were evaluated. The levels of ALP, osteocalcin, and Runx2 were all elevated in cells with overexpression of miR-2861 or miR-3960 (Figure 2(b)). These results indicated that overexpression of miR-2861 or miR-3960 promoted osteogenic transdifferentiation of VSMCs (Figures 2(b) and 2(c)). In contrast, knockdown of miR-2861 or miR-3960 reduced ALP, osteocalcin, and Runx2 levels induced by β-glycerophosphate (Figures 3(a) and 3(b)). All of these results suggest that both miR-2861 and miR-3960 act to promote osteogenic transdifferentiation of VSMCs.

3.3. miR-2861 and miR-3960 Posttranscriptionally Repress HDAC5 and Hoxa2 Expression. We previously identified that HDAC5 and Hoxa2 are the direct targets of miR-2861 and miR-3960, respectively, in a stromal cell line ST2 [13]. Here, we overexpressed miR-2861 or miR-3960 to directly identify their actions on HDAC5 or Hoxa2 in VSMCs. As expected, overexpression of miR-2861 or miR-3960 resulted in a decrease in the HDAC5 or Hoxa2 protein levels (Figures 4(a) and 4(b)). However, the HDAC5 or Hoxa2 mRNA levels were not affected (Figures 4(a) and 4(b)). These results revealed that miR-2861 and miR-3960 posttranscriptionally repressed HDAC5 and Hoxa2 protein expression by inhibiting mRNA translation in VSMCs.

3.4. Runx2 Stimulates miR-2861 and miR-3960 Expression in Vascular Smooth Muscle. We previously demonstrated that Runx2 directly induces the expression of the miR-3960/miR-2861 cluster by binding to the putative binding site of its promoter in ST2 cells [13]. Here, in order to identify the direct impact of Runx2 on miR-2861 and miR-3960 gene expression in VSMCs, we changed the functional levels of Runx2. First, we used a pcDNA-driven expression vector to overexpress Runx2 protein levels, as confirmed by Western blot (Figure 5(a)). The results showed that Runx2 overexpression could induce miR-2861 and miR-3960 expression in VSMCs (Figure 5(a)). Furthermore, we transfected siRNA designed against Runx2 into β-glycerophosphate-induced VSMCs to specifically silence Runx2 expression. Western blot analysis revealed that si-Runx2 blocked Runx2 expression compared with the control (Figure 5(b)). The expression of miR-3960 and miR-2861 was inhibited by the knockdown of Runx2 (Figure 5(b)). Thus, these results suggest a direct stimulatory action of Runx2 on the expression of miR-2861 and miR-3960 in VSMCs.

4. Discussion

In our previous study, we cloned both miR-2861 and miR-3960 and identified that miR-2861 induces osteoblast differentiation by repressing HDAC5, an enhancer of Runx2 degradation, and miR-3960 induces osteoblast differentiation by repressing Hoxa2, a repressor of Runx2 expression [13, 15]. miR-2861 binds to the coding domain sequence (CDS) of *HDAC5* mRNA with complementarity to the miR-2861 seed region and posttranscriptionally represses HDAC5 protein expression by inhibiting mRNA translation and not by mRNA degradation [15]. Similarly, miR-3960 directly targets *Hoxa2* by specifically binding with the target CDS of *Hoxa2* and represses Hoxa2 expression at the posttranscriptional level [13]. Thus, miR-3960 and miR-2861 can coregulate the Runx2 during osteoblast differentiation. In turn, Runx2 binds to the miR-2861/miR-3960 cluster promoter to transcriptionally induce the expression of miR-2861 and miR-3960 [13]. Runx2/miR-3960/miR-2861 is a critical positive feedback loop for osteoblast differentiation.

Recently, several miRNAs have been reported to be involved in osteogenic transdifferentiation and calcification of VSMCs [18–22]. It has been reported that miR-221 and miR-222 increase runt-related transcription factor 2 (Runx2) expression and calcium deposition in VSMCs [18]. On the contrary, miR-204, miR-133a, and miR-125b suppress osteoblastic differentiation of VSMCs by inhibiting Runx2 protein expression, whereas miR-204, miR-133a, and miR-125b inhibition enhance osteoblastic differentiation of VSMCs [19–22]. In this study, we show that Runx2/miR-3960/miR-2861 is also responsible for stimulating osteogenic transdifferentiation of VSMCs. At first, we demonstrated that the expression of both miR-2861 and miR-3960 was significantly increased during β-glycerophosphate-induced osteogenic transdifferentiation of VSMCs, indicating that miR-2861 and miR-3960 might play a role in vascular calcification. To prove whether miR-2861 and miR-3960 are directly associated with the osteogenic transdifferentiation of VSMCs, we evaluated the effect of miR-2861 and miR-3960 in the process of β-glycerophosphate-induced osteogenic transdifferentiation of VSMCs. Runx2 has been identified as a "master transcription factor" simulating osteogenic transdifferentiation of precursor cells [4, 23]. It plays an essential

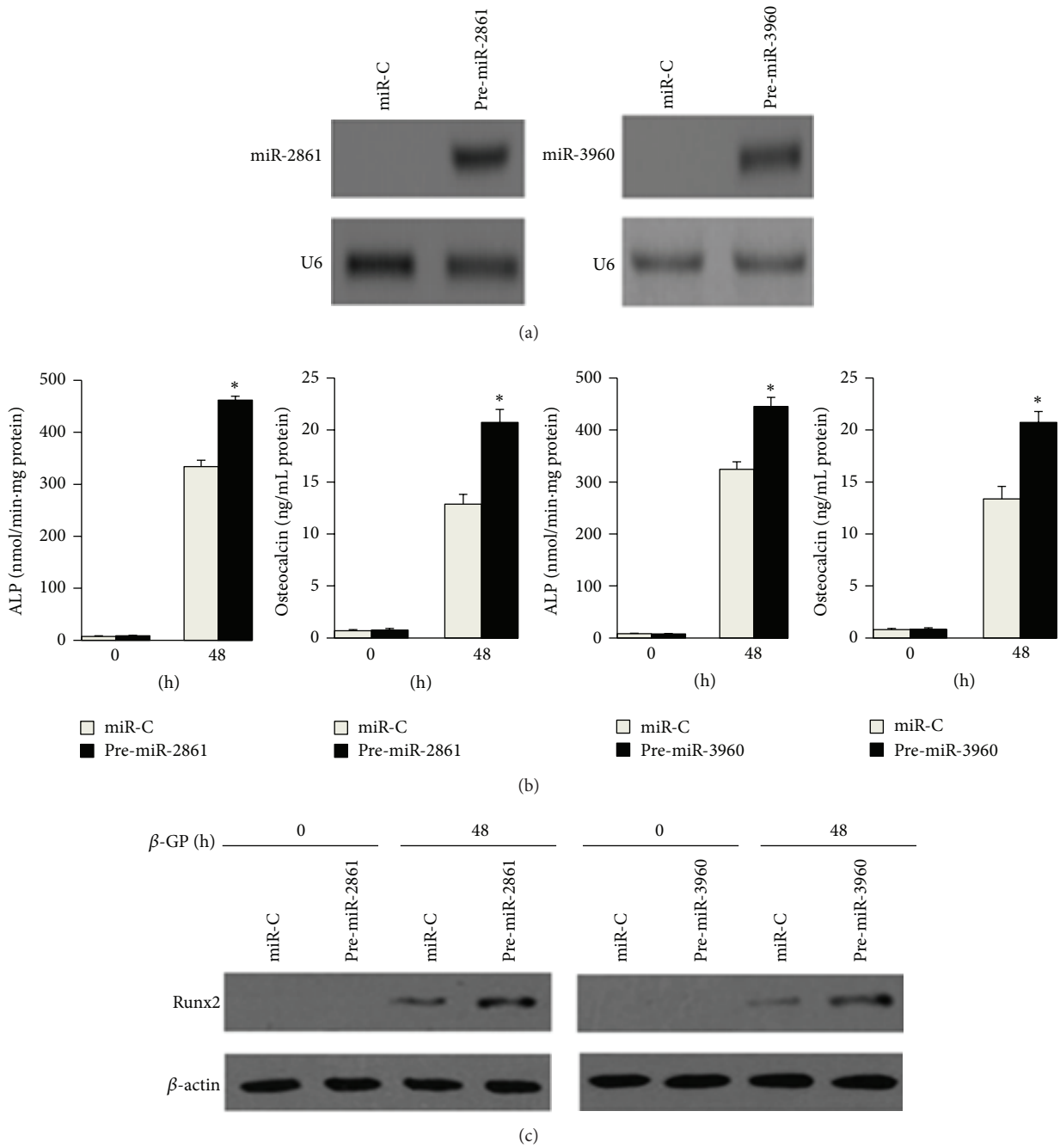


Figure 2: miR-2861 or miR-3960 promoted β-glycerophosphate-induced osteoblast differentiation of vascular smooth muscle cells. (a) Northern blot analysis of miR-2861 expression in VSMCs transfected with miR-C (control) or miR-2861 precursor (pre-miR-3861), miR-C, or pre-miR-3960. ((b) and (c)) ALP activity and osteocalcin secretion (b) and Western blot analysis of Runx2 protein expression (c) in 10 mM β-glycerophosphate- (β-GP-) treated VSMCs stably transfected with miR-C or pre-miR-2861, miR-C, or pre-miR-3960. Data are shown as mean ± s.e.m. $^{*}P < 0.05$.

role in the osteogenic transdifferentiation of VSMCs [24, 25]. Knockdown of Runx2 significantly inhibits the ALP expression and the calcification in the senescent VSMCs [26]. ALP is a prophase (proliferative phase) marker of osteoblast differentiation. Osteocalcin is a metaphase (bone matrix formation period) marker of osteoblast differentiation. Both miR-2861 and miR-3960 promoted osteogenic transdifferentiation of VSMCs, as indicated by increased levels of ALP activity, osteocalcin secretion, and Runx2 protein expression.

HDAC5 is an enhancer of Runx2 degradation. It deacetylates Runx2, allowing the protein to undergo Smurf-mediated degradation, and inhibition of HDAC5 increases Runx2 acetylation [27, 28]. Hoxa2, a member of the Hox homeodomain family of transcription factors that regulate skeletal

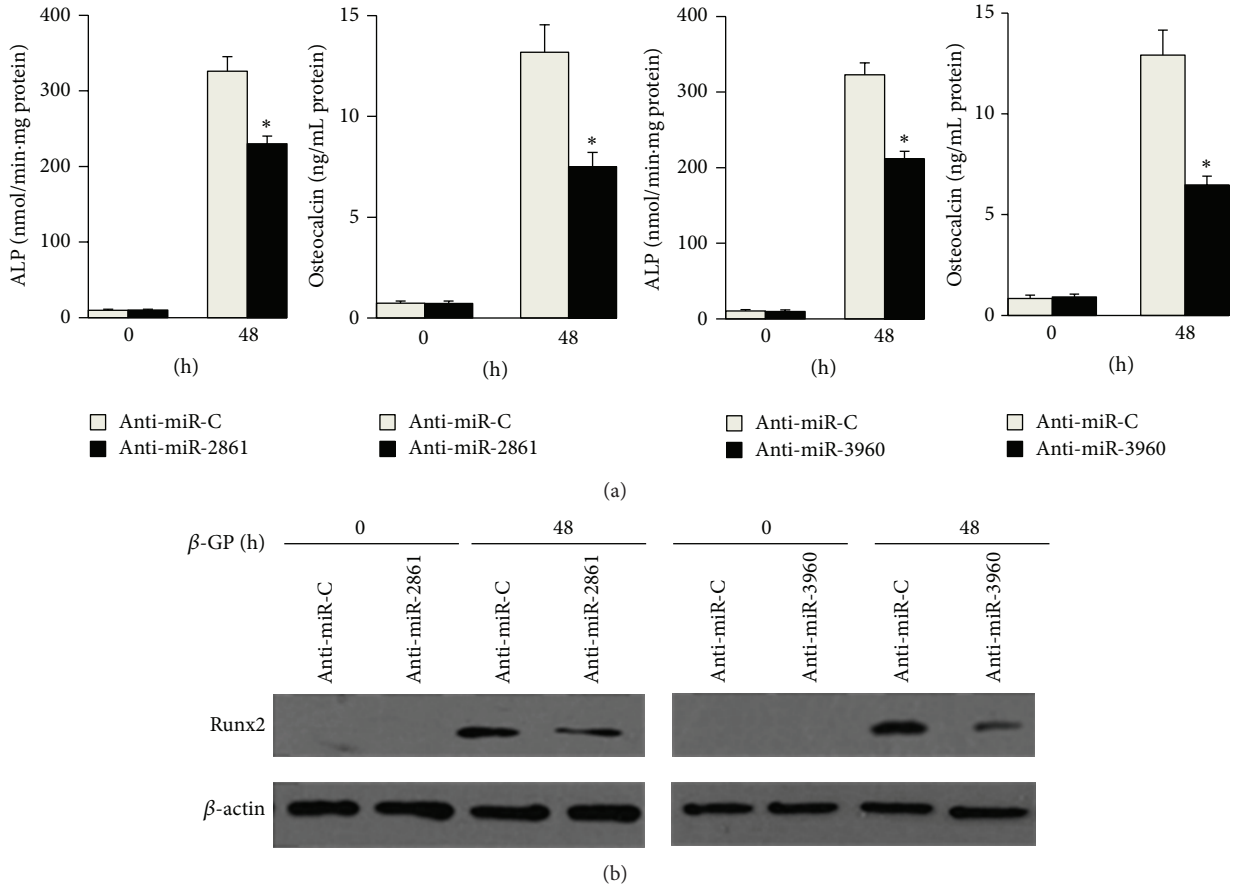


Figure 3: Inhibition of miR-2861 or miR-3960 inhibited β-glycerophosphate-induced osteoblast differentiation of vascular smooth muscle cells. ALP activity and osteocalcin secretion (a) and Western blot analysis of Runx2 protein expression (b) in 10 mM β-glycerophosphate-(β-GP-) treated VSMCs transfected with anti-miR-C (control) or anti-miR-2861, anti-miR-C, or anti-miR-3960. Data are shown as mean ± s.e.m. $^{*}P < 0.05$.

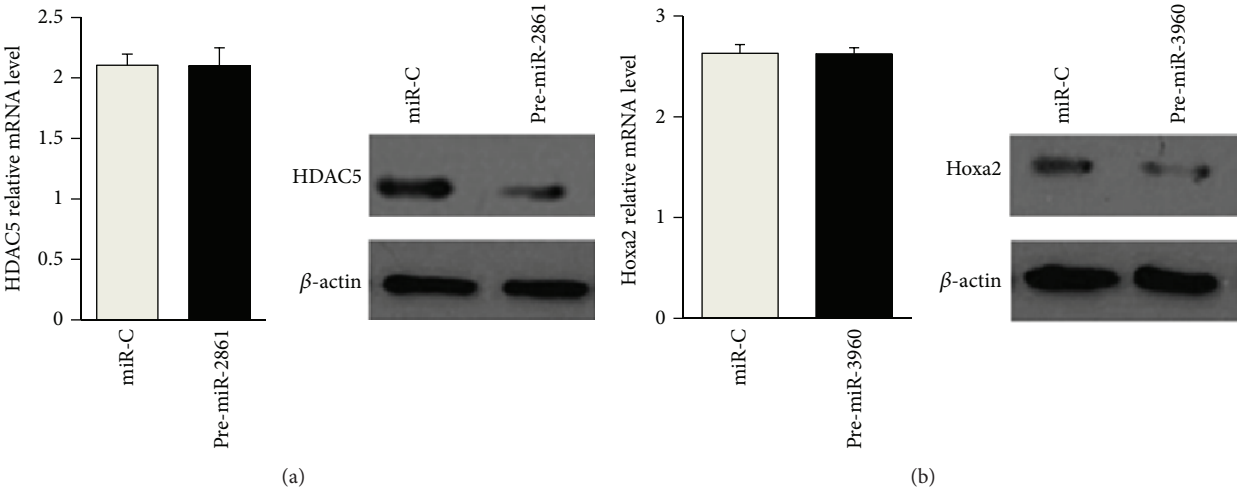


Figure 4: miR-2861 and miR-3960 posttranscriptionally repressed HDAC5 and Hoxa2 expression, respectively. ((a) and (b)) Quantitative RT-PCR analysis of mRNA levels of *HDAC5* and *Hoxa2* (left) and Western blot analysis of protein levels of HDAC5 and Hoxa2 (right) in VSMCs transfected with miR-C (control) or pre-miR-3960 (a), miR-C, or miR-3960 (b). For quantitative RT-PCR analysis, mRNA levels normalized to GAPDH. Data are shown as means ± s.e.m. For Western blot analysis, β-actin acts as a loading control.

