

Exosomal miRNAs in Heart Disease

Micro-RNAs (miRNAs) are small noncoding RNAs involved in the posttranscriptional regulation of gene expression. Exosomes have recently emerged as novel elements of intercellular communication in the cardiovascular system. Exosomal miRNAs could be key players in intercellular cross-talk, particularly during different diseases such as myocardial infarction (MI) and heart failure (HF). This review addresses the functional role played by exosomal miRNAs in heart disease and their potential use as new biomarkers.

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Cardiovascular diseases (CVDs) are the major cause of death worldwide, particularly in the elderly population. The Global World Health Organization (WHO) reported that 17.5 million people died from CVDs in 2012, accounting for 31% of all deaths, which is more than all of the other causes of death combined and double the number of deaths from cancer (51), despite the fact that continuous improvements have profoundly improved healthcare quality in recent years. Hence, a better comprehension of the fine mechanisms underlying CVDs could propel the development of novel and more efficient therapeutic strategies.

The heart is composed of diverse cellular components, including fibroblasts, cardiomyocytes, endothelial cells, and smooth muscle cells. In addition, there are also resident stem cells and transient cell types such as lymphocytes, mast cells, and macrophages. All of these cell types contribute to structural, biochemical, and electrical properties of the functional heart (2). A number of studies have demonstrated the existence of a complex network of well organized interactions between different cell populations. In fact, intercellular interactions play a pivotal role in several pathophysiological processes through different aspects such as direct cell-to-cell contact, cell-matrix interaction, and extracellular electric, chemical, or biological signals (69, 62, 43).

Exosomes are endogenous nano-vesicles (30–100 nm) that have been shown to carry biological information and modulate signaling pathways in target cells (47). A key role in CV pathophysiology has been credited to exosomes, which may participate as mediators of intercellular communication by delivering different types of signal molecules such as proteins and RNAs to recipient cells (64, 83).

Micro-RNAs (miRNAs) are small, noncoding RNA molecules that regulate gene expression at the posttranscriptional level (34). Our research group and others have recently studied the involvement of miRNAs in the development and progression of cardiovascular diseases (71, 35, 10, 33, 70, 66, 74).

In addition to their role in regulating gene expression, miRNAs can be used as potential novel biomarkers for determining different disease statuses. In fact, several groups have reported that circulating miRNAs are stable and that their expression profile can change under a great variety of physiological and pathological conditions, which make them promising candidate biomarkers (57, 38, 61). This review summarizes the current understanding of the functional role played by exosomal miRNAs in heart disease and their potential application as clinical biomarkers.

Exosomes as a Carrier of miRNAs

miRNAs are endogenous, small, highly conserved noncoding RNA molecules that have emerged as fundamental posttranscriptional regulators of gene expression (12, 87). miRNAs function by imperfectly base-pairing with target mRNAs to negatively impact their expression. Genes encoding miRNAs are transcribed by RNA polymerase II (Pol-II) and generate stem-loop primary transcripts (pri-miRNA) that undergo a series of cleavage events by components of the nucleus and cytoplasm to yield mature miRNA. In the nucleus, pri-miRNA is cleaved by the microprocessor complex formed by the enzyme Drosha and the RNA-binding protein DGCR8 to yield a 70-nt hairpin precursor miRNA (pre-miRNA) that is then exported to the cytoplasm by Exportin-5. In the cytoplasm, pre-miRNA is further processed by Dicer to yield ~22-nucleotide miRNA/miRNA*(star) duplexes, where miRNA is the guide strand and miRNA* is the passenger strand. Finally, Argonaute (AGO) proteins recruit miRNA duplexes to form a single-stranded miRNA, which is subsequently incorporated into the RNA-induced silencing complex (RISC). However, miRNA can also be transcribed by RNA polymerase III (25), and non-canonical pathways for miRNA biogenesis have also been described (30).

Since their discovery, miRNAs have provided a new perspective for understanding the molecular

mechanisms that underlie a range of cardiovascular disorders.

