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# microRNAs-Mediated MMPs Regulation: Novel Mechanism for Cardiovascular Diseases

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## Abstract

Cardiac remodeling and its role in disease progression is a multimechanistic and complex process. Disruption of normal extracellular matrix homeostasis is the most important event responsible for cardiac remodeling, altering heart structure and function. Therefore, targeting extracellular matrix remodeling enzymes such as matrix metalloproteinases has received much interest in terms of developing novel therapeutics strategies. Recent findings of microRNAs (endogenous, non-coding, ~22 nucleotide small RNA) in the cardiac tissue as dynamic modifiers of disease pathogenesis have provided glimpses of undiscovered regulatory mechanisms underlying cardiovascular diseases. The implication of several microRNAs in targeting extracellular matrix components (microRNA-29: collagen, fibrillin, elastin) and matrix metalloproteinases (microRNA-21: MMP-2; microRNA-320: MMP-9) has been well documented. The combined strategy of manipulating extracellular matrix remodeling through targeting matrix metalloproteinases by microRNAs has produced encouraging results in preclinical studies. This chapter reviews the potential of microRNA as

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therapeutics tool for cardiovascular diseases through direct and indirect interactions with the matrix metalloproteinases.

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**Keywords**

Cardiovascular diseases · Extracellular matrix · Matrix metalloproteinases · microRNAs

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## 1 Introduction

Cardiovascular diseases (CVD) are the leading cause of human morbidity and mortality worldwide. Adverse myocardial remodeling in response to hemodynamic load and cardiac stress in association with the hormonal response is a significant cause of CVD. In almost all CVD, an increase in cardiac fibroblasts number (due to the transformation of bone marrow (BM)-derived monocytes, BM progenitors, and fibrocytes) and extracellular matrix (ECM) deposition occurs [1]. The ECM forms a milieu surrounding cells that reciprocally influences many aspects of normal cell behavior, including proliferation, adhesion, migration, and survival; thereby assuring the harmonic structure and function of the heart. To do so, ECM undergoes dynamic remodeling regulated by a number of ECM synthesizing as well as degrading enzymes such as matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs). MMPs are zinc-dependent endopeptidase that plays a fundamental role in remodeling of the ECM under both normal and pathological conditions. The proteolytic activity of MMPs is strictly controlled by tissue inhibitors of metalloproteinases (TIMPs) [2, 3]. Considerable evidence implicates that the expression level of MMPs deregulates in cardiovascular diseases such as hypertrophy, atherosclerosis, leading to myocardial infarction and heart failure. Nearby, 60 MMP inhibitors have been pursued as clinical candidates targeting multiple disorders such as cardiovascular diseases, arthritis, and cancer. Since, the actual molecular mechanisms underlying their role in onset and progression of disease are still uncertain, clinical developments of most of the drugs have been discontinued due to nontargeted approach. The aim of this chapter is to review the involvement of a novel class of small noncoding RNAs (microRNA) known to acts as fine-tuner of the protein expression, in the regulation of MMPs and their use as a potential therapeutic tool in CVD.

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## 2 Extracellular Matrix Remodeling in Cardiovascular Diseases

Every cell influences their surroundings and gets influenced from the same. In the human body, fibroblasts are present in every tissue bridging the voids and mediating the connections between other cell types. In myocardium, although cardiac