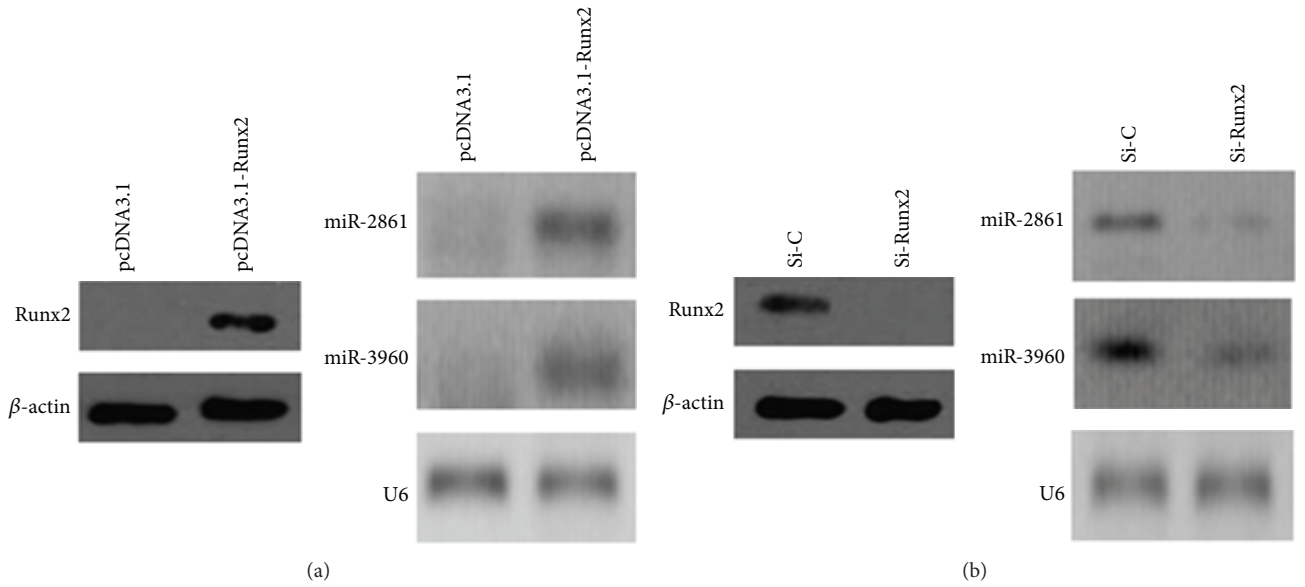


Figure 5: Runx2 stimulates miR-2861 and miR-3960 expression in vascular smooth muscle cells. (a) Western blot analysis of Runx2 protein levels (left) and Northern blot analysis of miR-2861 and miR-3960 levels (right) in VSMCs transfected with pcDNA3.1 vector control or Runx2 pcDNA3.1 vector (*pcDNA3.1-Runx2*) for 48 h. (b) Western blot analysis of Runx2 protein levels (left) and Northern blot analysis of miR-2861 and miR-3960 levels (right) in VSMCs transfected with siRNA control (*si-C*) or si-Runx2 and then cultured with β-glycerophosphate for 48 h. For Western blot analysis, β-actin acts as a loading control. For Northern blot analysis, U6 snRNA acts as a loading control.

patterning, is found to control Runx2 expression and is required in skeletal morphogenesis [29]. *Hoxa2*$^{-/-}$ mice show an upregulation of the Runx2 level, and Hoxa2 inhibits bone formation by repressing Runx2 expression [29]. In our previous study, ST2 cells transfected with a Hoxa2 expression vector showed reduced Runx2 expression and ALP activity, confirming that Hoxa2 can suppress Runx2 expression and further inhibits osteoblast differentiation [13]. In this study, we detected HDAC5 expression in VSMCs, inconsistent with several previous papers [29–32]. Moreover, we identified Hoxa2 expression in VSMCs. Thus, miR-2861 and miR-3960 directly target *HDAC5* and *Hoxa2*, respectively, to increase Runx2 levels in VSMCs.

Together, our study shows that Runx2/miR-3960/miR-2861 positive feedback loop plays an important role in osteogenic transdifferentiation of VSMCs and contributes to vascular calcification.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

This study was supported by the China National Natural Scientific Foundation (Grants nos. 81000122, 81370974, and 81400858); the Science and Technology Project of Changsha City (Grant no. K1207043-31); the Specialized Research Fund for the Doctoral Program of Higher Education (SRFDP; Grant no. 20120162110067); the National Basic Research Program of China (Grant no. 2014CB942903); the China National Funds for Distinguished Young Scientists (Grant no. 81125006).

References

[1] M. Wu, C. Rementer, and C. M. Giachelli, "Vascular calcification: an update on mechanisms and challenges in treatment," *Calcified Tissue International*, vol. 93, no. 4, pp. 365–373, 2013.

[2] D. A. Chistiakov, I. A. Sobenin, A. N. Orekhov, and Y. V. Bobryshev, "Mechanisms of medial arterial calcification in diabetes," *Current Pharmaceutical Design*, vol. 20, no. 37, pp. 5870–5883, 2014.

[3] M. R. Brodeur, C. Bouvet, M. Barrette, and P. Moreau, "Palmitic acid increases medial calcification by inducing oxidative stress," *Journal of Vascular Research*, vol. 50, no. 5, pp. 430–441, 2013.

[4] R. C. Johnson, J. A. Leopold, and J. Loscalzo, "Vascular calcification: pathobiological mechanisms and clinical implications," *Circulation Research*, vol. 99, no. 10, pp. 1044–1059, 2006.

[5] R. J. Guzman, "Clinical, cellular, and molecular aspects of arterial calcification," *Journal of Vascular Surgery*, vol. 45, no. 6, pp. A57–A63, 2007.

[6] L. L. Demer and Y. Tintut, "Vascular calcification: pathobiology of a multifaceted disease," *Circulation*, vol. 117, no. 22, pp. 2938–2948, 2008.

[7] D. P. Bartel, "MicroRNAs: genomics, biogenesis, mechanism, and function," *Cell*, vol. 116, no. 2, pp. 281–297, 2004.

[8] R. W. Carthew and E. J. Sontheimer, "Origins and mechanisms of miRNAs and siRNAs," *Cell*, vol. 136, no. 4, pp. 642–655, 2009.

[9] B. P. Lewis, C. B. Burge, and D. P. Bartel, "Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets," *Cell*, vol. 120, no. 1, pp. 15–20, 2005.

[10] V. Ambros and X. Chen, "The regulation of genes and genomes by small RNAs," *Development*, vol. 134, no. 9, pp. 1635–1641, 2007.

[11] J. W. Hagen and E. C. Lai, "MicroRNA control of cell-cell signaling during development and disease," *Cell Cycle*, vol. 7, no. 15, pp. 2327–2332, 2008.

[12] P. Trang, J. B. Weidhaas, and F. J. Slack, "MicroRNAs as potential cancer therapeutics," *Oncogene*, vol. 27, no. 2, pp. S52–S57, 2008.

[13] R. Hu, W. Liu, H. Li et al., "A Runx2/miR-3960/miR-2861 regulatory feedback loop during mouse osteoblast differentiation," *The Journal of Biological Chemistry*, vol. 286, no. 14, pp. 12328–12339, 2011.

[14] X. B. Liao, Y. Q. Peng, X. M. Zhou et al., "Taurine restores Axl/Gas6 expression in vascular smooth muscle cell calcification model," *Amino Acids*, vol. 39, no. 2, pp. 375–383, 2010.

[15] H. Li, H. Xie, W. Liu et al., "A novel microRNA targeting HDAC5 regulates osteoblast differentiation in mice and contributes to primary osteoporosis in humans," *The Journal of Clinical Investigation*, vol. 119, pp. 3666–3677, 2009.

[16] H. Xie, M. Sun, X.-B. Liao et al., "Estrogen receptor α36 mediates a bone-sparing effect of 17β-estrodiol in postmenopausal women," *Journal of Bone and Mineral Research*, vol. 26, no. 1, pp. 156–168, 2011.

[17] H. Xie, P.-L. Xie, X.-P. Wu et al., "Omentin-1 attenuates arterial calcification and bone loss in osteoprotegerin-deficient mice by inhibition of RANKL expression," *Cardiovascular Research*, vol. 92, no. 2, pp. 296–306, 2011.

[18] N. C. W. Mackenzie, K. A. Staines, D. Zhu, P. Genever, and V. E. MacRae, "miRNA-221 and miRNA-222 synergistically function to promote vascular calcification," *Cell Biochemistry and Function*, vol. 32, no. 2, pp. 209–216, 2014.

[19] P. Wen, H. Cao, L. Fang et al., "miR-125b/Ets1 axis regulates transdifferentiation and calcification of vascular smooth muscle cells in a high-phosphate environment," *Experimental Cell Research*, vol. 322, no. 2, pp. 302–312, 2014.

[20] X. B. Liao, Z. Y. Zhang, K. Yuan et al., "MiR-133a modulates osteogenic differentiation of vascular smooth muscle cells," *Endocrinology*, vol. 154, no. 9, pp. 3344–3352, 2013.

[21] R.-R. Cui, S.-J. Li, L.-J. Liu et al., "MicroRNA-204 regulates vascular smooth muscle cell calcification in vitro and in vivo," *Cardiovascular Research*, vol. 96, no. 2, pp. 320–329, 2012.

[22] C. Goettsch, M. Rauner, N. Pacyna, U. Hempel, S. R. Bornstein, and L. C. Hofbauer, "MiR-125b regulates calcification of vascular smooth muscle cells," *American Journal of Pathology*, vol. 179, no. 4, pp. 1594–1600, 2011.

[23] T. Komori, "Regulation of osteoblast differentiation by transcription factors," *Journal of Cellular Biochemistry*, vol. 99, no. 5, pp. 1233–1239, 2006.

[24] H. B. Chang, A. Javed, Q. Dai et al., "Oxidative stress induces vascular calcification through modulation of the osteogenic transcription factor Runx2 by AKT signaling," *The Journal of Biological Chemistry*, vol. 283, no. 22, pp. 15319–15327, 2008.

[25] N. X. Chen, D. Duan, K. D. O'Neill et al., "The mechanisms of uremic serum-induced expression of bone matrix proteins in bovine vascular smooth muscle cells," *Kidney International*, vol. 70, no. 6, pp. 1046–1053, 2006.

[26] R. Nakano-Kurimoto, K. Ikeda, M. Uraoka et al., "Replicative senescence of vascular smooth muscle cells enhances the calcification through initiating the osteoblastic transition," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 297, no. 5, pp. H1673–H1684, 2009.

[27] E.-J. Jeon, K.-Y. Lee, N.-S. Choi et al., "Bone morphogenetic protein-2 stimulates Runx2 acetylation," *Journal of Biological Chemistry*, vol. 281, no. 24, pp. 16502–16511, 2006.

[28] J. S. Kang, T. Alliston, R. Delston, and R. Derynck, "Repression of Runx2 function by TGF-β through recruitment of class II histone deacetylases by Smad3," *The EMBO Journal*, vol. 24, no. 14, pp. 2543–2555, 2005.

[29] B. Kanzler, S. J. Kuschert, Y.-H. Liu, and M. Mallo, "*Hoxa-2* restricts the chondrogenic domain and inhibits bone formation during development of the branchial area," *Development*, vol. 125, no. 14, pp. 2587–2597, 1998.

[30] R. Ginnan, L. Y. Sun, J. J. Schwarz, and H. A. Singer, "MEF2 is regulated by CaMKIIδ_2 and a HDAC4-HDAC5 heterodimer in vascular smooth muscle cells," *Biochemical Journal*, vol. 444, no. 1, pp. 105–114, 2012.

[31] J. Pang, C. Yan, K. Natarajan et al., "GIT1 mediates HDAC5 activation by angiotensin II in vascular smooth muscle cells," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 28, no. 5, pp. 892–898, 2008.

[32] X. Xu, C.-H. Ha, C. Wong et al., "Angiotensin II stimulates protein kinase D-dependent histone deacetylase 5 phosphorylation and nuclear export leading to vascular smooth muscle cell hypertrophy," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 27, no. 11, pp. 2355–2362, 2007.

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 263151, 13 pages
http://dx.doi.org/10.1155/2015/263151

Research Article

A MicroRNA-Transcription Factor Blueprint for Early Atrial Arrhythmogenic Remodeling

Mario Torrado,[1] Diego Franco,[2] Estefanía Lozano-Velasco,[2] Francisco Hernández-Torres,[2] Ramón Calviño,[3] Guillermo Aldama,[3] Alberto Centeno,[3] Alfonso Castro-Beiras,[1] and Alexander Mikhailov[1]

[1]*Institute of Health Sciences, University of La Coruña, 15006 La Coruña, Spain*
[2]*Department of Experimental Biology, University of Jaén, Jaén, Spain*
[3]*University Hospital Center of La Coruña, La Coruña, Spain*

Correspondence should be addressed to Alexander Mikhailov; margot@udc.es

Received 20 February 2015; Revised 22 April 2015; Accepted 23 April 2015

Academic Editor: Chi-Hsiao Yeh

Spontaneous self-terminating atrial fibrillation (AF) is one of the most common heart rhythm disorders, yet the regulatory molecular mechanisms underlying this syndrome are rather unclear. MicroRNA (miRNA) transcriptome and expression of candidate transcription factors (TFs) with potential roles in arrhythmogenesis, such as *Pitx2*, *Tbx5*, and myocardin (*Myocd*), were analyzed by microarray, qRT-PCR, and Western blotting in left atrial (LA) samples from pigs with transitory AF established by right atrial tachypacing. Induced ectopic tachyarrhythmia caused rapid and substantial miRNA remodeling associated with a marked downregulation of *Pitx2*, *Tbx5*, and *Myocd* expression in atrial myocardium. The downregulation of *Pitx2*, *Tbx5*, and *Myocd* was inversely correlated with upregulation of the corresponding targeting miRNAs (miR-21, miR-10a/10b, and miR-1, resp.) in the LA of paced animals. Through *in vitro* transient transfections of HL-1 atrial myocytes, we further showed that upregulation of miR-21 did result in downregulation of *Pitx2* in cardiomyocyte background. The results suggest that immediate-early miRNA remodeling coupled with deregulation of TF expression underlies the onset of AF.

1. Introduction

Atrial fibrillation (AF), a heart rhythm disorder dubbed as "the epidemic of the 21st century" [1], represents nowadays a serious clinical problem making the development of novel treatment approaches highly desirable (recently reviewed in [2]). AF is generally considered to be a progressive condition, occurring first in a paroxysmal form (short-lived self-terminating episodes) and then in persistent and eventually long-lasting permanent forms. Recent growing evidence indicates that the underlying molecular mechanisms are distinct in each form of AF [3, 4]. Proarrhythmogenic molecular remodeling, broadly defined as any change in atrial gene regulation that promotes atrial conduction disturbances, is potentially crucial for unraveling the onset mechanisms of atrial tachyarrhythmias.

There is emerging evidence that microRNAs (miRNAs) can modulate a diverse spectrum of cardiac functions through their ability to remodel cardiac transcriptional circuits (reviewed in [5–7]). The tight regulation of the levels of miRNAs is critical for maintaining normal physiological conditions, and dysregulated miRNA levels contribute to the development of heart diseases (reviewed in [8–10]). In relevance to this study, miRNAs have been demonstrated to be essential in regulating atrial excitability and can be involved, directly or indirectly, in increased atrial arrhythmogenicity and AF (reviewed in [11–15]). In this context, there is growing evidence regarding the aberrant miRNA expression in chronic AF conditions in patients [16–22], while only a few papers have focused on miRNA transcriptomic profiling of atrial samples from patients with paroxysmal AF [23,

24]. Specifically, these papers demonstrated that a miRNA-mediated imbalance in the gonadotropin releasing hormone receptor/p53 [23] and metalloproteinase/inhibitor [24] pathways may potentially be involved in atrial remodeling. As it is mentioned in the reports, fibrillating atrial samples were collected during cardiac surgery in hypertonic patients [23] or in patients with long-lasting (years) paroxysmal AF [24]. Each of these clinical conditions is known to be associated with a chronic atrial stretch and consequent atrial structural remodeling. In this sense, left atrial enlargement was observed in patients in the study by [24].

Although these studies provide evidence in support of the importance of miRNAs in long-lasting AF, the use of miRNA expression profiling as a tool to assess the very-early atrial remodeling in animal models of self-terminating atrial tachyarrhythmias (resembling early-onset lone paroxysmal AF in humans) is still missing. It is important to highlight that the left (LA) and right (RA) atria have different susceptibilities towards developing arrhythmias, given the fact that the pulmonary veins and surrounding myocardial regions of the LA constitute the most frequent foci of AF initiation.

In the current study, we report the changes in miRNA expression in the LA in response to ectopic tachyarrhythmia episodes induced by short-term atrial pacing in pigs. 38 microRNAs were differentially expressed, 18 of which were upregulated in the LA of paced versus sham (control) animals. In addition, we identified expression differences for novel miRNAs that have not previously been annotated as either cardiac- or AF-associated.

Alterations in atrial miRNAs can contribute to transcription factor (TF) deregulations underlying early atrial remodeling. Genome-wide association studies and other approaches have identified the TFs, *Pitx2* and *Tbx5* as candidate genes linked to susceptibility for early development of AF (reviewed in [41, 42]) and myocardin (*Myocd*) as a transcription cofactor linked with LA functional and structural remodeling [43]. Interestingly, *Pitx2*, *Tbx5*, and *Myocd* expression is markedly downregulated in the LA in response to ectopic tachyarrhythmia. Particularly intriguing is that the downregulation of these TFs inversely correlates with upregulation of the corresponding targeting miRNAs in the LA of paced animals.

2. Materials and Methods

2.1. Animals and Experimental Design. "Large white" 3-month-old pigs were obtained from a local commercial breeder (La Coruña, Spain) and randomly divided into two groups: (1) sham ($n = 5$; mean body weight, 25.2 ± 1.2 kg) and (2) atrial tachypacing ($n = 5$; mean body weight, 25.7 ± 0.8 kg). A close-chest tachyarrhythmia model was established in animals via rapid atrial electrical stimulation with a controlled ventricular response rate. To control anaesthesia, ventilation, and oxygenation, ECG, heart rhythm, and blood pressure were continuously monitored. The right femoral vein was dissected and cannulated with a multielectrode catheter (Medtronic, Minneapolis, USA) connected with an external pulse stimulator (A-M Systems, UK) for programmed pacing rates. Under fluoroscopic guidance, the catheter was passed into the right atrium (Figure 1). To avoid tachycardia-induced ventricular dysfunction (ventricular arrhythmias), esmolol hydrochloride (a beta adrenergic receptor blocker) was given before pacing. After a 5-minute stabilization of sinus rate, a three-burst-pacing (at 800 bpm) protocol was performed in each animal of the paced group. Occurrence and duration of rhythm disturbances were characterized by ECG (Figure 1). The duration of induced self-terminating paroxysms of AF was measured from the end of the last stimulus of the burst pacing to the first P-wave upon spontaneous reversion to normal sinus rhythm. Burst pacing was not performed in the sham-operated group. Animals were euthanized 20–24 hours after cessation of pacing to harvest cardiac tissues for RNA and protein isolation and histology. Animals were used in accordance with the European Commission Directive 86/609/EEC and all protocols were approved by the Institutional Animal Care and Use Ethics Committee (permit number: CE 012/2012; University of La Coruña, La Coruña, Spain).