It has been shown that miRNAs are aberrantly expressed in the cardiovascular system under some pathological conditions, including myocardial infarction (19, 45), heart failure (6, 77), cardiac hypertrophy (36, 44), and proliferative thickening of the vessel known as restenosis (15, 55).

Interestingly, miRNAs have recently been discovered in extracellular vesicles, which are small, membrane-derived particles classified by size, origin, and function. Exosomes represent a well characterized subtype of secreted extracellular vesicles. Exosomes, endogenous nanovesicles of 30–100 nm in diameter (7, 58), are secreted by multiple cell types into the extracellular space after fusion with the plasma membrane.

Although the biogenesis of these extracellular vesicles is complex and incompletely understood, evidence indicates that they are originally formed by endocytosis.

Initially, the formation of early endosomes (EEs) originates from primary endocytic vesicles that fuse with each other (68). EEs then mature into late endosomes (LEs) by changes in their protein composition and formation of intraluminal vesicles (ILV) inside the lumen of the endosome (50). The LEs contain these multivesicular ultrastructures that are also called multivesicular bodies (MVBs). Finally, MVBs may fuse with the plasma membrane releasing its vesicles (exosomes).

This mechanism facilitates the transfer of exosomes containing various molecules including bioactive proteins, lipids, DNA, mRNAs, and miRNAs carrying biological information. The heterogeneous molecular contents of exosomes have been shown to function as signaling molecules that influence target cell biology (5).

Exosomes are constitutively released from many cardiac cell types, including cardiomyocytes, fibroblasts, endothelial cells, and resident stem cells. A growing body of evidence suggests that exosomes derived from cardiac cells are responsible for cell-to-cell communication in the heart under both physiological and in pathological conditions (60, 54, 64).

It has been shown that exosomal miRNAs can target mRNAs in recipient cells (73, 31). This adds a new level of complexity to the role played by miRNAs in cardiovascular diseases. **FIGURE 1** depicts miRNA biogenesis and their sorting into exosomes.

Exosomal miRNAs and Cell-to-Cell Communication in Heart Disease

The role played by exosomal miRNAs in different pathological settings of heart disease is still incom-

pletely understood. However, growing evidence is being reported on their key involvement in important disease processes. **FIGURE 2** summarizes the role of exosomal miRNAs in cardiac cell communication in the heart.

Pathological remodeling of the heart is responsible for the progression toward heart failure (HF). It typically features an increase in cardiomyocyte size and myocardial fibrosis (40). Cardiomyocytes and cardiac fibroblasts, the most prevalent cell types in the heart, play a key role in cardiac remodeling.

Fibroblasts can influence cardiomyocytes through direct cell-to-cell contact and autocrine or paracrine factors (39, 65, 75). For example, hypoxia modulates the expression profile of several paracrine factors released by fibroblasts with a direct impact on cardiomyocyte phenotype (63). In addition, cardiomyocytes show cellular hypertrophy and electrophysiological alterations when treated with conditioned fibroblast media (20, 75). Altogether, these findings strongly support the idea that cardiac fibroblast-derived biological signals mediate intercellular communication in specific diseases and are active partakers in the pathophysiological mechanism underlying progression in the heart.

The release of extracellular vesicles, such as exosomes, with interaction and uptake by recipient cells is a common way of communication between cardiac fibroblasts and cardiomyocytes. However, less information is available on which biological signal is carried around in exosomes (73). Among the possible options, one interesting hypothesis is that exosomes carry well defined quantities of miRNAs that transport specific biological information from one cell to the other. This intriguing possibility is gaining increased support by recent experimental evidence. For example, Bang and colleagues (3) demonstrated a cross-talk between cardiac fibroblasts and cardiomyocytes via exosomal miRNAs. In that study, analysis of small RNAs from fibroblast-derived exosomes by deep sequencing revealed that cardiac fibroblasts retain many miRNAs passenger strands (miRNAs*), which usually undergo intracellular degradation. Among these, miR-21* had been previously found to be highly expressed in failing human hearts (82). The authors demonstrated that cardiac fibroblast selectively package miR-21* into exosomes that are actively secreted. Most interesting, these miR-21*-enriched exosomes can reach cardiomyocytes and, once taken up, can enhance cellular hypertrophy via repression of the SH3 domain containing 2 (SORB2) and PDZ and LIM domain 5 (PDLIM5) target genes. Interestingly, the authors were able to obtain a significant improvement in cardiac function and a parallel regression of hypertrophy in a