myocytes constitute nearly three-fourth of normal myocardial tissue volume, cardiac fibroblast accounts for more than two-third of cell population [2]. Fibroblast is cells of mesenchymal origin, predominantly responsible for synthesizing and secreting a diverse group of proteins and nonprotein components which constitute a dynamic, noncellular, and three-dimensional support structure called the ECM. Nearly 300 proteins which constitute the ECM are fibrillar, non-fibrillar as well as glycosylated in nature. They include 43 subunits of collagens, 36 glycosylated proteins, and nearly 200 glycoproteins complex. The nonprotein component includes water, minerals, and polysaccharides such as glycosaminoglycans [3]. Depending upon their origin, composition, location, and interactions among different cellular components (epithelial-endothelial elements, fibroblast, myocytes, and adipocyte), ECM forms different specialized structures unique to each tissue. In the cardiac matrix, collagen (types I, III, IV, V, and VI), glycoproteins (fibronectin, laminins, periostin, fibromodulin, and vitronectin), proteoglycans (versican, lumican, and biglycan), and glycosaminoglycans (hyaluronic acid and dermatan sulfate) are the major constituents. The components such as collagen IV, laminin, nidogen, and heparan sulfate synthesized by epithelial and endothelial cells forms the basement membrane. Whereas, the fibrillar collagens, fibronectin, and proteoglycans primarily synthesized by stromal cells (fibroblasts, pericytes) forms the interstitial matrix. Combined together in a systematic manner, the ECM component regulates the cardiac cells behavior biochemically, biophysically, and biomechanically. The biochemical regulations enable the cells to interact with their surroundings through direct and indirect signaling. Whereas, biophysical and biomechanical regulation is the support provided for tissue architecture and integrity which further decides cell proliferation, differentiation, migration, polarity, adhesion, and survival. These dynamic interactions between ECM and intracellular events are mediated by a family of transmembrane receptors known as integrins, discoidin domain receptors, and syndecans [4]. The substitution of even a single amino acid in any ECM component can alter the cell structure as well as its behavior. Therefore, under normal development and physiology, ECM modifying enzymes maintains a precise equilibrium between ECM synthesis and degradation. This equilibrium insures harmonic structure and function of every tissue including myocardium. However, if unconstrained, it leads to accumulation of ECM resulting in fibrosis or disruption of ECM network altering heart structure and function. As discussed above, the arterial wall consists of collagen types I and III, macrophages, and smooth muscle cells. Precursors released during biosynthesis of collagen type I and III such as carboxy-terminal or the amino-terminal propeptides (PICP, PINP, PIIICP, PIIINP) as well as during degradation such as carboxy-terminal or the amino-terminal telopeptide for type I and III collagen (CITP, NITP, CIIITP, PIIINP) have been studied as serological marker of ECM turnover in cardiac patients. A reduced serum level of CITP as well as increased level of CITP, PICP, and TIMP1 has been observed in hypertensive patients' suggesting both elevated synthesis and degradation occurs during high blood pressure [5–7]. Similarly, elevated levels of PIIINP and PICP (fibrosis) have been reported in patients with congestive heart failure and acute myocardial infarction (MI) [8]. These studies

vividly illustrate that dysregulation in ECM rebuilding and remodeling contributes to several pathological conditions as severe as heart failure and MI. Majorly, ECM remodeling is mediated by families of metalloproteinases viz., matrix metalloproteinase (MMPs), meprins, a disintegrin and metalloproteinase (ADAMs), and a disintegrin and metalloprotease with thrombospondin motifs (ADAMTS) [9, 10]. In addition, serine, cysteine, heparanases, sulphatases, and cathepsin proteases also degrade ECM resulting in the release of growth factors and cytokines. Alterations in the level of proteases may lead to different types of heart diseases. Therefore, regulating MMPs may prevent the onset or progression of disease.

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### 3 Matrix Metalloproteinases-Mediated Cardiac Remodeling

MMPs are the major class of proteases involved in ECM remodeling, therefore; playing a key role in biological (morphogenesis, angiogenesis, and wound healing) and pathological (tumor growth, arthritis, tissue ulceration, and cardiovascular disease) processes [11]. In 1962, a study on collagen remodeling during tadpole tail metamorphosis leads to the discovery of MMPs [12]. Since then, 25 vertebrate MMPs and 23 human homologues have been identified. Although human carries 24 MMP genes, chromosome 1 possesses two identical genes for MMP-23 making a total 23 MMP proteins [13]. On the basis of substrate specificity, domain organization and sequence similarity they are classified into four groups namely: (a) archetypal MMPs that includes collagenases (MMP-1, -8, -13), stromelysins (MMP-3, -10), and other archetypal MMPs (MMP-12, -19, -20, -27), (b) matrilysins (MMP-7, -26), (c) gelatinases (MMP-2, 9), and (d) furin-activatable MMPs such as secreted MMPs (MMP-11, -21, and -28), membrane-type MMPs (MMP-14, -15, -16, -17, -24, and -25), and type II transmembrane MMPs (MMP-23A and -23B). Generally, MMPs possesses four domains structure: a  $\sim$ 80-residue pro-peptide at the amino terminus, a  $\sim$ 165-residue catalytic domain with zinc localized at the active site, a  $\sim$ 200-residue hemopexin-like carboxyl-terminal domain linked to the catalytic domain by 15–65 residue linker region and a transmembrane domain. MMP-7 and MMP-26 lacks hemopexin domain whereas, MMP-23 has unique cysteine-rich, proline-rich, and IL-1 type II receptor-like domains instead of a hemopexin domain. Due to the interaction of a cysteine residue of the pro-peptide domain with the zinc ion of the catalytic site, the MMPs are proteolytically inactive [14]. The proteolytic activity of MMPs is strictly controlled and activated at three different levels: (a) transcriptional and posttranscriptional level (by several cytokines (IL-1, TNF- $\alpha$ ), growth factors (TGF- $\beta$ ), glucocorticoids, interferons), (b) zymogen activation (by other already activated MMPs, several serine proteinases or auto-proteolysis), and (c) by tissue inhibitors of metalloproteinases (TIMPs) [15, 16]. These regulatory measures insure the presence of MMPs in precise amount, at the right time and precise location, and in appropriate state (activated or inactive). However, cardiac or hemodynamic load