2.2. Cell Culture and Transfection In Vitro. The mouse atrial cardiomyocyte cell line, HL-1 (provided by Professor William C. Claycomb), was cultured in Claycomb's growth medium. HL-1 cells (6×10^5 cells per well) were transfected with pre-miR-21 (Ambion, USA) and anti-miR-21 (Eurogentec, Belgium) at 10–50 nM using Lipofectamine 2000 (Invitrogen, Barcelona, Spain) according to manufacturer's guidelines. Negative controls included nontransfected (mock) cells as well as $5'$ carboxyfluorescein- (FAM-) labeled pre-miR negative control transfected cells, which also allowed transfection efficiency evaluation. In each assay, transfections were performed in triplicate. The transfection efficiency was around 60% in all experiments. After 4 hours of posttransfection, HL-1 cells were cultured in fresh medium for 48 hours and then harvested and processed for RNA and protein extraction as described [44].

2.3. Microarray. Purification of total RNA, including small RNAs, from LA samples was performed using miRNeasy Mini Kit (Qiagen, USA), including the on-column DNase digestion with RNase-free DNase, as described in the Qiagen Manual. Deep-frozen LA samples were directly homogenized in QIAzol lysis reagent (Qiagen) using a high-speed rotor-stator homogenizer (Ultra-Turrax T8, Germany). RNA was quantified by spectrophotometry and quality was evaluated by gel electrophoresis. Microarray analysis of RNAs isolated from LA biopsies of three paced and three sham pigs was performed by a service provider (LC Sciences, Houston, USA) using Pig miRNA Array (MRA-1013, version miRPig_20), which contains 322 unique probes (9 repeats each) of pig mature miRNAs. The probe content comes from version 20 of the miRBase sequence database which was updated on June 24, 2013. Briefly, the detection probes were made by *in situ* synthesis using PGR (photogenerated reagent) chemistry. Hybridization was performed overnight on a μParaflo microfluidic chip using a microcirculation pump (Atactic Technologies). Fluorescence images were collected using a

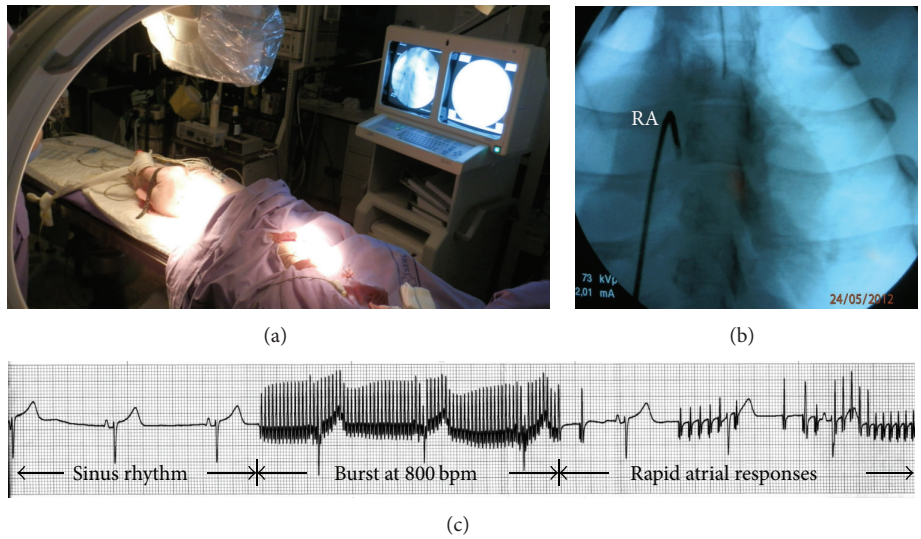


Figure 1: An electrical pacing assay. (a) Anaesthetized piglet (with automatic artificial lung ventilation) in the animal operating room. (b) Placing a pacing catheter in the right atrium (RA) using fluoroscopic guidance. (c) Representative telemetry ECG recordings monitored before, during, and after RA burst pacing.

laser scanner (GenePix 4000B, Molecular Device) and digitized using Array-Pro image analysis software (Media Cybernetics). *t*-test was performed between control and experimental sample groups. *T*-values were calculated for each miRNA, and *p* values were computed from the theoretical *t*-distribution. The clustering was done using the hierarchical method and performed with average linkage and Euclidean distance metric. The complete microarray data is available at NCBI through GEO (Gene Expression Omnibus) accession number GSE65330 (http://www.ncbi.nlm.nih.gov/geo).

2.4. Real-Time Quantitative PCR (qRT-PCR). qRT-PCR was performed on Bio-Rad IQ5 instrument (Bio-Rad, Madrid, Spain) and MxPro Mx3005p qPCR thermal cycler (Stratagene, Madrid, Spain) using, respectively, SYBR Green (Bio-Rad, Madrid, Spain) and Dynamo SYBR Green (Finnzymes, Finland) master mix as described previously [44, 45]. The primer pairs were located in different exons to rule out genomic DNA amplification. Each primer pair used yielded a single peak of dissociation on the melting curve and a single band with the expected size on PAGE gels. Identity of the PCR products was confirmed by sequencing. NT and non-RT RNA template reactions were used as negative controls. All PCR setups were performed at least in triplicate. Relative quantifications were calculated with the comparative ΔCt cycle method with normalization to the expression of housekeeping genes coding for ribosomal protein L19 (*Rpl19*), *β*-actin, glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), and *β-D*-glucuronidase (*Gusb*). The efficiency of target and reference amplifications was tested to be approximately equal. miRNA qRT-PCR was performed using Exiqon LNA microRNA qRT-PCR primers and detection kit (Exiqon, Madrid, Spain) according to manufacturer's guidelines. All reactions were run in triplicate using 5S as normalizing control. Primer sequences and additional data are available upon request.

2.5. Antibodies. The following primary antibodies were used: (1) rabbit polyclonal antibodies to PITX2A,B,C (Capra Science, Ängelholm, Sweden; at 1 : 2000 dilution): the specificity of the anti-PITX2 antibodies was independently validated by Western blot analysis of COS-7 cells expressing each PITX2 isoform [46]; (2) rabbit polyclonal antibodies to TBX5 (Abcam, Cambridge, UK; at 1 : 500 dilution); (3) rabbit polyclonal antibodies to myocardin which were generated by Davids Biotechnologie (Regensburg, Germany) using the recombinant TAD-containing fragment of porcine MYOCD as immunogen (at 1 : 500 dilution): these antibodies were shown to be specific for both MYOCD-A (minor) and MYOCD-B (major) variants expressed in pig cardiac tissues [47]; (4) rabbit polyclonal antibodies to cardiac troponin I (Abcam, Cambridge, UK; at 1 : 40000 dilution); (5) rabbit polyclonal antibodies to cardiac calsequestrin-2 (Abcam, Cambridge, UK; at 1 : 10000 dilution); (6) rabbit monoclonal antibodies to sarcoplasmic reticulum ATPase (SERCA-2A; Abcam, Cambridge, UK; at 1 : 10000 dilution); (7) rabbit polyclonal antibodies to caspase-3 (Cell Signaling, Leiden, Netherlands; at 1 : 1000); (8) rabbit monoclonal antibodies to caspase-9 (Abcam, Cambridge, UK; at 1 : 1000 dilution); and (9) mouse monoclonal anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) antibody (Sigma, Madrid, Spain; at 1 : 10000 dilution). Secondary peroxidase conjugated anti-rabbit and anti-mouse IgG (Fab-specific) antibodies were purchased from Sigma (Madrid, Spain).

2.6. SDS-PAGE and Western Blotting. Tissue samples were homogenized in standard 2x Laemmli buffer (Invitrogen, Barcelona, Spain) supplemented with complete protease

inhibitor cocktail (Roche, Madrid, Spain) as previously described [45, 46]. Following centrifugation at 20000 g for 30 minutes, the concentration of supernatant proteins was analyzed using the Bio-Rad DC Protein Assay Kit (Bio-Rad, Hercules, USA) according to the manufacturer's protocol. The protein extracts were normalized to total protein concentration; the results of normalization were confirmed by SDS-PAGE and Coomassie staining before Western blotting analysis [46]. Protein supernatants (loading range of 5–15 mg/run) were resolved on a 12% SDS-PAGE (Mini-Protean-III, Bio-Rad, Hercules, USA) and blotted onto PVDF-membranes (Hybond-P, Amersham Biosciences, Barcelona, Spain). Molecular weight (MW) standards (Precision Plus Protein WesternC Standards from Bio-Rad and SeeBlue Plus2 Pre-Stained Standard from Invitrogen) were included on each gel. Blots were probed with the antibodies indicated above and visualized by the Super-Signal West Pico chemiluminescent substrate (Pierce Biotechnology, Madrid, Spain). Equivalence of protein loading was confirmed by Amido Black 10B (Merck, Barcelona, Spain) staining of blots after immunodetection. The blots were reprobed with anti-GAPDH antibodies as additional control for loading. Quantification of Western blot signals was obtained by using a Bio-Rad GS800 calibrated densitometer with Quantity One software.

2.7. Histochemical Staining. Fragments of LA samples were fixed with 4% paraformaldehyde (in PBS, 1 hour at room temperature), paraffin-embedded, 5 μm sectioned, stained with Picrosirius Red (Sigma, Madrid, Spain) as described previously [48], and visualized with bright-field "Nikon Eclipse 600" microscopy by an experienced technician blinded to the experimental design. The area of red-stained pixels was quantified in three zones of LA free wall sections from three paced versus three sham animals by ROI (Region of Interest) analysis using ACT-2U Image software (version 1.4, Nikon, Japan).

2.8. Statistical Analysis. Values are expressed as mean ± S.E.M. All comparisons between groups were performed using unpaired Student's t-test. Differences were considered statistically significant for p value ≤ 0.05.

3. Results

3.1. Burst Tachypacing Results in Self-Terminating Paroxysms of Atrial Fibrillation. Brief self-terminating episodes of AF were induced in postnatal pigs by repetitive burst pacing of the RA via an implanted catheter electrode (see Figure 1). Hemodynamic parameters were comparable in the paced versus sham animals throughout the experiment. Before and after catheter insertion, the ventricular rate means were similar at baseline in both sham and pacing groups but increased in the pacing group compared with baseline upon electrical stimulation (150 ± 6 bpm versus 130 ± 3 bpm). Every burst was followed by consecutive (3–5) AF episodes, each lasting 20–30 sec. On average, a total duration of AF episodes (per animal) was 600 ± 120 sec. No AF episodes were detected by ECG-tracing at least 1 hour after the tachyarrhythmia protocol was terminated. On the next day, the ECG examination (prior to sacrifice of animals and excision of the heart) showed normal heart rhythm (with normal QRS duration) in both paced and sham pigs. Picrosirius red staining revealed no signs of fibrosis in LA samples from paced or sham animals (data not shown).

These results suggest that episodes of self-terminating AF observed in our porcine model are reminiscent of those which occur in patients with asymptomatic paroxysmal AF.

3.2. Paroxysmal Atrial Tachyarrhythmia Leads to a Rapid Change in Atrial miRNA Transcriptome. Since the RA might have been damaged by catheter insertion, we studied miRNA expression changes associated with AF in the LA from paced versus sham pigs 20–24 hours after pacing. Our initial goal was to analyze AF-induced miRNA transcriptome changes by microarray approach. The results of microarray experiments demonstrated that transient episodes of AF alter the miRNA expression profiles of the LA. Of 322 porcine mature miRNA sequences analyzed by microarrays, the expression of 29 miRNAs was significantly up- (12 miRNAs) or downregulated (17 miRNAs) in the LA from paced versus sham animals (Figure 2; Table 1). Seven miRNAs (i.e., miR-7140-3p, miR-4335, miR-129b, miR-296-5p, miR-4334-5p, miR-4339, and miR-182) showed 2-fold or greater increases after LA pacing. The biggest difference was observed for miR-7140-3p, which was expressed approximately 4-fold higher in paced than in sham animals. The known cardiac-expressed miRNAs (i.e., miR-24-2-5p, miR-542-5, miR-27b-5p, miR-7, and miR-30c-3p; see Table 1) were prominent among the miRNAs that showed a significant downregulated expression (~2-fold decrease) in the paced group compared with controls. Of particular note, the microarray profiling unveiled a low-to-moderate downregulation of three miRNAs (i.e., miR-143-3p, miR-363, and miR-18b) previously detected in patients with chronic AF. Pacing-induced alterations in the expression of other miRNAs with particular concern for their involvement in AF could not be determined by microarray hybridizations due to a high variability among replicates (miR-1, miR-21, miR-23a/b, miR-29a, and miR-133a) or very low hybridization signals (miR-10a, miR-10b; see the complete microarray data at NCBI through GEO accession number GSE65330).

Consequently, our second goal was to study whether the expression of these and other AF-associated miRNAs (not revealed by microarray evaluation) could be altered in our porcine model. To this end, the expression of 11 miRNAs, selected on the basis of published reports [7–9, 13, 14, 21] (and references wherein), was analyzed by qPCR in the LA from paced versus sham pigs (Figure 3). Six miRNAs (miR-1, miR-10a-5p, miR-10b, miR-21, miR-29a, and miR-208a) showed a higher expression in paced as compared with nonpaced animals. The expression of miR-23a, miR-23b, and miR-24 was significantly reduced following pacing, while that of miR-133a, miR-125-3p, and miR-141 remained unchanged.

Taken together, the results indicate that short-term atrial tachyarrhythmia episodes lead to a rapid miRNA remodeling of atrial myocardium.

Table 1: The miRNAs significantly up- or downregulated in the paced LA, ordered by the fold change (FC).

miRNA	FC	Direction	p value	Reported expression in*	Reference
ssc-miR-7140-3p	3,92	Up	0,0199	Porcine lung	[25]
ssc-miR-4335	3,22	Up	0,0540	Porcine intestine	[26]
ssc-miR-129b	2,90	Up	0,0520	Catfish heart	[27]
ssc-miR-296-5p	2,35	Up	0,0530	Human placenta	[28]
ssc-miR-4334-5p	2,22	Up	0,0072	Porcine intestine	[26]
ssc-miR-4339	2,16	Up	0,0499	Porcine intestine	[26]
ssc-miR-182	2,13	Up	0,0021	Human skeletal muscles	[29]
ssc-miR-4332	1,95	Up	0,0339	Porcine intestine	[26]
ssc-miR-1249	1,90	Up	0,0550	Rat pancreas	[30]
ssc-miR-1307	1,83	Up	0,0542	Human blood cells	[31]
ssc-miR-671-3p	1,63	Up	0,0492	Human atria	[23]
ssc-miR-423-3p	1,61	Up	0,0025	Rat hippocampus	[32]
ssc-miR-23b	−1,10	Down	0,0146	Human atria, AF	[20]
ssc-miR-22-3p	−1,19	Down	0,0181	Human atria, AF	[20]
ssc-miR-143-3p	−1,26	Down	0,0402	Human atria, AF	[20]
ssc-miR-324	−1,30	Down	0,0050	Human blood cells	[33]
ssc-miR-455-5p	−1,42	Down	0,0551	Human meningeal arteries	[34]
ssc-miR-326	−1,46	Down	0,0546	Human brain tissues	[35]
ssc-miR-362	−1,57	Down	0,0069	Human skeletal muscles	[36]
ssc-miR-363	−1,62	Down	0,0069	Human atria, AF	[21]
ssc-miR-18b	−1,67	Down	0,0550	Human atria, AF	[21]
ssc-miR-17-3p	−1,68	Down	0,0336	Mouse heart	[37]
ssc-miR-24-2-5p	−1,79	Down	0,0187	Human atria, AF	[23]
ssc-miR-542-5p	−1,85	Down	0,0318	Human atria, AF	[23]
ssc-miR-486	−1,88	Down	0,0365	Mouse skeletal muscles	[38]
ssc-miR-27b-5p	−1,96	Down	0,0509	Human atria, AF	[23]
ssc-miR-7	−2,37	Down	0,0211	Porcine heart	[39]
ssc-miR-30c-3p	−2,55	Down	0,0345	Porcine heart	[39]
ssc-miR-381	−2,69	Down	0,0540	Human skeletal muscles	[40]

*Except the expression in malignant tumors and model cell lines. Bold, detected in AF patient samples.

3.3. Paroxysmal Atrial Tachyarrhythmia Leads to Downregulation of Transcriptional Factors Related to Atrial Arrhythmia Susceptibility. TFs, such as *Pitx2* and *Tbx5*, are increasingly recognized as potentially important contributors to atrial arrhythmia susceptibility (recently reviewed in [42]). Enlargement of the LA is widely regarded to be an epidemiological risk indicator for AF, and a transcription cofactor, myocardin (*Myocd*), has been identified as a possible susceptibility gene affecting LA size and function [43]. Our objective in this regard was to study whether these genes (with potential roles in arrhythmogenesis) are involved in early response of LA myocardium to induction of paroxysmal-like AF.

We evaluated transcript levels of *Pitx2*, *Tbx5*, and *Myocd* in the LA myocardium from paced versus nonpaced control pigs by qRT-PCR (Figure 4(a)). While the level of *Myocd* mRNA expression was reduced following pacing, *Pitx2* and *Tbx5* remained statistically unchanged between control and paced groups. At the protein level, however, a marked decline in expression of not only *Myocd* but also *Pitx2* and *Tbx5* was revealed in the LA of paced animals by Western blot (Figure 4(b)).

Signs of increased apoptosis were detected in atrial tissue in the canine tachypacing model of AF [49]. In this sense, the possibility that apoptosis might be responsible for the observed *Pitx2*, *Tbx5*, and *Myocd* protein downregulation could not be excluded. Western blot analysis did not reveal differences in LA expression levels of pro-caspase-3 and pro-caspase-9 between paced and control pigs, nor signs of activation of these key apoptotic enzymes in any of LA samples studied. In addition, LA levels of cardiac calcium-handling proteins (calsequestrin-2 and sarcoplasmic reticulum Ca^{2+} ATPase) and troponin I were not affected by pacing *per se* (Figure 4(b)). Therefore, it is doubtful that apoptosis is the principal factor responsible for the observed *Pitx2*, *Tbx5*, and *Myocd* protein downregulation in the LA following pacing.