mouse model of ang-II-induced cardiac hypertrophy with left ventricular pressure overload and through antagonization of miR-21*. In contrast, another research group recently found that overexpression of miR-21* resulted in inhibition of cardiac hypertrophy by suppressing HDAC8 expression (81). Hence, further studies are necessary to define the role of miR-21* in the hypertrophy-specific signaling pathway.

Several groups have shown that cardiac endothelial cells play key roles in heart biology and diseases through dynamic interactions with different cardiac cell types. For example, endothelial cells can interact with fibroblasts and cardiomyocytes through gap junctions, cell surface mole-

cules, and release of a variety of auto- and paracrine agents, which directly influence the cardiac function of the adult heart (24, 48, 69). Cardiac endothelial cells also contain different miRNAs involved in intercellular communication. It has been demonstrated that endothelial cells can secrete exosomes (31) and uptake exosomes secreted by other cell types.

A recent study by Halkein et al. (29) reported the presence of signaling between myocytes and the endothelium mediated by exosomal miRNAs. They showed that a proteolytic fragment of the full-length, 23-kDa PRL polypeptide, termed 16K PRL, stimulated endothelial cells to release miR-146a-loaded exosomes. These miRNA-containing vesi-

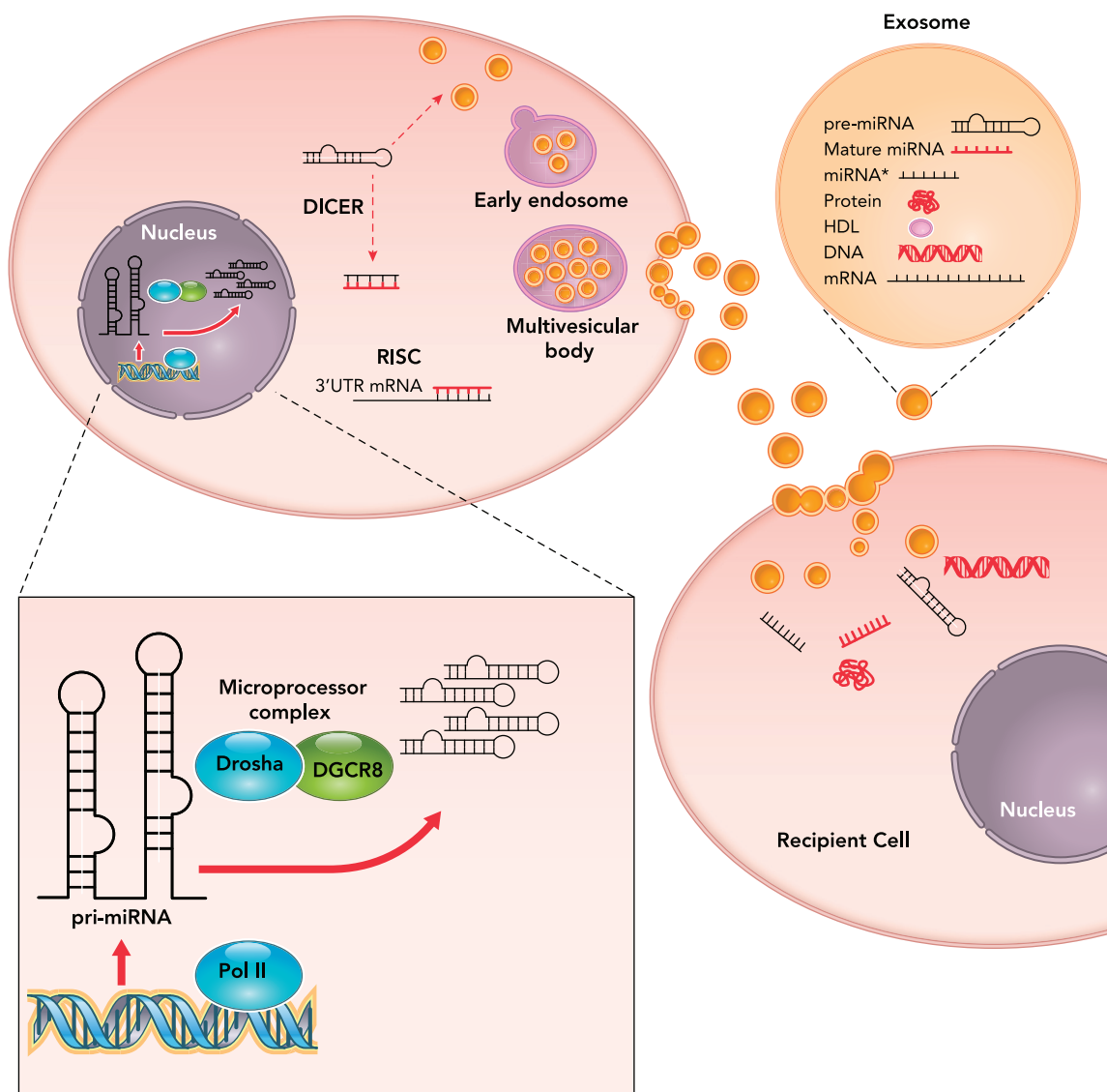


FIGURE 1. Biogenesis of miRNAs and their sorting into exosomes

Genes encoding miRNAs are transcribed by RNA polymerase II and are processed in the nucleus by the microprocessor complex into pre-miRNAs. In the cytoplasm, pre-miRNAs are cleaved by Dicer to yield mature miRNA duplexes. The functional strand of mature miRNA is incorporated into the RNA-induced silencing complex (RISC). In the cytoplasm, pre-miRNAs, mature miRNAs, and miRNA* can also be incorporated into exosomes, which are derived from the internal ultrastructure of multivesicular bodies (MVBs). Exosomes are released to the extracellular space when intracellular MVBs fuse with the plasma membrane.