(hypertension), hyperhomocysteinemia, myocarditis, and RAAS-mediated cardiac stress alters the level of MMPs and their regulators leading to morphological, structural, and functional changes of the heart, referred to as cardiac remodeling. Cardiac remodeling includes degradation or deposition of extracellular matrix (ECM), myocardiocytes hypertrophy, abnormal angiogenesis, coronary collateralization, alterations in receptor signaling cascade, apoptosis, abnormal differentiation, and survival of cardiac stem cell, differential expressions of miRNAs and epigenetic modifications.

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## 4 Matric Metalloproteinases and Cardiovascular Diseases

In heart, the function of MMPs extends well beyond ECM degradation, such as, stimulation of multiple biological effects through liberating and mobilizing growth factors, cytokines [17]. Since all cardiac remodeling and myocardial architecture is associated with a change in the ECM composition; MMPs and their tissue inhibitors (TIMPs) are involved in the pathogenesis of a wide spectrum of cardiovascular disorders including atherosclerosis, restenosis, cardiomyopathy, congestive heart failure, myocardial infarction, and aortic aneurysm. Out of 23 MMPs identified in human, MMP-1, -2, -3, -7, -8, -9, -12, -13, -14, and TIMPs-1, -2, -3, -4 have been evaluated to respond to cardiac tissue repair stimuli [18]. The studies on patients tissue extracts with dilated cardiomyopathy revealed a decrease in MMP-1, -12, and increase in MMP-3 level [19, 20]. Similarly, post-MI studies on murine detected an increased level of MMP-8, -9, -13, and -14 [21–23]. Out of 23 MMPs, MMP-9 is surfaced as a leading candidate for having direct effects on cardiac remodeling. Its level increases post-MI, its inhibition improves post-MI outcomes and patients with the highest MMP-9 levels at baseline showed the greatest cardiovascular mortality [24]. A study on rat MI model also demonstrated time-dependent increase in relative TIMP-1 and TIMP-2 mRNA levels and decrease in TIMP-4 level [25]. Later, several clinical studies have utilized plasma samples to profile TIMP levels [26, 27].

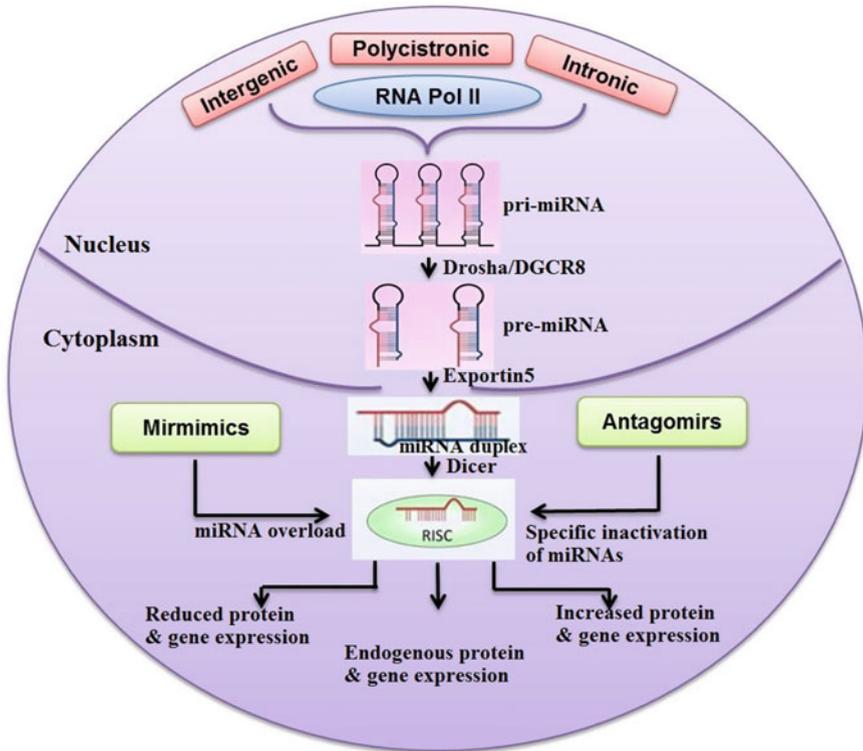
These observations have led to the recognition of MMPs and TIMPs as potential therapeutic targets through the development of pharmacological reagents. Previous studies revealed that MMP inhibition have reduced the accumulation of myocardial ECM (fibrosis), LV dilation as well as preserved cardiac function and geometry in ischemic cardiomyopathy [25]. A number of pharmacological MMP inhibitors such as trandolapril and ramipril studied in vitro as well as in rat model have found to control MMP level but at the expense of induction or reduction of other MMPs and TIMPS [28–30]. Lately, though peptides were found to be selective inhibitors of MMP-9 and MMP-2, their clinical usage is highly questionable because of their susceptibility to proteolysis [31]. In addition, clinical trials of other MMP inhibitors like PG-116800 failed to reduce cardiac remodeling or improve clinical outcomes after MI [32]. Therefore, novel molecular therapies have been investigated to more effectively treat or prevent CVDs. RNA-based epigenetics mechanisms including those controlled by small noncoding micro-RNAs (miRNAs) plays a pivotal role in