It then became our working hypothesis that inhibition of translation by miRNAs can be responsible, at least in part, for the decrease in protein expression of *Pitx2*, *Tbx5*, and *Myocd* in paced pigs. There is growing evidence that

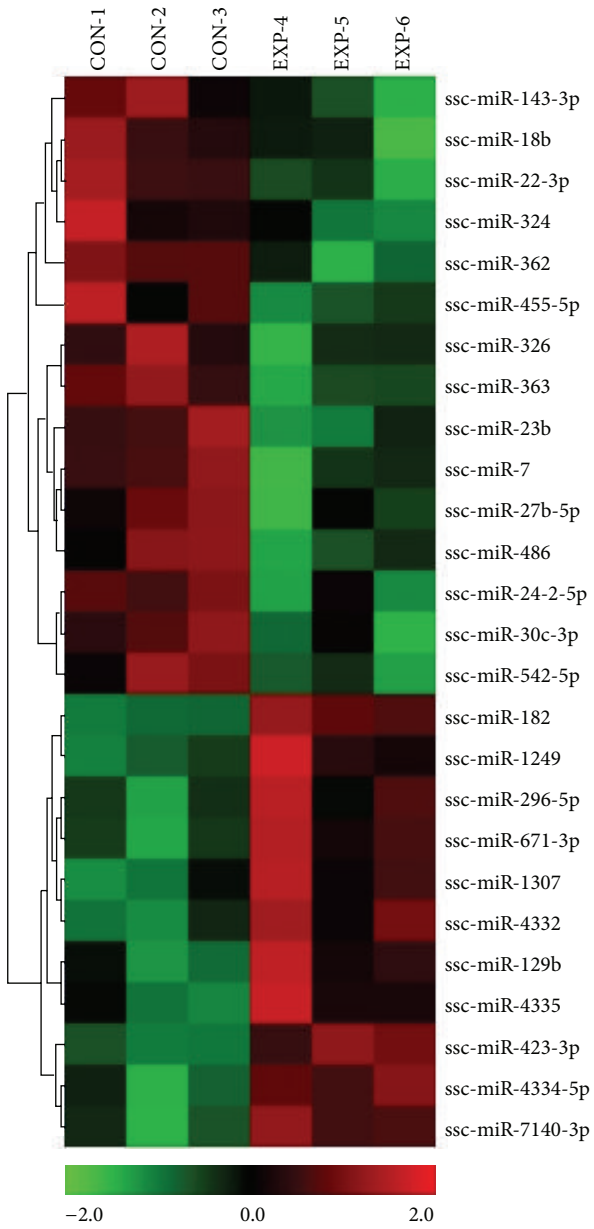


FIGURE 2: MicroRNA microarray expression profiling of the left atrium from paced versus nonpaced pigs. Heat map of the most differentially expressed (statistically significant) miRNAs in the left atrium of three paced (4–6) compared to three sham (1–3) animals. Relative expression is $\log_2$.

miRNAs induce gene silencing with translational repression occurring first and then followed by mRNA decay (reviewed in [50, 51]). Our qPCR analysis showed that pacing resulted in a significant upregulation of a set of AF-associated miRNAs (i.e., miR-1, miR-10a, miR-10b, miR-21, miR-29a, and miR-208a) in the LA compared with the control (see Figure 3). Computational analysis (by miRanda and Targetscan) of the 3′ untranslated region of pig *Pitx2*, *Tbx5*, *and Myocd* genes revealed consensus sites for binding of, respectively, miR-21, miR-10a/miR-10b, and miR-1 (Figure 5(a)). Some of these predicted miRNA/mRNA pairings were in line with the available experimental evidence. In transfection assays, miR-1 inhibits mouse *Myocd* expression at the transcript [52] and protein level [53], while both miR-10a and miR-10b negatively modulate human *Tbx5* expression at the protein level [54].

In this study, we attempted to validate, for the first time, whether miR-21 can alter *Pitx2* expression in a cardiomyocyte background. To this end, miR-21 mimic and anti-miR-21 inhibitor were transfected into atrial HL-1 cardiomyocytes and the expression of *Pitx2c* gene was detected by qRT-PCR and Western blot (Figures 5(b), 5(c), and 5(d)). Transfection of HL-1 cardiomyocytes with miR-21 mimic silenced *Pitx2c* expression to a certain, but statistically significant, extent, whereas anti-miR-21 induced a marked increase in the *Pitx2c* transcript without affecting the expression of *Mef2c* (myocyte enhancer factor 2c) TF. Of note, endogenous miR-21, abundantly expressed in HL-1 cells [55], may interfere with *Pitx2*-silencing by miR-21 mimic transfection. An efficient *Pitx2c* activation by anti-miR-21, as observed in transfection assays, is concordant with this suggestion. A complimentary Western blot analysis (Figures 5(c) and 5(d)) revealed that PITX2C protein levels were significantly decreased in miR-21 transfected cells, being only slightly upregulated after anti-miR-21 delivery. The latter could probably be the result of additional mechanisms influencing the *Pitx2c* transcript versus protein ratio in anti-miR-21 transfected cells.

Consistent with the results obtained and other reports [53, 54], the histogram plot (Figure 5(e)) exhibits a clear inverse correlation in the protein expression of *Pitx2* versus miR-21, *Tbx5* versus miR-10b, and *Myocd* versus miR-1 in the LA of paced animals, confirming thus our hypothesis.

Together these data show that sporadic AF paroxysms are associated with marked downregulation of these TFs involved in AF susceptibility of atrial myocardium.

4. Discussion

In this study we provide evidence for two major findings. On the one hand, self-terminating paroxysms of AF caused a rapid and substantial miRNA remodeling of the LA myocardium in our animal model. On the other hand, this miRNA remodeling is associated with a marked downregulation of *Pitx2*, *Tbx5*, and *Myocd* TFs in paced atria.

The expression of 38 miRNAs was significantly altered due to burst pacing-induced paroxysms of AF, as revealed by microarray (29 miRNAs, see Table 1) and qPCR analysis (9 miRNAs, see Figure 3), and this number is obviously superior to that found in microarray study of LA samples from patients with paroxysmal AF (10 miRNAs [24]). They can be divided, in terms of expression patterns, into three categories as (1) miRs that have not previously been reported as expressed in the heart, (2) miRs with known expression in cardiac and skeletal muscles but not yet associated with AF, and (3) miRs with well-established AF-associated expression. Although this is by no means an exclusive list, it certainly contains some of the promising miRs to be further evaluated for their potential in AF. Of particular note, in this context, are miR-182 and miR-486, which were found to attenuate atrophy-related gene expression in skeletal muscle [38, 56]. Albeit the

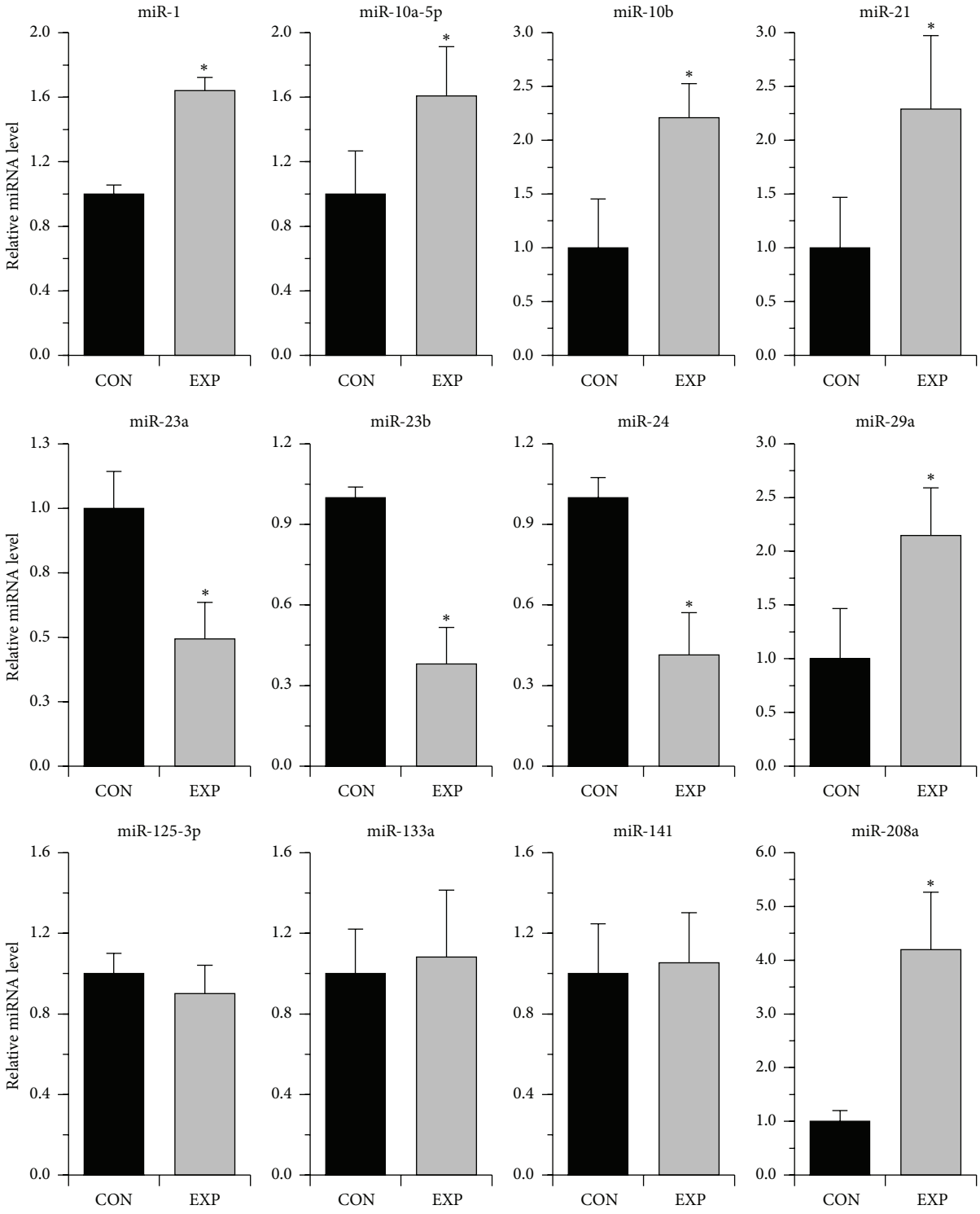


Figure 3: Expression of AF-associated miRNAs in the left atrium from paced (EXP) versus nonpaced (CON) pigs as revealed by qRT-PCR analysis. $^{*}p \leq 0.05$ ($n = 5$ for each group).

assayed tissue was skeletal muscle, both these miRs target the FoxO1 TF which has an important function in mediating apoptosis in the heart [57]. AF is strongly associated with changes in the heart that occur with aging (reviewed in [58]). In this respect, miR-17-3p (see Table 1), a negative modulator of cardiac aging [37], would also be a good candidate to be studied for a potential AF meaning.

The expression signature of known AF-associated miRNAs in our model of paroxysmal-like AF (bold-marked in Table 1 and listed in Figure 3) appears to be different from that

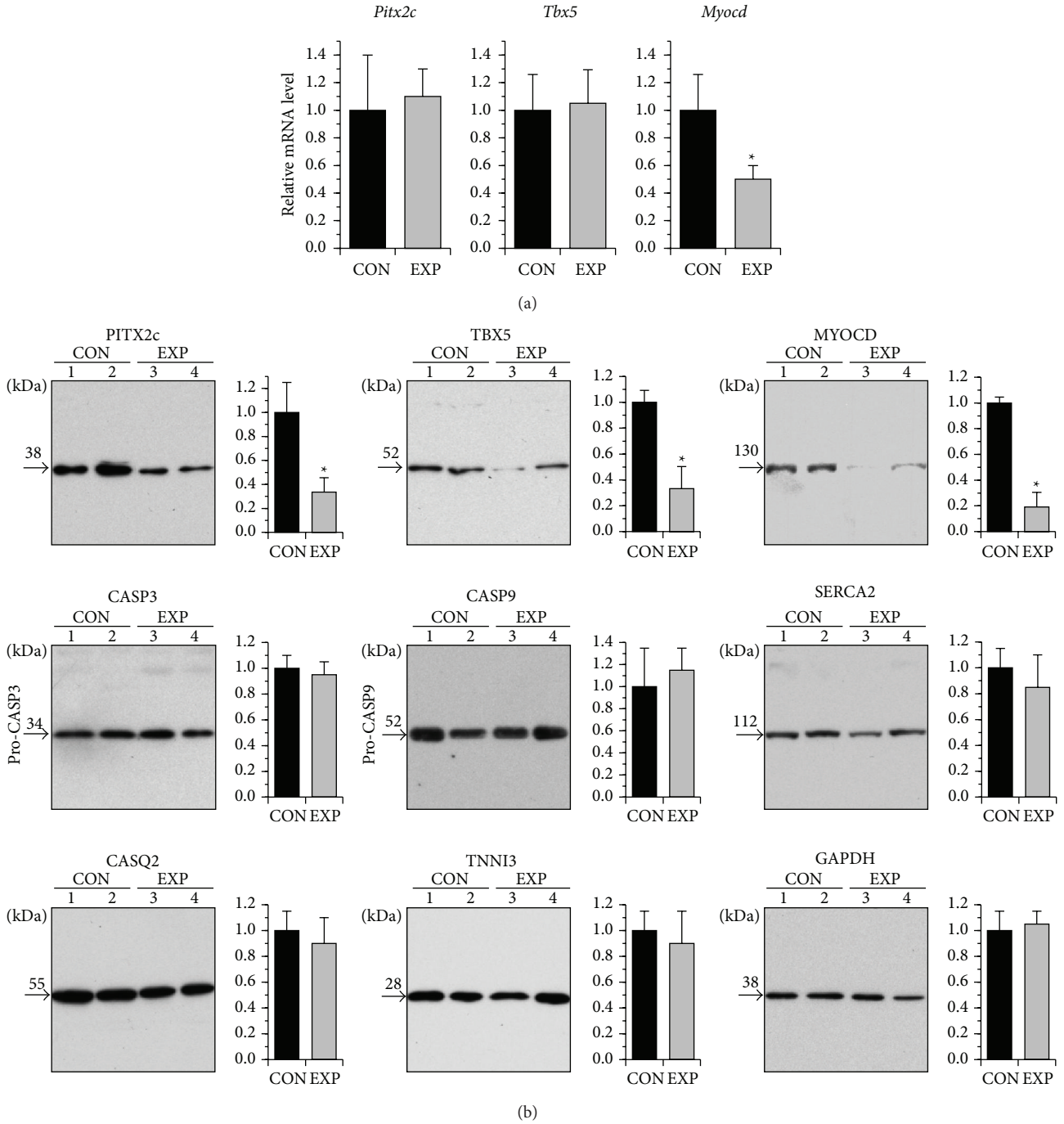


Figure 4: Altered expression of *Pitx2, Tbx5*, and *Myocd* in response to atrial short-term tachyarrhythmia. (a) Average relative values of transcript levels of *Pitx2c*, *Tbx5*, and *Myocd* in the left atrium from paced (EXP) versus sham (CON) animals. $^{*}p \leq 0.05$ ($n = 5$ for each group). (b) Representative Western blots of left atrium samples from sham-operated (CON, lines 1-2) and paced (EXP, lines 3 and 4) animals and overall relative levels of the proteins as based on average values from each group. $^{*}p \leq 0.05$ ($n = 5$ for each group). Western blot replicates were probed with antibodies against PITX2A,B,C, TBX5, MYOCD (myocardin), CASP3 (caspase-3), CASP9 (caspase-9), SERCA2 (cardiac sarcoplasmic reticulum Ca(2+)-ATPase 2a), CASQ2 (cardiac calsequestrin), TNNI3 (cardiac troponin I), and GAPDH (glyceraldehyde-3-phosphate dehydrogenase). MW values of the bands detected are shown.

found in chronic AF in patients and animal models. Downregulation of miR-1, miR-10a, miR-29a, and miR-208a has been implicated in chronic AF [7, 13, 59], while these miRNAs were highly upregulated in the porcine LA in response to brief paroxysms of AF. In the same way, the levels of miR-133 expression significantly downregulated in chronic AF [13, 14] were unchanged in our model. Upregulation of miR-18b and miR-363 was found to be associated with chronic AF

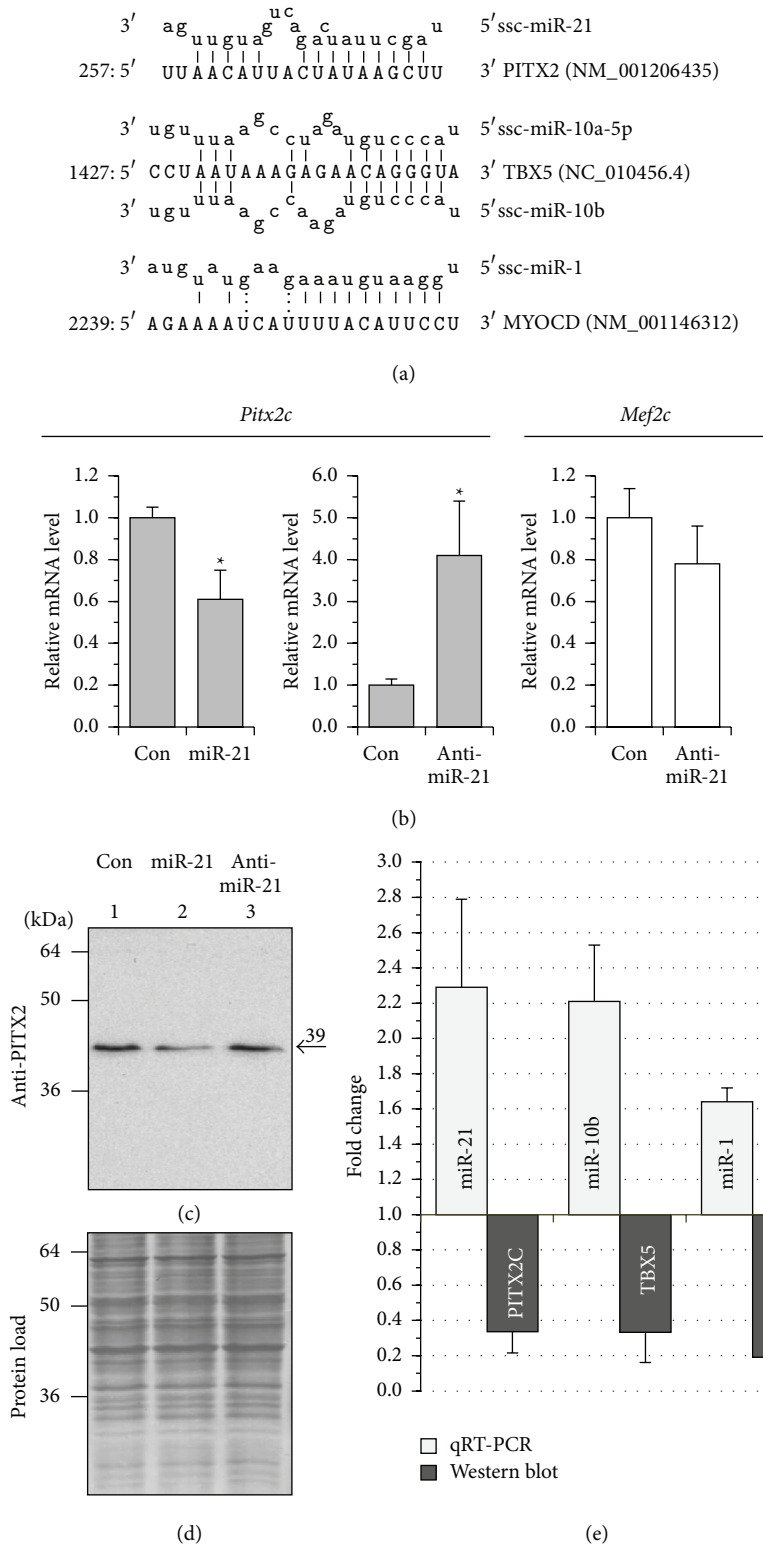


FIGURE 5: MicroRNAs which can be potentially responsible for the downregulation of *Pitx2*, *Tbx5*, and *Myocd* expression in atrial myocardium after pacing. (a) miRNA:mRNA alignments. Shown are the schematics of porcine *Pitx2* 3′-UTR sequence targeted by miR-21, porcine *Tbx5* 3′-UTR sequence targeted by miR-10a and miR-10b, and human *Myocd* 3′-UTR sequence targeted by miR-1. The porcine *Tbx5* 3′-UTR was identified within the pig genomic chromosome-14 sequence (NC_010456.4), starting from nt 40259322. (b) Overall relative levels of *Pitx2c* (grey boxes) and *Mef2c* (white boxes) transcripts in HL-1 cells transfected with 50 nM miR-21 mimic, anti-miR-21 inhibitor, and FAM-labeled pre-miR negative control (Con). The results of qRT-PCR analysis are shown. Data from 3 replicates of each transfection were pooled and averaged. $^{*}p \leq 0.05$. (c) Control and transfected cells were pooled (from triplicate wells in each setup), lysed, electrophoresed, and immunoblotted with antibodies against PITX2A,B,C. MW values (kDa) of the bands detected are shown. (d) Membrane stained with Amido Black 10B. (e) The histogram demonstrating inverse expression correlations of selected miRNA/target gene pairs in paced pigs as revealed by qRT-PCR (miR-21, miR-10b, and miR-1) and Western blot (PITX2C, TBX5, and MYOCD). See text for details.

in patients [21], albeit these miRNAs were downregulated in paced pigs (see Table 1). Overall, it is tempting to speculate that the expression of the same set of AF-associated miRNAs can be up- or downregulated as a function of progression from paroxysmal to chronic AF. What is of note in this regard, however, is that the expression of miR-22-3p, miR-23b, and miR-143-3p was similarly downregulated in both patients with chronic AF [20] and our paroxysmal-like AF model (see Table 1). Likewise, expression of miR-21 was upregulated in the LA of patients with long-term persistent AF [60], as well as after short-term AF in pigs (this work). These results suggest that the expression of a subgroup of AF-associated microRNAs could be similarly altered in both paroxysmal and chronic AF states, indicating that congruent miRNA remodeling may occur within different AF contexts.

There are a few data [23, 24] which instead suggest that microRNA remodeling is quite distinct in paroxysmal AF in comparison with that in chronic AF. In one of these reports [23], RA biopsies were obtained from hypertonic patients with paroxysmal AF secondary to or associated with organic heart diseases, suggesting that the observed alterations in miRNA expression may have been attributable to the underlying heart disease rather than paroxysmal AF *per se*. In the other study [24], patients with paroxysmal AF had no obvious clinical signs of cardiovascular disease but LA biopsies taken from them for miRNA microarray analysis showed a markedly increased fibrosis as compared to those from patients with sinus rhythm. Our study is advantageous to the above-mentioned reports in the sense that we performed the miRNA microarray analysis, using LA biopsies from paced animals without pathological cardiovascular background. In addition, biochemical and histochemical analyses did not reveal signs of proapoptotic and profibrotic activation in paced atrial samples.

An aspect of particular interest of our study was the finding that the upregulation of miR-1, miR-10a, miR-10b, and miR-21 is negatively correlated with downregulation of, respectively, MYOCD, TBX5, and PITX2c in the paced LA (see Figure 5(e)). The results of transient transfection assays demonstrated that (1) miR-21 negatively regulates the expression of *Pitx2c* (this work), (2) miR-1 suppresses *Myocd* expression [53], and (3) miR-10a and miR-10b both inhibit *Tbx5* expression [54]. The functional relevance of these miRNA/TF relations (established through our model) to AF development remains elusive and thus future experimental approaches using knockdown and overexpression strategies are highly desirable.

There are lines of evidence that implicate downregulated expression of *Pitx2*, *Tbx5*, and, probably, *Myocd* in a predisposition to AF progression. In fact, several studies revealed an association between LA *Pitx2* downregulation and increased atrial arrhythmogenicity (reviewed in [61]). More recent research implicates *Pitx2* and *Pitx2*-mediated signaling in prevention of AF [62, 63]. In some patients with Holt-Oram syndrome and AF, the disease is caused by *Tbx5* mutations that create a premature termination codon, which is predicted to result in haploinsufficiency. It should be noted, however, that increased *Tbx5* dosage (gain-of-function mutations) was also associated with Holt-Oram syndrome and paroxysmal AF (reviewed in [41, 42]). *Myocd* is regarded as the most likely candidate among the genes affecting LA size [43], which is a clinical marker for risk stratification of AF development [64]. Cardiac-restricted inactivation of the *Myocd* gene in the adult heart resulted in development of severe four-chambered heart failure as a result of massive myocyte loss via apoptosis and replacement fibrosis (reviewed in [65, 66]).

The proarrhythmogenic mechanisms leading to the spontaneously self-terminating AF episodes typical of patients with paroxysmal AF remain elusive and not yet studied as hoped. Our results provide suggestive evidence for a significant *Pitx2*, *Tbx5*, and *Myocd* downregulation in a specific microRNA-upregulation context in response to tachycardia-induced AF. From this perspective, our data would suggest that higher levels of miR-21, miR-10b, and miR-1 in the LA myocardium contribute to arrhythmogenesis via perturbation of *Pitx2*, *Tbx5*, and *Myocd* signaling pathways, respectively.

5. Conclusions

In conclusion, we herein demonstrate that even short-lasting atrial tachyarrhythmia is associated with significant miRNA remodeling coupled with decreased expression of TFs with potential roles in arrhythmogenesis. We propose that the downregulation of *Pitx2c, Tbx5*, and *Myocd* may be permissive for future AF development. This study provides, therefore, previously unknown insights into interrogating miRNA-TF regulations underlying paroxysmal AF development in a large animal model, with potentially important implications from a biomedical perspective.

Conflict of Interests

The authors declare that there is no conflict of interests for this study.

Acknowledgments

This work was carried out within the framework agreement between the Institute of Health Sciences, University of La Coruña, and Department of Experimental Biology, University of Jaén, Spain. The HL-1 cell line was kindly provided by Dr. W. C. Claycomb (Louisiana State University Medical Center, USA). Alexander Mikhailov, Mario Torrado, and Alfonso Castro-Beiras were supported by funds from the Institute of Health Sciences and by a grant (GRC 2013/061) from the Autonomic Government of Galicia, Spain. Diego Franco, Estefanía Lozano-Velasco, and Francisco Hernández-Torres were supported by the Excellence Project of the Consejería de Innovación, Ciencia y Empresa of the Junta de Andalucía (Grant no. CTS1416) and a CNIC-T transactional grant (CNIC-T 2009/08). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the paper.

References

[1] S. S. Chugh, J. L. Blackshear, W.-K. Shen, S. C. Hammill, and B. J. Gersh, "Epidemiology and natural history of atrial fibrillation: clinical implications," *Journal of the American College of Cardiology*, vol. 37, no. 2, pp. 371–378, 2001.

[2] K. Nishida and S. Nattel, "Atrial fibrillation compendium: historical context and detailed translational perspective on an important clinical problem," *Circulation Research*, vol. 114, no. 9, pp. 1447–1452, 2014.

[3] A. Deshmukh, J. Barnard, H. Sun et al., "Left atrial transcriptional changes associated with atrial fibrillation susceptibility and persistence," *Circulation: Arrhythmia and Electrophysiology*, vol. 8, no. 1, pp. 32–41, 2015.

[4] N. Voigt, J. Heijman, Q. Wang et al., "Cellular and molecular mechanisms of atrial arrhythmogenesis in patients with paroxysmal atrial fibrillation," *Circulation*, vol. 129, no. 2, pp. 145–156, 2014.

[5] V. Divakaran and D. L. Mann, "The emerging role of micrornas in cardiac remodeling and heart failure," *Circulation Research*, vol. 103, no. 10, pp. 1072–1083, 2008.

[6] S. V. N. Prasad and S. S. Karnik, "MicroRNAs-regulators of signaling networks in dilated cardiomyopathy," *Journal of Cardiovascular Translational Research*, vol. 3, no. 3, pp. 225–234, 2010.

[7] D.-Z. Wang, "MicroRNAs in cardiac development and remodeling," *Pediatric Cardiology*, vol. 31, no. 3, pp. 357–362, 2010.

[8] E. Orenes-Piñero, S. Montoro-García, J. V. Patel, M. Valdés, F. Marín, and G. Y. H. Lip, "Role of microRNAs in cardiac remodelling: new insights and future perspectives," *International Journal of Cardiology*, vol. 167, no. 5, pp. 1651–1659, 2013.

[9] G. H. Kim, "MicroRNA regulation of cardiac conduction and arrhythmias," *Translational Research*, vol. 161, no. 5, pp. 381–392, 2013.

[10] R. L. Neppl and D. Wang, "The myriad essential roles of microRNAs in cardiovascular homeostasis and disease," *Genes & Diseases*, vol. 1, no. 1, pp. 18–39, 2014.

[11] Z. Wang, Y. Lu, and B. Yang, "MicroRNAs and atrial fibrillation: new fundamentals," *Cardiovascular Research*, vol. 89, no. 4, pp. 710–721, 2011.

[12] K.-H. Shi, H. Tao, J.-J. Yang, J.-X. Wu, S.-S. Xu, and H.-Y. Zhan, "Role of microRNAs in atrial fibrillation: new insights and perspectives," *Cellular Signalling*, vol. 25, no. 11, pp. 2079–2084, 2013.

[13] G. Santulli, G. Iaccarino, N. De Luca, B. Trimarco, and G. Condorelli, "Atrial fibrillation and microRNAs," *Frontiers in Physiology*, vol. 5, article 15, 2014.

[14] S. Lee, E. Choi, M. J. Cha, and K. C. Hwang, "Looking into a conceptual framework of ROS-miRNA-atrial fibrillation," *International Journal of Molecular Sciences*, vol. 15, no. 12, pp. 21754–21776, 2014.

[15] X. Luo, B. Yang, and S. Nattel, "MicroRNAs and atrial fibrillation: mechanisms and translational potential," *Nature Reviews Cardiology*, vol. 12, no. 2, pp. 80–90, 2015.

[16] Z. Girmatsion, P. Biliczki, A. Bonauer et al., "Changes in microRNA-1 expression and IK1 up-regulation in human atrial fibrillation," *Heart Rhythm*, vol. 6, no. 12, pp. 1802–1809, 2009.

[17] Y. Lu, Y. Zhang, N. Wang et al., "MicroRNA-328 contributes to adverse electrical remodeling in atrial fibrillation," *Circulation*, vol. 122, no. 23, pp. 2378–2387, 2010.

[18] N. Cooley, M. J. Cowley, R. C. Y. Lin et al., "Influence of atrial fibrillation on microRNA expression profiles in left and right atria from patients with valvular heart disease," *Physiological Genomics*, vol. 44, no. 3, pp. 211–219, 2012.

[19] X. Luo, Z. Pan, H. Shan et al., "MicroRNA-26 governs profibrillatory inward-rectifier potassium current changes in atrial fibrillation," *The Journal of Clinical Investigation*, vol. 123, no. 5, pp. 1939–1951, 2013.

[20] H. Liu, H. Qin, G.-X. Chen et al., "Comparative expression profiles of microRNA in left and right atrial appendages from patients with rheumatic mitral valve disease exhibiting sinus rhythm or atrial fibrillation," *Journal of Translational Medicine*, vol. 12, no. 1, article 90, 2014.

[21] K. H. Slagsvold, A. B. Johnsen, O. Rognmo, M. Hoydal, U. Wisloff, and A. Wahba, "Comparison of left versus right atrial myocardium in patients with sinus rhythm or atrial fibrillation—an assessment of mitochondrial function and microRNA expression," *Physiological Reports*, vol. 2, no. 8, Article ID e12124, 2014.

[22] A. Barana, M. Matamoros, P. Dolz-Gaiton et al., "Chronic atrial fibrillation increases microRNA-21 in human atrial myocytes decreasing L-type calcium current," *Circulation: Arrhythmia and Electrophysiology*, vol. 7, no. 5, pp. 861–868, 2014.

[23] D. Y. Chiang, M. Zhang, N. Voigt et al., "Identification of microRNA-mRNA dysregulations in paroxysmal atrial fibrillation," *International Journal of Cardiology*, vol. 184, pp. 190–197, 2015.

[24] J. Wang, Y. Wang, J. Han et al., "Integrated analysis of microRNA and mRNA expression profiles in the left atrium of patients with nonvalvular paroxysmal atrial fibrillation: role of miR-146b-5p in atrial fibrosis," *Heart Rhythm*, vol. 12, no. 5, pp. 1018–1026, 2015.

[25] A. Podolska, C. Anthon, M. Bak et al., "Profiling microRNAs in lung tissue from pigs infected with *Actinobacillus pleuropneumoniae*," *BMC Genomics*, vol. 13, article 459, 2012.

[26] S. Sharbati, M. R. Friedländer, J. Sharbati et al., "Deciphering the porcine intestinal microRNA transcriptome," *BMC Genomics*, vol. 11, no. 1, article 275, 2010.

[27] Z. Xu, J. Chen, X. Li, J. Ge, J. Pan, and X. Xu, "Identification and characterization of microRNAs in channel catfish (*Ictalurus punctatus*) by using Solexa sequencing technology," *PLoS ONE*, vol. 8, no. 1, Article ID e54174, 2013.

[28] S.-Y. Choi, J. Yun, O.-J. Lee et al., "MicroRNA expression profiles in placenta with severe preeclampsia using a PNA-based microarray," *Placenta*, vol. 34, no. 9, pp. 799–804, 2013.

[29] F. Olivieri, M. Ahtiainen, R. Lazzarini et al., "Hormone replacement therapy enhances IGF-1 signaling in skeletal muscle by diminishing miR-182 and miR-223 expressions: a study on postmenopausal monozygotic twin pairs," *Aging Cell*, vol. 13, no. 5, pp. 850–861, 2014.

[30] A. Castell-Auví, L. Cedó, J. Movassat et al., "Procyanidins modulate microRNA expression in pancreatic islets," *Journal of Agricultural and Food Chemistry*, vol. 61, no. 2, pp. 355–363, 2013.

[31] C. V. Collares, A. F. Evangelista, D. J. Xavier et al., "Identifying common and specific microRNAs expressed in peripheral blood mononuclear cell of type 1, type 2, and gestational diabetes mellitus patients," *BMC Research Notes*, vol. 6, no. 1, article 491, 2013.

[32] M.-M. Li, T. Jiang, Z. Sun et al., "Genome-wide microRNA expression profiles in hippocampus of rats with chronic temporal lobe epilepsy," *Scientific Reports*, vol. 4, article 4734, 2014.

[33] C. Chang, C. Lin, W. Hsieh, H. Lai, C. Tsai, and Y. Cheng, "MicroRNA expression profiling in PBMCs: a potential diagnostic biomarker of chronic hepatitis C," *Disease Markers*, vol. 2014, Article ID 367157, 9 pages, 2014.

[34] Y. Jiang, M. Zhang, H. He et al., "MicroRNA/mRNA profiling and regulatory network of intracranial aneurysm," *BMC Medical Genomics*, vol. 6, no. 1, article 36, 2013.

[35] A. Junker, M. Krumbholz, S. Eisele et al., "MicroRNA profiling of multiple sclerosis lesions identifies modulators of the regulatory protein CD47," *Brain*, vol. 132, no. 12, pp. 3342–3352, 2009.

[36] P. Dmitriev, L. Stankevicins, E. Ansseau et al., "Defective regulation of MicroRNA target genes in myoblasts from facioscapulohumeral dystrophy patients," *Journal of Biological Chemistry*, vol. 288, no. 49, pp. 34989–35002, 2013.

[37] W. W. Du, X. Li, T. Li et al., "The microRNA miR-17-3p inhibits mouse cardiac fibroblast senescence by targeting Par4," *Journal of Cell Science*, vol. 128, no. 2, pp. 293–304, 2015.

[38] M. S. Alexander, J. C. Casar, N. Motohashi et al., "MicroRNA-486–dependent modulation of DOCK3/PTEN/AKT signaling pathways improves muscular dystrophy–associated symptoms," *The Journal of Clinical Investigation*, vol. 124, no. 6, pp. 2651–2667, 2014.

[39] A. M. Reddy, Y. Zheng, G. Jagadeeswaran et al., "Cloning, characterization and expression analysis of porcine microRNAs," *BMC Genomics*, vol. 10, article 65, 2009.