the regulation of cardiovascular diseases [33]. As a result, miRNAs have emerged as potential diagnostic and therapeutic tool for cardiovascular disease.

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## 5 Small Non-coding RNAs with Big Potential

The existence of miRNAs came into picture nearly two decades ago (1993) when Victor Ambros, Rosalind Lee, and Rhonda Feinbaum discovered that *lin-4*, a gene involved in development of *C. elegans* larvae does not code for protein but instead codes a pair of small RNAs [34]. These small RNAs are highly conserved, endogenously expressed, and noncoding RNAs that negatively modulate gene expression by either promoting the degradation of mRNA or downregulating the protein production by translational repression. They are transcribed from either intergenic, intronic, or polycistronic regions of the genome previously thought to be the junk region (~97%). Since their discovery, more than 2000 human miRNAs have been cataloged in mirBase (miRNAs database) (<http://www.mirbase.org>) predicted to regulate expressions of at least 30% of all human protein-encoding genes. The biogenesis of miRNAs is a complex process where every step is subjected to tight molecular regulation (Fig. 1). It begins within nucleus by transcription of miRNAs genes by RNA polymerase II into ~2000 nucleotides primary transcripts (pri-miRNAs) that can encode one or more miRNAs. A combination of Drosha (an RNase III enzyme) and Pasha also known as DGCR8 (an RNA-binding protein) cleaves primary miRNAs into precursor miRNAs (60–100 nucleotides) which gets transported to cytoplasm in a Ran/GTP/Exportin-5-dependent manner. Once in the cytoplasm, another cleaving enzyme Dicer (RNase III) modifies the precursor miRNAs and generates double-stranded miRNA duplexes of 19–23 nucleotides containing functional guide strand and a passenger strand. The duplex miRNA gets incorporated into RNA-induced silencing complex (RISC) by associating with Argonaute proteins (Ago). The passenger strand gets degraded while the guide strands remains intact and binds the 3' untranslated region (UTR) of target messenger RNA (mRNA). Pairing across all 22 nucleotides rarely occurs in mammalian cells instead, the seed region of the miRNA (nucleotides 2–7 or 8) appears most important for targeting mRNAs. If the base pair at 3' UTR matches perfectly, the mRNA gets cleaved and degraded by Ago2-RISC complex. Otherwise, it causes downregulating of protein production through translational repression [35, 36]. miRNAs not only target single gene but often functionally related genes networks thus maintain optimal amount of cellular proteins and hence play a crucial role in the regulation of biological functions. In human cardiac tissue, miRNAs have been identified at all stages of development (for example, miR-1, miR-16, miR-27b, miR-30d, miR-126, miR-133, miR-143, miR-208, and the let-7 family) [37]. Our previous study has characterized several known and novel miRNAs in chicken cardiac tissue at all stages of development. Using next-generation sequencing, we identified a total of 1056 microRNAs, out of which 353 were known (for example, let-7, miR-140, miR-181, miR-30,



**Fig. 1** Schematic representation of miRNA biogenesis as well as therapeutics. Inside nucleus, either of the intergenic, polycystronic or intronic regions of DNA gets transcribed by RNA Polymerase II (RNA Pol II) into nearly 2 kb long primary transcripts (pri-miRNAs). Drossha/DGCR8 cleaves pri-miRNAs into precursor miRNAs (pre-miRNA) of 60–100 bp which gets transported to cytoplasm by exportin 5. In cytoplasm, Dicer modifies pre-miRNA into double-stranded miRNA duplexes. One of the strand (guide strand) activates the RNA-induced silencing complex (RISC) which in turn causes translational repression as well as mRNA cleavage. mirmimics (*left*) activates RISC and reduce protein and gene expression while, antagomirs (*right*) reduce RISC activation resulting in translational repression/mRNA cleavage and in the end increase protein and gene expression