[40] S. Greco, A. Perfetti, P. Fasanaro et al., "Deregulated microRNAs in myotonic dystrophy type 2," *PLoS ONE*, vol. 7, no. 6, Article ID e39732, 2012.

[41] A. V. Postma, V. M. Christoffels, and C. R. Bezzina, "Developmental aspects of cardiac arrhythmogenesis," *Cardiovascular Research*, vol. 91, no. 2, pp. 243–251, 2011.

[42] S. Mahida, "Transcription factors and atrial fibrillation," *Cardiovascular Research*, vol. 101, no. 2, pp. 194–202, 2014.

[43] L. Wang, M. R. D. Tullio, A. Beecham et al., "A comprehensive genetic study on left atrium size in Caribbean Hispanics identifies potential candidate genes in 17p10," *Circulation: Cardiovascular Genetics*, vol. 3, no. 4, pp. 386–392, 2010.

[44] E. Lozano-Velasco, A. Contreras, C. Crist, F. Hernández-Torres, D. Franco, and A. E. Aránega, "Pitx2c modulates Pax3+/Pax7+ cell populations and regulates Pax3 expression by repressing miR27 expression during myogenesis," *Developmental Biology*, vol. 357, no. 1, pp. 165–178, 2011.

[45] M. Torrado, R. Iglesias, A. Centeno, E. López, and A. T. Mikhailov, "Targeted gene-silencing reveals the functional significance of myocardin signaling in the failing heart," *PLoS ONE*, vol. 6, no. 10, Article ID e26392, 2011.

[46] M. Torrado, D. Franco, F. Hernańdez-Torres et al., "Pitx2c is reactivated in the failing myocardium and stimulates Myf5 expression in cultured cardiomyocytes," *PLoS ONE*, vol. 9, no. 3, Article ID e90561, 2014.

[47] M. Torrado, A. Centeno, E. López, and A. T. Mikhailov, "*In vivo* forced expression of myocardin in ventricular myocardium transiently impairs systolic performance in early neonatal pig heart," *The International Journal of Developmental Biology*, vol. 53, no. 8–10, pp. 1457–1467, 2009.

[48] M. S. C. Fontes, A. J. A. Raaijmakers, T. van Doorn et al., "Changes in Cx43 and $Na_V1.5$ expression precede the occurrence of substantial fibrosis in calcineurin-induced murine cardiac hypertrophy," *PLoS ONE*, vol. 9, no. 1, Article ID e87226, 2014.

[49] Y. Li, Z.-H. Gong, L. Sheng et al., "Anti-apoptotic effects of a calpain inhibitor on cardiomyocytes in a canine rapid atrial fibrillation model," *Cardiovascular Drugs and Therapy*, vol. 23, no. 5, pp. 361–368, 2009.

[50] M. R. Fabian, N. Sonenberg, and W. Filipowicz, "Regulation of mRNA translation and stability by microRNAs," *Annual Review of Biochemistry*, vol. 79, pp. 351–379, 2010.

[51] W. Hu and J. Coller, "What comes first: translational repression or mRNA degradation? the deepening mystery of microRNA function," *Cell Research*, vol. 22, no. 9, pp. 1322–1324, 2012.

[52] A. Heidersbach, C. Saxby, K. Carver-Moore et al., "microRNA-1 regulates sarcomere formation and suppresses smooth muscle gene expression in the mammalian heart," *Elife*, vol. 2, Article ID e01323, 2013.

[53] X. H. Liao, N. Wang, D. W. Zhao et al., "NF-κB (p65) negatively regulates myocardin-induced cardiomyocyte hypertrophy through multiple mechanisms," *Cell Signaling*, vol. 26, no. 12, pp. 2738–2748, 2014.

[54] F. Wang, X.-Y. Yang, J.-Y. Zhao et al., "MiR-10a and MiR-10b target the 3′-untranslated region of TBX5 to repress its expression," *Pediatric Cardiology*, vol. 5, no. 5, pp. 1072–1079, 2014.

[55] D. T. Humphreys, C. J. Hynes, H. R. Patel et al., "Complexity of murine cardiomyocyte miRNA biogenesis, sequence variant expression and function," *PLoS ONE*, vol. 7, no. 2, Article ID e30933, 2012.

[56] M. B. Hudson, J. A. Rahnert, B. Zheng, M. E. Woodworth-Hobbs, H. A. Franch, and S. Russ Price, "miR-182 attenuates atrophy-related gene expression by targeting $FoxO_3$ in skeletal muscle," *The American Journal of Physiology—Cell Physiology*, vol. 307, no. 4, pp. C314–C319, 2014.

[57] P. Puthanveetil, A. Wan, and B. Rodrigues, "FoxO1 is crucial for sustaining cardiomyocyte metabolism and cell survival," *Cardiovascular Research*, vol. 97, no. 3, pp. 393–403, 2013.

[58] P. Kazemian, G. Oudit, and B. I. Jugdutt, "Atrial fibrillation and heart failure in the elderly," *Heart Failure Reviews*, vol. 17, no. 4-5, pp. 597–613, 2012.

[59] J. Xiao, D. Liang, Y. Zhang et al., "MicroRNA expression signature in atrial fibrillation with mitral stenosis," *Physiological Genomics*, vol. 43, no. 11, pp. 655–664, 2011.

[60] O. Adam, B. Löhfelm, T. Thum et al., "Role of miR-21 in the pathogenesis of atrial fibrosis," *Basic Research in Cardiology*, vol. 107, no. 5, article 278, 2012.

[61] D. Franco, A. Chinchilla, H. Daimi, J. N. Dominguez, and A. Arnega, "Modulation of conductive elements by Pitx2 and their impact on atrial arrhythmogenesis," *Cardiovascular Research*, vol. 91, no. 2, pp. 223–231, 2011.

[62] J. Wang, Y. Bai, N. Li et al., "Pitx2-microRNA pathway that delimits sinoatrial node development and inhibits predisposition to atrial fibrillation," *Proceedings of the National Academy of Sciences*, vol. 111, no. 25, pp. 9181–9186, 2014.

[63] L. A. Aguirre, M. E. Alonso, C. Badía-Careaga et al., "Long-range regulatory interactions at the 4q25 Atrial Fibrillation risk locus involve *PITX2c* and *ENPEP*," *BMC Biology*, vol. 13, no. 1, article 26, 2015.

[64] D. P. Leong and H. Dokainish, "Left atrial volume and function in patients with atrial fibrillation," *Current Opinion in Cardiology*, vol. 29, no. 5, pp. 437–444, 2014.

[65] A. T. Mikhailov and M. Torrado, "In search of novel targets for heart disease: myocardin and myocardin-related transcriptional cofactors," *Biochemistry Research International*, vol. 2012, Article ID 973723, 11 pages, 2012.

[66] J. M. Miano, "Myocardin in biology and disease," *Journal of Biomedical Research*, vol. 29, no. 1, pp. 3–19, 2015.

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 531740, 9 pages
http://dx.doi.org/10.1155/2015/531740

Review Article

MicroRNA-143/-145 in Cardiovascular Diseases

Wang Zhao, Shui-Ping Zhao, and Yu-Hong Zhao

Department of Cardiology, The Second Xiangya Hospital of Central South University, No. 139, People Street, Changsha, Hunan 410011, China

Correspondence should be addressed to Shui-Ping Zhao; zhaosp@medmail.com.cn

Received 3 October 2014; Accepted 2 April 2015

Academic Editor: Xiao-Bo Liao

MicroRNAs (miRNAs) play an essential role in the onset and development of many cardiovascular diseases. Increasing evidence shows that miRNAs can be used as potential diagnostic biomarkers for cardiovascular diseases, and miRNA-based therapy may be a promising therapy for the treatment of cardiovascular diseases. The microRNA-143/-145 (miR-143/-145) cluster is essential for differentiation of vascular smooth muscle cells (VSMCs) and determines VSMC phenotypic switching. In this review, we summarize the recent progress in knowledge concerning the function of miR-143/-145 in the cardiovascular system and their role in cardiovascular diseases. We discuss the potential role of miR-143/-145 as valuable biomarkers for cardiovascular diseases and explore the potential strategy of targeting miR-143 and miR-145.

1. Introduction

MicroRNAs (miRNAs) are short 18–24 nucleotide, single-stranded, noncoding RNAs that bind to the complementary target sites in 3′-untranslated regions (3′-UTRs) of specific mRNA targets to inhibit translation or to cause mRNA degradation [1]. It is estimated that the human genome contains more than 1,000 miRNAs, which regulate at least 30% of protein-coding genes [2–4]. In addition, one single miRNA can exert inhibitory effects on many mRNAs, whereas one single mRNA can be modulated by many miRNAs. Thus, the regulation of the expression of mRNAs by miRNAs is a complex process that involves diverse cellular functions: cell proliferation and differentiation, apoptosis, neuronal patterning, immunity, fat metabolism, and phenotypic switching of vascular smooth muscle cells (VSMCs) [5, 6]. Dysregulation of several miRNAs is associated with many diseases including cancer, cardiovascular diseases, and neurological disorders [7–10]. MiRNA-based therapy has become a promising treatment for many human diseases including cardiovascular diseases and cancer [11–13].

Cardiovascular diseases are the leading cause of morbidity and mortality worldwide. Although it has been well established that genetic mutations and cellular mechanisms contribute to the pathogenesis of several cardiovascular diseases, recent studies have shown that miRNAs play key roles in cardiovascular system development [4, 14–16]. It has been determined that miRNAs contribute to many cardiovascular processes, such as embryonic stem cell differentiation, cardiomyocyte proliferation, VSMC phenotypic switching, endothelial responses to shear stress, and erythropoiesis [4, 6, 17–21]. Dysregulation of miRNAs has been found in many cardiovascular diseases including heart failure, myocardial ischemia, congenital heart disease, atherosclerosis, and hypertension [22–27].

The microRNA-143/-145 (miR-143 and miR-145) encoding genes are located in close proximity with each other on human chromosome 5 and are believed to be cotranscribed in the same bicistronic transcript [28]. The miR-143/-145 gene cluster is expressed in the heart and in VSMCs [29–32]. MiR-143 is believed to play an essential role in the function and formation of the cardiac chamber via regulation of myocardial cell morphology [32]. MiR-143 and miR-145 are essential for VSMC differentiation [31] and are molecular keys to determine VSMC phenotypic switching [6, 30]. It is interesting that miR-143 and miR-145 can be upregulated in endothelial cells in response to shear stress and subsequently are exported in exosome-like vesicles that regulate VSMC phenotype [33]. In addition, it has been reported that circulating miR-145 levels differ in patients with coronary artery disease [34, 35]; in patients with acute myocardial infarction (AMI), the level of miR-145 in total peripheral

Table 1: Targets of miR-143/145 and associated function.

Target	Function	Reference
Ets-like gene 1 (for miR-143)	Proliferation and differentiation	[31]
KLF4 and myocardin (for miR-145)		[30, 31, 34]
MRTF-β	Actin modeling	[42]
Slingshot 2		[42]
KLF4 (for miR-145)		[42]
KLF5 (for miR-145)		[42]
Srgap1		[42]
Srgap2		[42]
ACE	Contractility	[29]

blood correlates with infarct size [36]. This suggests that miR-145 could be a valuable biomarker for cardiovascular diseases. Furthermore, several clinical studies have shown that miR-143/-145 dysregulation is associated with many cardiovascular diseases, including essential hypertension, atherosclerosis, pulmonary arterial hypertension, and coronary artery disease [35, 37–40]. Therefore, targeting miR-143/-145 may be a promising therapeutic strategy for the treatment of these cardiovascular diseases [41].

In this review, we describe the function of miR-143 and miR-145 in the cardiovascular system and discuss their roles in cardiovascular diseases. In addition, we explore whether miR-143 and miR-145 may be valuable biomarkers for cardiovascular diseases and whether targeting miR-143/-145 could be a potential strategy for the treatment of cardiovascular diseases.

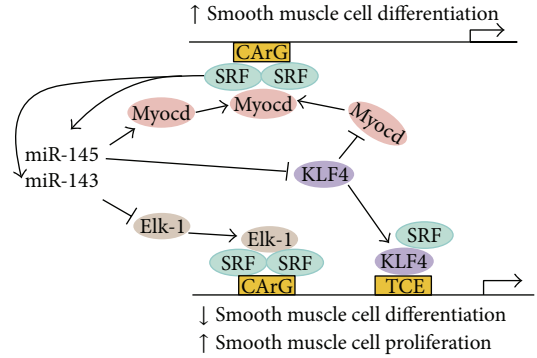


Figure 1: Roles of miR-143/-145 in the regulation of vascular smooth muscle cell differentiation and proliferation (adapted from Cordes et al. [31]). Expressions of miR-143/-145 are positively regulated by SRF with the functions to repress several factors, including KLF4 and Elk-1, which are reported to be involved in the regulation of smooth muscle cell proliferation, leading to promotion of smooth muscle cell differentiation and inhibition of proliferation. SRF: serum response factor; Myocd: myocardin; KLF4: Kruppel-like factor 4; Elk-1: Ets-like gene 1.

2. The Functions of MiR-143 and MiR-145 in the Cardiovascular System

2.1. VSMCs. MiR-143 and miR-145 are abundantly expressed in VSMCs [29–31, 43, 44]. Both miR-143 and miR-145 play crucial roles in VSMC differentiation [29, 31, 43]. Overexpression of miR-145 upregulates the expression of VSMC differentiation marker genes, such as smooth muscle alpha-actin, calponin, and smooth muscle-myosin heavy chain (SM-MHC), thus promoting differentiation of VSMCs into the contractile phenotype [30]. The effect of miR-145 on VSMC phenotypic modulation is through the suppression of Kruppel-like factor 5 (KLF5) and Kruppel-like factor 4 (KLF4) and subsequent stimulation of their downstream signaling molecule myocardin [30, 45] (Figure 1). Furthermore, miR-145 has been found to be sufficient to induce differentiation of multipotent neural crest stem cells into VSMCs [31]. Similarly, miR-143 can regulate VSMC phenotype similar to miR-145, but to a lesser extent [31]. In addition to KLF5, KLF4, and myocardin, many other targets of miR-143/-145 have been identified, including Slit-Robo GTPase-activating protein 1 (Srgap1), Srgap2, Adducin-3, Slingshot 2 (Ssh2), angiotensin converting enzyme (ACE), calmodulin kinase II δ, fascin, and myocardin-related transcription factor-β (MRTF-β) [29–31, 42, 46] (Table 1). Moreover, the expression of miR-143/-145 is induced by the serum response factor (SRF) and its coactivator myocardin and regulated by cytoskeletal dynamics and responses of VSMCs to injury. This suggests that they are an integral component of a complex regulatory network of SRF in the control of VSMC phenotypic switching [42].

The role of miR-143/-145 in VSMC was further investigated by studies in knockout mice. MiR-143/-145 knockout mice exhibit many abnormalities in VSMCs, including a reduction in marker expression, decreased contractile function, increased and dilated rough endoplasmic reticulum, thin smooth muscle layer, and decreased actin-based stress fibers [29, 42, 43]. These structural functional findings indicate that miR-143/-145 knockout results in a shift from a contractile to synthetic phenotype of VSMCs, suggesting that miR-143 and miR-145 are required for maintaining the contractile phenotype and function of VSMCs. Although miR-143/-145 play an important role in determining VSMC phenotype and function, it appears that they are not essential for cardiovascular development *in vivo*, since miR-143/-145 knockout mice are viable and do not have gross macroscopic alterations [29, 42, 43]. This is consistent with the finding that miR-143 is only active during the early stages of heart

Table 2: Aberrant expression of miR-143/145-associated diseases.

Disease	miR-143/145 expression	Source of miRNAs	Reference
Essential hypertension	Down	Peripheral blood mononuclear cells	[15]
Coronary artery disease	Down	Plasma	[35]
Unstable angina	Up	Plasma	[34]
Acute myocardial infarction	Up	Peripheral total blood	[36]
pulmonary arterial hypertension	Up	Lung tissues	[37]

development and disappears from cardiomyocytes at later stages [29]. In contrast, the Dicer-knockout mice are lethal by extensive internal hemorrhage at the later embryonic stage [47]. These findings suggest that other miRNAs, but not miRNA-143 and miRNA-145, are essential for VSMC development *in vivo*.

2.2. Endothelium. MiR-143 and miR-145 have been shown to be upregulated in endothelial cells in response to shear stress [33, 48]. In human aortic arterial endothelial cells, shear stress-induced upregulation of miR-145 decreases the expression of its target gene junctional adhesion molecule-A (JAM-A), a molecule that induces inflammatory cell entry into the atherosclerotic site [48]. MiR-143 and miR-145 have been known to control the expression of various proteins that are involved in the regulation of actin cytoskeleton [33, 42, 49]. Although it remains unclear whether miR-143 and miR-145 contribute to actin cytoskeleton rearrangement induced by shear stress in endothelial cells [50], miR-143 and miR-145 have been found to be upregulated in endothelial cells in response to KLF2 and shear stress [33]. MiR-143/-145 are released from endothelial cells in exosome-like vesicles and subsequently regulate target gene expression in VSMCs [33]. It appears that miR-143/-145 may function as a signaling molecule that communicates between endothelial cells and VSMCs.

2.3. Heart. MiR-143/-145 have been found to be expressed in the heart [31, 32, 51]. MiR-143 controls F-actin dynamics and cell morphology and is essential for cardiac chamber function and morphogenesis [32]. MiR-143 expression is found to be regulated by heart beat [52]. In addition, the expression of miR-143/-145 is restricted to an early stage of heart development and disappears from cardiomyocytes at E16.5 [29, 42]. MiR-145 has been found upregulated in the heart of mice after transverse aortic constriction and inhibits isoproterenol-induced cardiomyocyte hypertrophy [51]. The expression of miR-143/-145 in cardiomyocytes can be induced by activin A via the p38 mitogen-activated protein kinase (MAPK) signaling pathway, resulting in inhibition on insulin-mediated biological action [53]. Furthermore, the expression level of miR-145 exhibited a tendency toward a higher expression in patients with end-stage dilated cardiomyopathy compared with healthy controls, though no significant difference was found [54].

2.4. MiR-143 and MiR-145 in the Plasma. MiRNAs are present in the peripheral blood cells such as erythrocytes and mononuclear cells [19, 55]. The miR-143/-145 complex has been found to be downregulated in M1 microphages compared with M2 macrophages, suggesting that miR-143/-145 may play an important role in macrophage differentiation and polarized activation processes [56]. In addition, microRNAs can be stably present in the serum, and thus circulating miRNAs can be used for potential biomarkers for many diseases (Table 2). MiR-143/-145 in human peripheral blood mononuclear cells have been found to be upregulated in patients with essential hypertension [38]. Changes in circulating miR-145 levels were found in patients with stable coronary artery disease [35], stable or unstable angina [34], and acute myocardial infarction [36]. Therefore, miR-143 and miR-145 can be used for potential biomarkers for these cardiovascular diseases.

3. Role of MiR-143/-145 in Atherosclerosis and Hypertension

3.1. Atherosclerosis. Atherosclerosis is a chronic disease of the vascular wall involving multiple pathological processes, such as lipid retention and inflammations in many different types of cells such as various inflammatory cells, endothelial cells, and VSMCs [57]. Since miR-143 and miR-145 are expressed in endothelial cells, VSMCs, and inflammatory cells, it is not surprising that several clinical and animal studies have shown that miR-143 and miR-145 contribute to pathogenesis of atherosclerosis [33, 39, 58–60]. The role of miR-143/-145 in atherosclerosis may result from their regulation of endothelial cells, VSMCs, and circulating blood cells.

VSMCs play a role in the pathogenesis of atherosclerosis [61]. The miR-145 cluster has been found to be downregulated in the proliferative VSMCs of atherosclerotic arteries in ApoE-knockout mice [58]. This suggests that downregulation of miR-145 may contribute to atherogenesis. Consistent with this idea, miR-143/-145 knockout mice with normal serum concentration of triglycerides and lipoproteins exhibited spontaneous atherosclerotic lesion in the femoral artery [29]. Furthermore, overexpression of miR-145 in VSMCs reduced plague size in aortic sinuses, ascending aortas, and brachiocephalic arteries in ApoE-knockout mice [59]; this further confirms the atheroprotective role of miR-145. However, the atheroprotective mechanisms of miR-143/-145 have not been

well elucidated, even though many potential targets of miR-143/-145, such as ACE [29], are involved in the pathogenesis of atherosclerosis [62]. The atheroprotective role of miR-143 and miR-145 may be attributed to their ability to promote contractile VSMC phenotype and to inhibit the synthetic VSMC phenotype that is associated with atherosclerosis [29, 57]. In agreement with this hypothesis is that miRNAs, including miR-145 that are associated with the contractile VSMC phenotype, are downregulated and some miRNAs, such as miR-21, 146a and 221 that are connected to the synthetic VSMC phenotype, are upregulated in atherosclerotic plaques [63].

Endothelial cells are subject to different regimes of shear stress in distinct areas of blood vessels. Atherosclerotic lesions are susceptible to occurring in areas of disturbed flow and low shear stress, whereas unbranched portions of arteries with uniform laminar flow are protected from atherogenesis. It has been reported that miR-143/-145 are upregulated in human aortic arterial endothelial cells and human umbilical vein endothelial cells in response to atheroprotective laminar flow [33, 48]. The exact mechanisms underlying atheroprotective effect of miR-143/-145 remains unclear. Hergenreider et al. reported that KLF2 transduction and shear stress induced upregulation of miR-143/-145 in endothelial cells, which were released in exosome-like vesicles and inhibited the target gene expression in VSMCs [33]. Furthermore, extracellular vesicles derived from KLF2-expression endothelial cells reduced aortic atherosclerosis in ApoE-knockout mice [33], suggesting that miR-143/-145 may exert atheroprotective role via a route of communication between endothelial cells and VSMCs. In addition, Schmitt et al. found that upregulation of miR-145 repressed junctional adhesion molecule-A- (JAM-A-) induced entry of monocytes into the sites of atherosclerotic lesions, suggesting that miR-145 may inhibit atherosclerotic formation via downregulation of JAM-A in endothelial cells [48].

In clinical studies, miR-145 has been found to be overexpressed in carotid atherosclerotic plaques in patients with symptomatic stroke compared with plaques in patients with nonsymptomatic stoke [64]. Recently, Santovito et al. reported that miR-145 was upregulated in atherosclerotic plaques of hypertensive patients compared with control plaques of nonhypertensive patients [39], suggesting that hypertension may upregulate miR-145 expression in human atherosclerotic plaque. These clinical findings that miR-145 is upregulated in atherosclerotic plaques are apparently in contrast with the animal studies showing that upregulation of miR-145 is atheroprotective [59]. This discrepancy may be explained by compensatory upregulation of miR-145 in response to chronic stress in patients with chronic diseases.

3.2. Hypertension. VSMCs determine vascular tone and regulate vascular resistances and thus play a fundament role in the development of hypertension [65]. The transition of VSMCs from a state of differentiation to a state of proliferation contributes to the pathogenesis of hypertension [66]. MiR-143/-145 knockout mice have a reduced blood pressure due to reduced vascular tone [29, 42, 67], suggesting that miR-143/-145 are required for controlling blood pressure.

MiR-143 and miR-145 have been found to be downregulated in VSMCs in response to acute and chronic vascular stress [30, 43]. In hypertension, VSMCs are constantly exposed to excessive stretch due to persisting high blood pressure. It has been reported that miR-145 is important for the expression of the VSMC contractile phenotype, [29, 31, 43] as it mediates stretch-induced differentiation of VSMCs [68, 69]. Recently, Hu et al. found that stretch-induced activation of extracellular signal-regulated kinase 1/2 (ERK1/2) and upregulation of ACE contribute to reduced expression of miR-145 because of a reduction in mechanical stretch [68]. Stretch-induced downregulation of miR-145 resulted in reduced expression of VSMCs contractile markers, including KLR4 and myocardin [68]. Therefore, modulation of the VSMC phenotype by miR-145 in response to persistent hypertensive stretch may contribute to the pathogenesis of hypertension.

Many of potential targets of miR-143/-145, such as CAMK-II and ACE, are involved in blood pressure regulation [29, 69]. MiR-145 can increase the expression of L-type calcium channel via suppression of CAMK-II [69]. L-type calcium channels that couple blood pressure-induced membrane potential change to the myogenic response in VSMCs has been known to contribute to the pathogenesis of hypertension [70]. In addition, ACE, a regulator of blood pressure, is upregulated in VSMCs of miR-145 knockout mice, resulting in an increased generation of angiotensin II in blood vessel walls [29]. ACE in VSMCs is known to play an important role in vascular remodeling in hypertension [71]. Therefore, many signaling pathways that are targeted by miR-145 may underlie the miR-145-mediated pathogenesis of hypertension.

Recently, Kontaraki et al. found that the expression levels of miR-143/-145 in human peripheral blood mononuclear cells were lower in hypertensive patients than in healthy controls and the expression levels negatively correlated with 24 hr diastolic and mean blood pressure [38]. This clinical finding that miR-143/-145 is downregulated in hypertensive patients is consistent with animal studies showing that the expression of miR-143/-145 is decreased in vascular walls after balloon carotid artery injury and in ApoE-knockout mice [30, 43, 58]. It is possible that a fraction of the miR-145 levels in human peripheral blood mononuclear cells may be transferred from the vascular wall.

Many microRNAs, including miR-145, have an important role in pathogenesis of pulmonary arterial hypertension (PAH) [41, 72]. MiR-145 has been shown to be activated by transforming growth factor-β (TGF-β) signaling pathway in VSMCs [73, 74] and upregulation of miR-145 by TGF-β to promote differentiation in lung myofibroblasts [75]. Caruso et al. reported that miR-145 is upregulated in PAH patients and in a mouse model of hypoxia-induced PAH, as well as in the lungs of mice with mutant bone morphogenetic protein (BMP) receptor type-2 (BMPR2), a receptor for the TGF-β superfamily [37]. Furthermore, miR-145 knockout results in a significant protection from the development of PAH [37], suggesting that miR-145 plays an important role in the development of PAH.

4. MiR-143/-145 as Potential Diagnostic Markers for Cardiovascular Diseases

In addition to control cellular processes, miRNAs are released into the circulation and can be stably present in the plasma [76]. Since miRNAs can be detected in the plasma under diseased conditions, circulating miRNAs may function as potential diagnostic markers for cardiovascular diseases [55, 76, 77]. Increasing evidence has shown that circulating miRNAs may function as diagnostic markers for coronary artery disease (CAD), diabetic heart diseases, myocardial infarction, hypertension, and heart failure [34, 55, 76, 78, 79].

Considering the specificity of microRNAs, which are short, single-strand, and noncoded, their measurement would be different from other coded genes. Currently there are three primary methods for detection and quantification of microRNAs, Northern blot, PCR, and microarray. Among them, PCR is the mostly used approach for microRNAs studies especially in clinical studies, due to high sensitivity and specificity as well as the ability to quantify the number of microRNAs present in each sample. Through using quantitative PCR approach, several studies have investigated the circulating miR-145 levels in patients with coronary artery disease [34, 35]. Fichtlscherer et al. reported that circulating miR-145 was downregulated in patients with stable coronary artery disease compared with healthy controls [35]. However, D'Alessandra et al. found that circulating miR-145 was upregulated in patients with unstable angina compared with healthy controls [34]. This discrepancy may be associated with different disease status (stable CAD by Fichtlscherer et al. versus unstable angina by D'Alessandra et al.). More miR-145 may be released from vascular walls due to more severe injuries in patients with unstable angina compared with patients with stable CAD. This hypothesis agrees with the findings that plasma concentrations of miR-145 correlate with muscle injury in rodents [44]. In addition, both studies used a limited number of patients (19 patients with unstable angina versus 20 controls in D'Alessandra's study and 36 patients with CAD versus 17 controls in Fichtlscherer's study). Further studies with a large sample size are required to demonstrate the role of miR-145 as a potential diagnostic marker for CAD.

The miR-145 level in total peripheral blood has been found to be elevated in patients with acute myocardial infarction and correlate with the infarction size estimated by troponin-T release [36]. Since miR-145 is enriched in VSMCs, elevated miR-145 levels in AMI may reflect the vessel injury that occurs during atherosclerotic plague rupture. Consistent with this idea, upregulation of miR-145 expression is found in atherosclerotic plaques in hypertensive patients [39]. Although miR-145 levels correlated with infarction size, Meder et al. did not identify miR-145 as a good predictor for AMI using receiver operator characteristic curves [36].

Recently, Kontaraki et al. investigated the expression levels of many VSMC-modulating miRNAs including miR-143/-145 in human peripheral blood mononuclear cells in patients with essential hypertension in comparison with healthy controls. The expression levels of miR-143/-145 were decreased in hypertensive patients compared with healthy controls and the expression levels negatively correlated with 24 hr diastolic and mean blood pressure [38]. It remains to be determined whether changes in miR-143/-145 levels in human peripheral blood mononuclear cells are the results of hypertension in peripheral blood mononuclear cells or secondary to the vascular wall injury induced by hypertension. In addition, although Kontaraki et al. reported that diabetes did not affect the expression of miR-143/-145 in hypertensive patients [38], it has been reported that miR-145 was reduced in AMI patients with diabetes compared with AMI patients without diabetes [36]. Future studies with large cohorts of patients are required to confirm the role of miR-143/-145 as diagnostic markers for essential hypertension.

5. MiR-143/-145 as a Potential Therapeutic Targets for the Treatment of Cardiovascular Diseases

MiRNA dysregulation is linked to the development of cardiovascular diseases; restoration of dysregulated miRNAs to their normal levels can potentially reduce or even eliminate diseases, at least in animal models [80, 81]. MiRNA-based therapy has been regarded as a promising method for clinical applications in the treatment of cardiovascular diseases [13, 80]. Compared with traditional molecular targets, miRNAs have features that favor them as potential good therapeutic targets. MiRNAs may target many mRNAs that share common biological and cellular function and thus may produce a strong effect on end-point functions. In this way, targeting miRNAs may less likely cause desensitization, which occurs commonly when only one therapeutic target is aimed for in most classical drugs. In addition, targeting cell- and disease-specific expression of miRNAs may reduce off-target effects, which are severe problems for classical drugs. Therefore, identification of cell- and disease-specific miRNAs is important for successful miRNA-based therapy.

MiR-143/-145 are predominantly expressed in VSMCs and play a role in several cardiovascular diseases, such as essential hypertension [38], atherosclerosis [40], pulmonary arterial hypertension [37, 39], and coronary artery disease [35]. Targeting miR-143/-145 may be a promising therapy for these cardiovascular diseases. However, to date, no miRNA-based therapy has been developed to treat cardiovascular diseases in human clinical trials. Several animal studies have demonstrated that targeting miR-143/-145 may be a promising therapy for vascular diseases.

Given the association of aberrant expression of miR-143/-145 with the pathogenesis of many cardiovascular diseases, experimentally manipulating them to be either up or down expressed might be beneficial in the prophylaxis or treatment of these diseases. Currently, adenoviruses-mediated gene transfer system is a commonly used method which can experimentally make some target genes up or down expression *in vivo*. Through using the commercially available Adeno-X Expression Systems 2 kit, Cheng's group successfully constructed the adenoviruses which are expressing miR-145. Subsequent studies by implying this gene transfer system into rat model with carotid artery balloon injury reported that transient local adenoviral overexpression of miR-145

inhibited neointimal lesion formation in rat carotid arteries after vascular balloon injury [30], suggesting that restoration of downregulated miR-145 may be effective for the treatment of vascular diseases. Furthermore, adenoviral overexpression of smart miR-145 in which the flank sequence of miR-145 was switched with that of miR-31 results in a more inhibition on balloon injury-induced neointimal lesion formation in rat carotid arteries, compared with adenoviral overexpression of miR-145 [58], suggesting that modulation of the flank sequence may be an effective way to improve therapeutic efficacy of miRNAs. In addition, using a similar method, Elia et al. found that overexpression of either miR-143 or miR-145 reduced balloon injury-induced neointimal lesion formation in rat carotid arteries [43]. Furthermore, systemic injection of lentiviral miR-145 markedly reduced atherosclerotic volumes in aortic roots, ascending aortas, and brachiocephalic arteries of ApoE-knockout mice [59].

To date, most investigators have used virus-mediated gene transduction for overexpression of miRNAs in animals. Recently, Ohnaka et al. developed a nonviral method for transduction of miR-145 into a bypass graft, using an electroporator [82], involving the usage of an expression vector for human miR-145 (pMIW-cGFP-miR-145) which is commercially available. Their study demonstrated that MiR-145-overexpressing vein grafts inhibited neointimal lesions formation in the arterialized rabbit vein graft model [82]. This nonviral gene transfer method reduces the potential ethical issues and cytotoxicity owing to difficulties in virus handing and regulation and may be more appropriate for clinical application.

6. Conclusions and Perspectives

Recently, increasing evidence has shown that plasma miRNAs can be used as a potential disease biomarker [55, 76, 77]. MiR-143 and miR-145, important molecules that determine the phenotype of VSMCs, can be released into the plasma in response to vascular injury and thus may be used as potential diagnostic biomarkers for vascular diseases. Recent clinical findings have demonstrated that miR-143 and miR-145 levels in the plasma are associated with essential hypertension [38], coronary artery disease [34, 35], and AMI [36]; these findings highlight the importance of miR-143/-145 as potential biomarkers for cardiovascular diseases. However, one should keep in mind that the use of miR-143/-145 as biomarkers for cardiovascular diseases should be further investigated in comprehensive studies with a large sample size, since, to date, clinical studies have only included a small sample size. In addition, the expression levels of multiple miRNAs differ based upon the specific cardiovascular disease [78], and thus expression profiles of multiple miRNAs may be better used for diagnosis. Furthermore, it would be interesting to identify circulating miRNAs as biomarkers for predicting a response to a specific treatment in cardiovascular diseases.

MiRNAs play an essential role in the development of many cardiovascular diseases, and miRNA-based therapy has been recognized to be a promising novel therapeutic strategy for the treatment of cardiovascular diseases [13, 16, 80]. MiR-143/-145 are relatively specific for VSMCs and thus are attractive potential drug target for vascular diseases such as atherosclerosis, hypertension, and CAD. However, although the delivery of viral vectors expressing miR-143/-145 effectively inhibits neointimal lesion formation in the animal model, no clinical trials have demonstrated an effective miRNA-based therapy for the treatment of cardiovascular disease. The study with miRNA-based therapy for cardiovascular disease is just beginning. Future preclinical and clinical studies are warranted to evaluate the efficacy of miRNA-based therapy in patients with cardiovascular diseases.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

References

[1] D. P. Bartel, "MicroRNAs: genomics, biogenesis, mechanism, and function," *Cell*, vol. 116, no. 2, pp. 281–297, 2004.

[2] I. Bentwich, A. Avniel, Y. Karov et al., "Identification of hundreds of conserved and nonconserved human microRNAs," *Nature Genetics*, vol. 37, no. 7, pp. 766–770, 2005.

[3] R. C. Friedman, K. K.-H. Farh, C. B. Burge, and D. P. Bartel, "Most mammalian mRNAs are conserved targets of microRNAs," *Genome Research*, vol. 19, no. 1, pp. 92–105, 2009.

[4] E. M. Small and E. N. Olson, "Pervasive roles of microRNAs in cardiovascular biology," *Nature*, vol. 469, no. 7330, pp. 336–342, 2011.

[5] L. He and G. J. Hannon, "MicroRNAs: small RNAs with a big role in gene regulation," *Nature Reviews Genetics*, vol. 5, no. 7, pp. 522–531, 2004.

[6] A. Y. Rangrez, Z. A. Massy, V. M.-L. Meuth, and L. Metzinger, "MiR-143 and miR-145 molecular keys to switch the phenotype of vascular smooth muscle cells," *Circulation: Cardiovascular Genetics*, vol. 4, no. 2, pp. 197–205, 2011.

[7] F. de Santa, I. Iosue, A. Del Rio, and F. Fazi, "MicroRNA biogenesis pathway as a therapeutic target for human disease and cancer," *Current Pharmaceutical Design*, vol. 19, no. 4, pp. 745–764, 2013.

[8] S. Hagiwara, P. Kantharidis, and M. Cooper, "MicroRNA as biomarkers and regulator of cardiovascular development and disease," *Current Pharmaceutical Design*, vol. 20, no. 14, pp. 2347–2370, 2014.