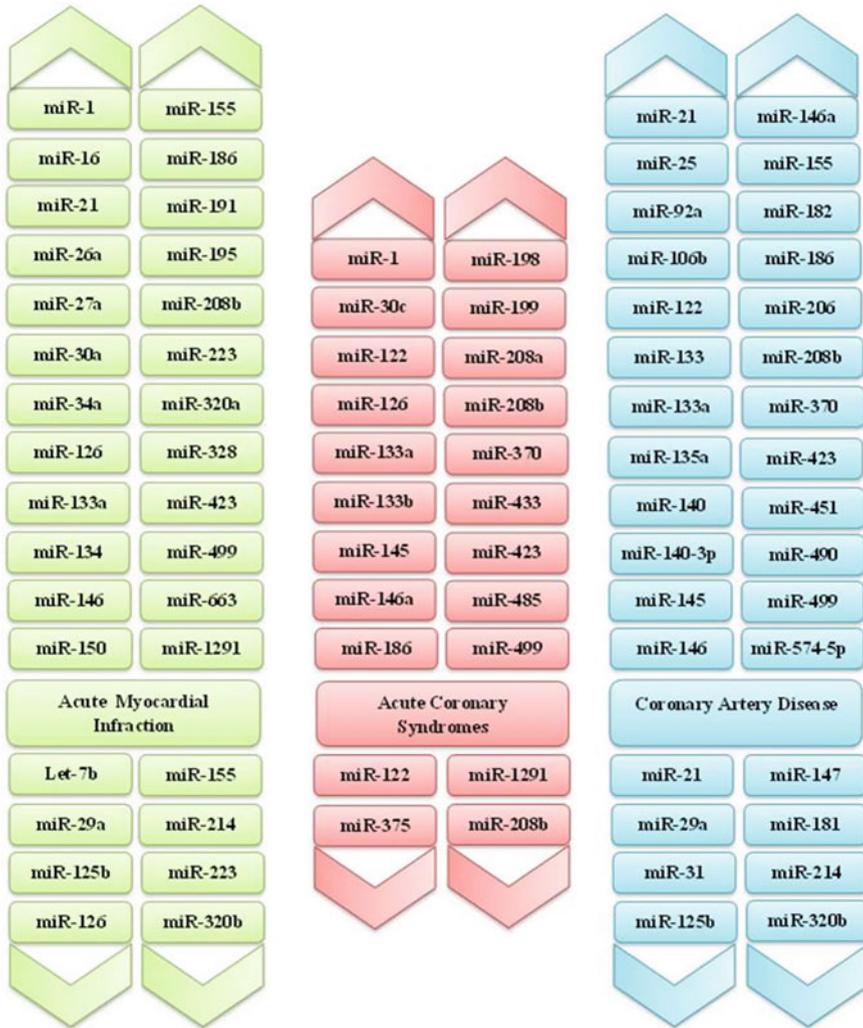
miR-205, miR-103, and miR-22) while 703 were novel miRNAs [38]. These finding implicate that the miRNAs are highly expressed in non-diseased cardiac tissue from embryogenesis to adult life and play a key role in both normal cardiac maintenance and disease. The role of miRNAs in cardiac remodeling of the adult heart was further established with Dicer deletion through the use of a tamoxifen-inducible Cre recombinase in the postnatal murine myocardium. In juvenile, it lead to premature death within 1 week due to atrial enlargement and mild ventricular remodeling whereas, in adult, it induced myocyte hypertrophy, fibrosis, biventricular enlargement, and induction of fetal transcription [39]. These phenotypes were consistent with the defects during heart development observed in zebrafish embryos devoid of Dicer

function [40]. Later, another study came up with the similar outcome but by deletion of the DiGeorge syndrome critical region gene 8 (DGCR8 instead of *dicer*) using muscle creatine kinase-Cre mice and a conditional floxed allele of the DGCR8 [41]. MicroRNA expression profiling studies revealed that, the 18 most strongly expressed miRNAs and miRNA families account for >90% of all miRNA expressed in the adult mouse heart. Surprisingly, most of them are differentially expressed during cardiac disease, indicating the importance of miRNAs as modifiers of gene expression programs in cardiovascular disease. However, not all differentially expressed miRNAs are implicated in the cardiac disease. For example, miR-214, which is most highly upregulated among the 24 miRNAs studied, failed to induce cardiac phenotypes in transgenic mice in ischemic cardiomyopathy, dilated cardiomyopathy, and aortic stenosis, [42]. Whereas, ablation of miR-133 via double knockout resulted in aberrant proliferation and apoptosis of myocytes, cardiac defects, suggesting their therapeutic application in heart disease [43].

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## 6 Role of microRNAs in Cardiovascular Diseases

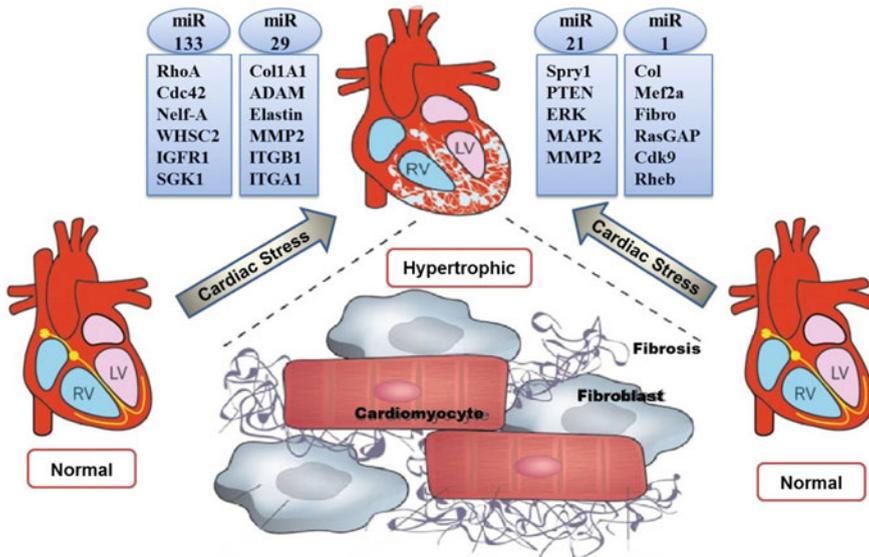
miRNAs are highly stable in blood circulation due to their incorporation into microvesicles, exosomes, their association with RNA-binding proteins, and their adherence to circulating lipoproteins. These protective mechanisms enable miRNAs to fulfill biological functions outside the cell and serve as potential prognostic or diagnostic biomarkers in CVD pathologies [44]. Because of their nucleic acid nature they can be detected with high sensitivity and specificity using various molecular techniques such as microarrays and quantitative real-time PCR. Previous studies have shown a number of miRNAs to be unregulated in plasma of cardiovascular disease patients (Fig. 2). miR-208b and miR-499 were highly elevated (1600-fold and 100-fold respectively) in plasma from acute myocardial infarction (AMI) patients as compared with patients presenting with atypical chest pain without AMI [45]. Similarly, elevated miR-208a was detected in plasma of ~90% AMI patients whereas; it was undetectable in patients with non-AMI coronary heart disease or patients with other cardiovascular diseases [46]. However, unlike miR-208a which is specifically expressed in the heart, not all miRNAs are tissue specific. For example, miR-208b and miR-499 are also expressed in skeletal muscle therefore confounding the interpretation of miRNAs as diagnostic biomarkers in cardiac diseases [47]. Although recent advances in prognostics and/or diagnostics have increased the survival rate of patients with AMI, no significant change in mortality rates in patients who survive the initial AMI is observed. In a recent cohort study of 7733 AMI patients, 71% of AMI survival developed heart failure within 5 years [48]. Therefore, an expanded understanding of the function of miRNAs in gene regulatory networks engaged in MMP mediated cardiac remodeling is looked upon as a novel therapeutic strategy for CVD.



**Fig. 2** Overview of upregulated and downregulated miRNAs in acute myocardial infarction (AMI), acute coronary syndromes (ACS) and coronary artery disease (CAD). Information is extracted from multiple studies included in references section [37, 42, 78, 80, 81]

## 7 microRNAs-Mediated Matrix Metalloproteinases Regulation

Recent studies have predicted/validated multiple gene targets of several miRNAs implicated in the pathophysiology of cardiovascular diseases [49] (Fig. 3). However, a major deficiency in the available literature on miRNA alterations occurring



**Fig. 3** Schematic representation of miRNAs, involved in development of cardiac hypertrophy and myocardial fibrosis. Cardiac stress leads to the dysregulation of miRNAs (few of them are represented: miR133, miR29, miR21 and miR21) as well as their target genes/proteins (few of them are represented). *Rho A* Ras homolog gene family, member A, *Cdc42* Cell division control protein 42, *Nelf-A* Negative elongation factor A, *WHSC2* Wolf–Hirschhorn syndrome candidate 2, *IGFR1* Insulin-like growth factor receptor 1, *SGK1* Serum-and glucose- regulated kinase, *Col1A1* Collagen type 1  $\alpha$ 1, *ADAM* A disintegrin and metalloproteinase, *MMP-2* Matrix metalloproteinase-2, *ITGB1* Integrin beta-1, *ITGA1* Integrin Alpha 1, *Spry1* Sprouty RTK Signaling Antagonist 1, *PTEN* Phosphatase and tensin homolog, *ERK* Extracellular signal-regulated kinases, *MAPK* Mitogen-activated protein kinases, *Col* Collagen, *Mef2a* Myocyte Enhancer Factor 2A, *Fibro* Fibronectin, *Ras-GAP* GTPase-Activating Protein, *Cdk9* Cyclin-dependent kinase 9, *Rheb* Ras homolog enriched in brain