[9] L. A. MacFarlane and P. R. Murphy, "MicroRNA: biogenesis, function and role in cancer," *Current Genomics*, vol. 11, no. 7, pp. 537–561, 2010.

[10] C. Wang, B. Ji, B. Cheng, J. Chen, and B. Bai, "Neuroprotection of microRNA in neurological disorders (Review)," *Biomedical Reports*, vol. 2, no. 5, pp. 611–619, 2014.

[11] D. S. Greenberg and H. Soreq, "MicroRNA therapeutics in neurological disease," *Current Pharmaceutical Design*, vol. 20, no. 38, pp. 6022–6027, 2014.

[12] M. V. Iorio and C. M. Croce, "MicroRNA dysregulation in cancer: Diagnostics, monitoring and therapeutics. A comprehensive review," *EMBO Molecular Medicine*, vol. 4, no. 3, pp. 143–159, 2012.

[13] E. van Rooij and E. N. Olson, "MicroRNA therapeutics for cardiovascular disease: opportunities and obstacles," *Nature Reviews Drug Discovery*, vol. 11, no. 11, pp. 860–872, 2012.

[14] K. R. Cordes and D. Srivastava, "MicroRNA regulation of cardiovascular development," *Circulation Research*, vol. 104, no. 6, pp. 724–732, 2009.

[15] M. V. G. Latronico and G. Condorelli, "MicroRNAs and cardiac pathology," *Nature Reviews. Cardiology*, vol. 6, no. 6, pp. 419–429, 2009.

[16] K. C. Vickers, K.-A. Rye, and F. Tabet, "MicroRNAs in the onset and development of cardiovascular disease," *Clinical Science*, vol. 126, no. 3, pp. 183–194, 2014.

[17] P. Jakob and U. Landmesser, "Role of microRNAs in stem/progenitor cells and cardiovascular repair," *Cardiovascular Research*, vol. 93, no. 4, pp. 614–622, 2012.

[18] S. Kumar, C. W. Kim, R. D. Simmons, and H. Jo, "Role of flow-sensitive microrna in endothelial dysfunction and atherosclerosis: mechanosensitive athero-miRs," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 34, no. 10, pp. 2206–2216, 2014.

[19] M. A. Listowski, E. Heger, D. M. Bogusławska et al., "MicroRNAs: fine tuning of erythropoiesis," *Cellular and Molecular Biology Letters*, vol. 18, no. 1, pp. 34–46, 2013.

[20] T. Marin, B. Gongol, Z. Chen et al., "Mechanosensitive microRNAs—role in endothelial responses to shear stress and redox state," *Free Radical Biology and Medicine*, vol. 64, pp. 61–68, 2013.

[21] M. S. Parmacek, "MicroRNA-modulated targeting of vascular smooth muscle cells," *Journal of Clinical Investigation*, vol. 119, no. 9, pp. 2526–2528, 2009.

[22] T. Alexy, A. M. James, and C. D. Searles, "Shear sensitive microRNAs and atherosclerosis," *Biorheology*, vol. 51, no. 2-3, pp. 147–158, 2014.

[23] I. Elzenaar, Y. M. Pinto, and R. J. Van Oort, "MicroRNAs in heart failure: new targets in disease management," *Clinical Pharmacology and Therapeutics*, vol. 94, no. 4, pp. 480–489, 2013.

[24] V. Sala, S. Bergerone, S. Gatti et al., "MicroRNAs in myocardial ischemia: Identifying new targets and tools for treating heart disease. New frontiers for miR-medicine," *Cellular and Molecular Life Sciences*, vol. 71, no. 8, pp. 1439–1452, 2014.

[25] A. Synetos, K. Toutouzas, K. Stathogiannis et al., "MicroRNAs in arterial hypertension," *Current Topics in Medicinal Chemistry*, vol. 13, no. 13, pp. 1527–1532, 2013.

[26] L. N. L. van Aelst and S. Heymans, "MicroRNAs as biomarkers for ischemic heart disease," *Journal of Cardiovascular Translational Research*, vol. 6, no. 4, pp. 458–470, 2013.

[27] H.-J. Xing, Y.-J. Li, Q.-M. Ma et al., "Identification of microRNAs present in congenital heart disease associated copy number variants," *European Review for Medical and Pharmacological Sciences*, vol. 17, no. 15, pp. 2114–2120, 2013.

[28] C. Weber, A. Schober, and A. Zernecke, "MicroRNAs in arterial remodelling, inflammation and atherosclerosis," *Current Drug Targets*, vol. 11, no. 8, pp. 950–956, 2010.

[29] T. Boettger, N. Beetz, S. Kostin et al., "Acquisition of the contractile phenotype by murine arterial smooth muscle cells depends on the Mir143/145 gene cluster," *Journal of Clinical Investigation*, vol. 119, no. 9, pp. 2634–2647, 2009.

[30] Y. Cheng, X. Liu, J. Yang et al., "MicroRNA-145, a novel smooth muscle cell phenotypic marker and modulator, controls vascular neointimal lesion formation," *Circulation Research*, vol. 105, no. 2, pp. 158–166, 2009.

[31] K. R. Cordes, N. T. Sheehy, M. P. White et al., "miR-145 and miR-143 regulate smooth muscle cell fate and plasticity," *Nature*, vol. 460, no. 7256, pp. 705–710, 2009.

[32] D. C. Deacon, K. R. Nevis, T. J. Cashman et al., "The miR-143-adducin3 pathway is essential for cardiac chamber morphogenesis," *Development*, vol. 137, no. 11, pp. 1887–1896, 2010.

[33] E. Hergenreider, S. Heydt, K. Tréguer et al., "Atheroprotective communication between endothelial cells and smooth muscle cells through miRNAs," *Nature Cell Biology*, vol. 14, no. 3, pp. 249–256, 2012.

[34] Y. D'Alessandra, M. C. Carena, L. Spazzafumo et al., "Diagnostic potential of plasmatic microRNA signatures in stable and unstable angina," *PLoS ONE*, vol. 8, no. 11, Article ID e80345, 2013.

[35] S. Fichtlscherer, S. De Rosa, H. Fox et al., "Circulating microRNAs in patients with coronary artery disease," *Circulation Research*, vol. 107, no. 5, pp. 677–684, 2010.

[36] B. Meder, A. Keller, B. Vogel et al., "MicroRNA signatures in total peripheral blood as novel biomarkers for acute myocardial infarction," *Basic Research in Cardiology*, vol. 106, no. 1, pp. 13–23, 2011.

[37] P. Caruso, Y. Dempsie, H. C. Stevens et al., "A role for miR-145 in pulmonary arterial hypertension: evidence from mouse models and patient samples," *Circulation Research*, vol. 111, no. 3, pp. 290–300, 2012.

[38] J. E. Kontaraki, M. E. Marketou, E. A. Zacharis, F. I. Parthenakis, and P. E. Vardas, "Differential expression of vascular smooth muscle-modulating microRNAs in human peripheral blood mononuclear cells: novel targets in essential hypertension," *Journal of Human Hypertension*, vol. 28, no. 8, pp. 510–516, 2014.

[39] D. Santovito, C. Mandolini, P. Marcantonio et al., "Overexpression of microRNA-145 in atherosclerotic plaques from hypertensive patients," *Expert Opinion on Therapeutic Targets*, vol. 17, no. 3, pp. 217–223, 2013.

[40] Y. Wei, M. Nazari-Jahantigh, P. Neth, C. Weber, and A. Schober, "MicroRNA-126,-145, and-155: a therapeutic triad in atherosclerosis?" *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 33, no. 3, pp. 449–454, 2013.

[41] S. Albinsson and K. Swärd, "Targeting smooth muscle microRNAs for therapeutic benefit in vascular disease," *Pharmacological Research*, vol. 75, pp. 28–36, 2013.

[42] M. Xin, E. M. Small, L. B. Sutherland et al., "MicroRNAs miR-143 and miR-145 modulate cytoskeletal dynamics and responsiveness of smooth muscle cells to injury," *Genes and Development*, vol. 23, no. 18, pp. 2166–2178, 2009.

[43] L. Elia, M. Quintavalle, J. Zhang et al., "The knockout of miR-143 and -145 alters smooth muscle cell maintenance and vascular homeostasis in mice: correlates with human disease," *Cell Death & Differentiation*, vol. 16, no. 12, pp. 1590–1598, 2009.

[44] K. Endo, H. Weng, Y. Naito et al., "Classification of various muscular tissues using miRNA profiling," *Biomedical Research*, vol. 34, no. 6, pp. 289–299, 2013.

[45] S. Yamaguchi, K. Yamahara, K. Homma et al., "The role of microRNA-145 in human embryonic stem cell differentiation into vascular cells," *Atherosclerosis*, vol. 219, no. 2, pp. 468–474, 2011.

[46] M. Quintavalle, L. Elia, G. Condorelli, and S. A. Courtneidge, "MicroRNA control of podosome formation in vascular smooth muscle cells in vivo and in vitro," *Journal of Cell Biology*, vol. 189, no. 1, pp. 13–22, 2010.

[47] S. Albinsson, Y. Suarez, A. Skoura, S. Offermanns, J. M. Miano, and W. C. Sessa, "MicroRNAs are necessary for vascular smooth muscle growth, differentiation, and function," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 30, no. 6, pp. 1118–1126, 2010.

[48] M. M. N. Schmitt, R. T. A. Megens, A. Zernecke et al., "Endothelial junctional adhesion molecule-a guides monocytes into flow-dependent predilection sites of atherosclerosis," *Circulation*, vol. 129, no. 1, pp. 66–76, 2014.

[49] N. Ma and X. Gao, "β-actin is predicted as one of the potential targets of miR-145: choose internal control gene in verification of microRNA target," *Carcinogenesis*, vol. 34, no. 1, article 236, 2013.

[50] R. A. Boon, T. A. Leyen, R. D. Fontijn et al., "KLF2-induced actin shear fibers control both alignment to flow and JNK signaling in vascular endothelium," *Blood*, vol. 115, no. 12, pp. 2533–2542, 2010.

[51] R. Li, G. Yan, Q. Zhang et al., "miR-145 inhibits isoproterenol-induced cardiomyocyte hypertrophy by targeting the expression and localization of GATA6," *FEBS Letters*, vol. 587, no. 12, pp. 1754–1761, 2013.

[52] K. Y. Miyasaka, Y. S. Kida, T. Banjo et al., "Heartbeat regulates cardiogenesis by suppressing retinoic acid signaling via expression of miR-143," *Mechanisms of Development*, vol. 128, no. 1-2, pp. 18–28, 2011.

[53] M. Blumensatt, S. Greulich, D. Herzfeld De Wiza et al., "Activin A impairs insulin action in cardiomyocytes via up-regulation of miR-143," *Cardiovascular Research*, vol. 100, no. 2, pp. 201–210, 2013.

[54] S. V. Naga Prasad, Z.-H. Duan, M. K. Gupta et al., "Unique MicroRNA profile in end-stage heart failure indicates alterations in specific cardiovascular signaling networks," *The Journal of Biological Chemistry*, vol. 284, no. 40, pp. 27487–27499, 2009.

[55] A. J. Tijsen, Y. M. Pinto, and E. E. Creemers, "Circulating microRNAs as diagnostic biomarkers for cardiovascular diseases," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 303, no. 9, pp. H1085–H1095, 2012.

[56] Y. Zhang, M. Zhang, M. Zhong, Q. Suo, and K. Lv, "Expression profiles of miRNAs in polarized macrophages," *International Journal of Molecular Medicine*, vol. 31, no. 4, pp. 797–802, 2013.

[57] A. Rudijanto, "The role of vascular smooth muscle cells on the pathogenesis of atherosclerosis," *Acta Medica Indonesiana*, vol. 39, no. 2, pp. 86–93, 2007.

[58] X. Liu, Y. Cheng, J. Yang et al., "Flank sequences of miR-145/143 and their aberrant expression in vascular disease: mechanism and therapeutic application," *Journal of the American Heart Association*, vol. 2, no. 6, Article ID e000407, 2013.

[59] F. Lovren, Y. Pan, A. Quan et al., "MicroRNA-145 targeted therapy reduces atherosclerosis," *Circulation*, vol. 126, no. 11, supplement 1, pp. S81–S90, 2012.

[60] F. Sala, J. F. Aranda, N. Rotllan et al., "MiR-143/145 deficiency attenuates the progression of atherosclerosis in Ldlr-/-mice," *Thrombosis and Haemostasis*, vol. 112, no. 4, pp. 796–802, 2014.

[61] A. C. Doran, N. Meller, and C. A. McNamara, "Role of smooth muscle cells in the initiation and early progression of atherosclerosis," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 28, no. 5, pp. 812–819, 2008.

[62] E. Ambrosioni, S. Bacchelli, D. Degli Esposti, and C. Borghi, "ACE-inhibitors and experimental atherosclerosis," *Clinical and Experimental Hypertension*, vol. 15, supplement 1, pp. 157–172, 1993.

[63] E. Raitoharju, N. Oksala, and T. Lehtimäki, "MicroRNAs in the atherosclerotic plaque," *Clinical Chemistry*, vol. 59, no. 12, pp. 1708–1721, 2013.

[64] F. Cipollone, L. Felicioni, R. Sarzani et al., "A unique MicroRNA signature associated with plaque instability in humans," *Stroke*, vol. 42, no. 9, pp. 2556–2563, 2011.

[65] R. M. K. W. Lee, G. K. Owens, T. Scott-Burden, R. J. Head, M. J. Mulvany, and E. L. Schiffrin, "Pathophysiology of smooth muscle in hypertension," *Canadian Journal of Physiology and Pharmacology*, vol. 73, no. 5, pp. 574–584, 1995.

[66] E. M. Rzucidlo, K. A. Martin, and R. J. Powell, "Regulation of vascular smooth muscle cell differentiation," *Journal of Vascular Surgery*, vol. 45, supplement, pp. A25–A32, 2007.

[67] G. D. Norata, C. Pinna, F. Zappella et al., "MicroRNA 143-145 deficiency impairs vascular function," *International Journal of Immunopathology and Pharmacology*, vol. 25, no. 2, pp. 467–474, 2012.

[68] B. Hu, J. T. Song, H. Y. Qu et al., "Mechanical stretch suppresses microRNA-145 expression by activating extracellular signal-regulated kinase 1/2 and upregulating angiotensin-converting enzyme to alter vascular smooth muscle cell phenotype," *PLoS ONE*, vol. 9, no. 5, Article ID e96338, 2014.

[69] K. M. Turczyńska, M. K. Sadegh, P. Hellstrand, K. Swärd, and S. Albinsson, "MicroRNAs are essential for stretch-induced vascular smooth muscle contractile differentiation via microRNA (miR)-145-dependent expression of L-type calcium channels," *The Journal of Biological Chemistry*, vol. 287, no. 23, pp. 19199–19206, 2012.

[70] S. Moosmang, V. Schulla, A. Welling et al., "Dominant role of smooth muscle L-type calcium channel Cav1.2 for blood pressure regulation," *The EMBO Journal*, vol. 22, no. 22, pp. 6027–6034, 2003.

[71] H. D. Intengan and E. L. Schiffrin, "Vascular remodeling in hypertension: roles of apoptosis, inflammation, and fibrosis," *Hypertension*, vol. 38, no. 3, part 2, pp. 581–587, 2001.

[72] J. Meloche, R. Paulin, S. Provencher, and S. Bonnet, "Therapeutic potential of microRNA modulation in pulmonary arterial hypertension," *Current Vascular Pharmacology*. In press.

[73] B. N. Davis-Dusenbery, M. C. Chan, K. E. Reno et al., "Down-regulation of Krüppel-like Factor-4 (KLF4) by microRNA-143/145 is critical for modulation of vascular smooth muscle cell phenotype by transforming growth factor-β and bone morphogenetic protein 4," *The Journal of Biological Chemistry*, vol. 286, no. 32, pp. 28097–28110, 2011.

[74] X. Long and J. M. Miano, "Transforming growth factor-β1 (TGF-β1) utilizes distinct pathways for the transcriptional activation of microRNA 143/145 in human coronary artery smooth muscle cells," *The Journal of Biological Chemistry*, vol. 286, no. 34, pp. 30119–30129, 2011.

[75] S. Yang, H. Cui, N. Xie et al., "MiR-145 regulates myofibroblast differentiation and lung fibrosis," *The FASEB Journal*, vol. 27, no. 6, pp. 2382–2391, 2013.

[76] J. Xu, J. Zhao, G. Evan, C. Xiao, Y. Cheng, and J. Xiao, "Circulating microRNAs: novel biomarkers for cardiovascular diseases," *Journal of Molecular Medicine*, vol. 90, no. 8, pp. 865–875, 2012.

[77] F. Russo, S. Di Bella, V. Bonnici et al., "A knowledge base for the discovery of function, diagnostic potential and drug effects on cellular and extracellular miRNAs," *BMC Genomics*, vol. 15, supplement 3, p. S4, 2014.

[78] E. Bronze-da-Rocha, "MicroRNAs expression profiles in cardiovascular diseases," *BioMed Research International*, vol. 2014, Article ID 985408, 23 pages, 2014.

[79] S. Rawal, P. Manning, and R. Katare, "Cardiovascular microRNAs: As modulators and diagnostic biomarkers of diabetic heart disease," *Cardiovascular Diabetology*, vol. 13, no. 1, article 44, 2014.

[80] S. Dangwal and T. Thum, "MicroRNA therapeutics in cardiovascular disease models," *Annual Review of Pharmacology and Toxicology*, vol. 54, pp. 185–203, 2014.

[81] F. Wahid, A. Shehzad, T. Khan, and Y. Y. Kim, "MicroRNAs: synthesis, mechanism, function, and recent clinical trials," *Biochimica et Biophysica Acta—Molecular Cell Research*, vol. 1803, no. 11, pp. 1231–1243, 2010.

[82] M. Ohnaka, A. Marui, K. Yamahara et al., "Effect of microRNA-145 to prevent vein graft disease in rabbits by regulation of smooth muscle cell phenotype," *Journal of Thoracic and Cardiovascular Surgery*, vol. 148, no. 2, pp. 676.e2–682.e2, 2014.