during cardiovascular diseases is that very few studies have assessed miRNA/MMP interactions. Since all the MMPs have not been explored to the same intensity, especially in the context of miRNA/MMP interactions as it relates to onset and progression of cardiovascular diseases, this part of discussion is skewed in favor of those miRNAs/MMPs and ECM components where the literature is more abundant. Roy et al. published the first report showing that miR-21 regulates MMP-2 expression in cardiac fibroblasts in response to myocardial ischaemia–reperfusion via PTEN (phosphatase and tensin homologue) pathway [50]. Later, Chen et al. found that miR-29b epigenetically regulates MMP-2/MMP-9 genes expression in the context of atherosclerosis. They observed that oxidized low-density lipoprotein (oxLDL) reduces methylation (DNA methyltransferases) of the MMP-2/MMP-9 genes via upregulation of miRNA-29b leading to the increase MMP-2/MMP-9 gene expression which is known to have a profound influence on cardiovascular diseases [51]. The member of miR-29 family suppresses the expression of several ECM proteins and has been predicted to downregulate during myocardial infarction.

Abonnenc et al. confirmed the role of miR29 in ECM remodeling through pre-miR-29b and anti-miR-29b transfection in murine cardiac fibroblast (CF) [52]. Based on CF secretome data, they concluded that pre-miR-29b treatment reduced the collagen and MMP-2 secretion while anti-miR-29b did not stimulate their secretion [52, 53]. Lately, Chen et al. examined miRNA changes in different heart-cell compartments using congestive heart-failure experimental model. They observed that with decrease expression of miR-29b, the expression of MMP-2 did not change significantly in fibroblasts of left atrial. However, with increases expression miR-21 in left ventricular fibroblasts, the expression of MMP-2 did change [54]. These studies clearly suggest that aberrant posttranscriptional regulation of matrix metalloproteinases (MMPs) by miRNA is an important factor in cardiovascular diseases. Considering, the possibility of exploiting small noncoding RNAs as therapeutic tool for cardiovascular pathology, few studies used exogenous small interfering RNA (siRNA) which follow the same mechanism as miRNA to targets MMP-2 and -9. As described previously, MMP-2 is known to involve in various vascular complications including plaque destabilization and restenosis. Due to its constitutive expression by vascular smooth muscle cells, MMP-2 can facilitate vascular SMCs migration through hydrolysis of numerous extracellular matrix components leading to atherosclerosis. Transfection of vascular smooth muscle cells with MMP-2 siRNA has significantly decreased MMP-2 gene expression, resulting in the suppression of cell migration [55]. Similarly, atherosclerotic plaque stability has been obtained through silencing of MMP-9 gene using MMP-9 siRNA in apolipoprotein E (ApoE)-/- mice, suggesting that miRNAs and/or siRNAs represents potential pharmacological tool to target MMPs and their associated proteins [56].

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## 8 microRNA Research and Therapeutics

Despite tremendous efforts in the traditional approach of drug design involving enzymes, cell surface receptors, increasing number of mortality and morbidity due to cardiovascular disease poses a great challenge to the therapeutic strategy adopted until now. Because of their specificity to their targets in a particular cellular pathway, miRNAs are rapidly becoming the most promising pharmacological tool to diagnose and treat cardiovascular disease. Therefore, it is necessary to understand methods, material, and tools required to resolve complex findings of miRNA research. miRNAs represent only about 0.01% of total cell RNA requiring extremely precise and sensitive instruments often with additional purification and enrichment steps. For visualization of individual pre-miRNA or mature miRNA expression in cells or tissues, quantitative real-time PCR (qRT-PCR), northern blotting, and in situ hybridization (ISH) in combination with immunohistochemistry are widely used. Northern blotting and ISH are very simple yet sensitive techniques but consumes relatively more time and sample since they do not involve miRNA amplification. qRT-PCR represents a balance of cost, precision, and sample size

using modified way of reverse transcribing individual miRNA from a pool of miRNAs present in isolated total mRNAs. For screening of genome-wide miRNA expression, high-throughput molecular biology techniques such as miRNA microarray, deep sequencing are followed by validation with qRT-PCR. While miRNA microarray and qRT-PCR are two of the most common methods for evaluating known miRs, there are still contrasting reports on the correlation between microarray and qRT-PCR [57, 58]. To study novel miRNAs whose complement has not been ascertained, a more sensitive technique for small RNA sequences, which provides absolute abundance values of miRNAs is next-generation sequencing. It is known that a specific miRNA can regulate the expression of multiple genes (targets) while the expression of individual gene can be regulated by multiple miRNAs [59]. Therefore, for a comprehensive understanding of miRNA function and potential therapeutic use in heart disease, identification and validation of miRNA targets is of fundamental importance. There are several *in silico* target prediction programs such as TargetScan, PicTar, MiRanda/mirSVR, miRBase/MicroCosm, RNA22, and PITA. These databases use common feature like complementarity between the 3' UTR of the target mRNA and the 5'-seed of the miRNA, conservation among species, the presence of several miRNA target sites and pattern recognition [60–62]. The predicted target needs to be further validated which can be achieved using biochemical methods, proteomic/transcriptome analysis, and RISCome analysis. Biochemical methods include qRT-PCR, western blotting, reporter assays, hybrid PCR, cytoplasm/nucleus ratio of mRNA, affinity purification, biotin-tagged miRNAs, and labeled microRNA pull-down assay (LAMP) [63–68]. Proteome analysis includes stable isotope labeling with amino acids in cell culture (SILCA) along with mass spectrometry to identify miRNA–mRNA interactions [69, 70]. Lastly, RISCome analysis includes target identification by sequencing of RNA-induced silencing complex and Argonaute. Other novel high-throughput assays based on immunoprecipitation are RIP-Chip (RNA-Binding Protein Immunoprecipitation-Microarray Profiling), PAR-CLIP (Photoactivatable-Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation), and HITS-CLIP (High-throughput Sequencing of RNAs isolated by cross-linking immunoprecipitation [46, 71–76]. Not all these methods have been applied for cardiovascular research yet, but since miRNA research is a growing field, these methods could be adapted because of their accurate and quantitative estimation of miRNA profiles and expression.

The general strategy of therapy for any disease is to reverse the pathological changes that resulted due to disease. Therefore, those MMPs and miRNAs whose overexpression is responsible for cardiovascular disease should be suppressed and vice versa. The suppression of overexpressed MMPs/miRNAs can be achieved by miRNA mimic technology (miR-mimic) which is an approach for gene silencing by generating artificial double-stranded miRNA-like RNA fragments. These RNA fragments mimic endogenous miRNAs and bind specifically to its target mRNA activating the RNA-induced silencing complex (RISC), downregulating specific mRNAs and thus induce gene suppression [77]. On the contrary, the expression of MMPs can be induced by anti-miRs (cholesterol-conjugated, 2-O-methyl-modified

antimiRs, called antagomiRs), which are a class of chemically engineered oligonucleotides specifically silencing single endogenous miRNA (Fig. 1). They competitively inhibit specific miRNA by binding to the target mature miRNA and lead to a reduced activation of RISC and consequently to an upregulation of specific mRNA and gene expression [78]. As mentioned above, few studies have demonstrated the value of mimic in downregulating the expression of MMP-9 in mouse model of atherosclerosis as well as *in vitro*. Besides, to promote miRNAs as a viable therapeutic target there are several difficulties to overcome such as, difference in predicted and actual physiological targets, potential off-target effects of antimiRs, and global effect on ECM of other organs. Further studies are required to elucidate the exact methods by which miRNAs are able to repress translation and initiate mRNA degradation of selected targets. The recent success of the first human clinical trial of a miravirsin (SPC3649) therapeutic for suppression of hepatitis C virus (HCV) replication has raised possibilities for the use miRNAs as a therapeutic target in CVD and clinical trials are eagerly awaited [79].

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## 9 Conclusion

Recent studies have established a cause-and-effect relationship between the induction and activation of MMPs and cardiovascular diseases raising great expectations for MMPs as promising target. Also, miRNAs targeting MMPs and/or their regulators, as directly or indirectly discussed above are sufficient to draw a plausible inference that miRNA regulation of MMPs expression is an important mechanism for causing CVD. Since the activity of MMPs relies upon several complex interconnected molecular interactions, many clinical trials of MMP inhibitors failed to improve clinical outcomes. miRNA-based therapeutics under pre-clinical trials have shown promising results in numerous animal models of CVD such as cardiac hypertrophy, fibrosis, and MI. Till now, the role of less than 12 MMPs have been investigated post-myocardial infarction and very few MMPs have been predicted to be the targets of miRNAs. Therefore, extensive studies analyzing the complex interactions between specific miRNAs and MMPs in context with CVD will definitely bridge this gap and provide novel opportunities for diagnosis and therapy of CVD.

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