cles can be transferred from endothelial cells into neighboring cardiomyocytes, leading to a subsequent decrease in metabolic activity and decreased expression of specific miR-146a-target mRNAs. The 16K PRL is an antiangiogenic peptide that has been discovered as an important factor in initiating and driving peripartum cardiomyopathy (PPCM). Interestingly, knockdown of miR-146a in STAT3 conditional knockout mice by infusion of an anti-sense RNA oligonucleotide appeared sufficient to induce significant attenuation of PPCM clinical features compared with saline-treated mice.

A very recent report by the Ong et al. (52) analyzed the exosome-mediated cross-talk between ECs and transplanted CPCs in a mouse model of MI. The authors showed that co-delivery of CPCs with a plasmid carrying hypoxia-inducible factor-1 (HIF1) into the ischemic myocardium could improve the survival of transplanted CPCs. Using in vitro studies, these authors observed that cardiac ECs produced exosomes, which were actively internalized by recipient CPCs. Interestingly, Ong et al. showed that exosomes from cardiac ECs that

overexpress HIF1 contained miR-126 and miR-210, suggesting that miR-126 and miR-210 can be transferred via exosomes from ECs to CPCs where they modulate the expression of the pro-survival kinases and induce a glycolytic switch, which eventually leads to an increase in survival of the transplanted CPCs. In fact, when the effects of the miR-126 and miR-210 were inhibited, the protective effects on CPCs induced by the exosomes were prevented. Hence, these findings indicate that the exosomal communication between ECs and CPCs is crucial in restoring and maintaining optimal function of damaged cardiac tissue after acute myocardial infarction and could be a potential target in cell therapy.

Recent evidence suggests a role for cardiomyocyte-derived exosomes in mediating signal transduction to target cells. Gupta et al. (28) were the first to report that adult cardiomyocytes release exosomes. In fact, they showed that exosomes released by primary cultures of adult rat cardiomyocytes under hypoxic conditions were loaded with a large amount of heat shock protein (HSP) 60,

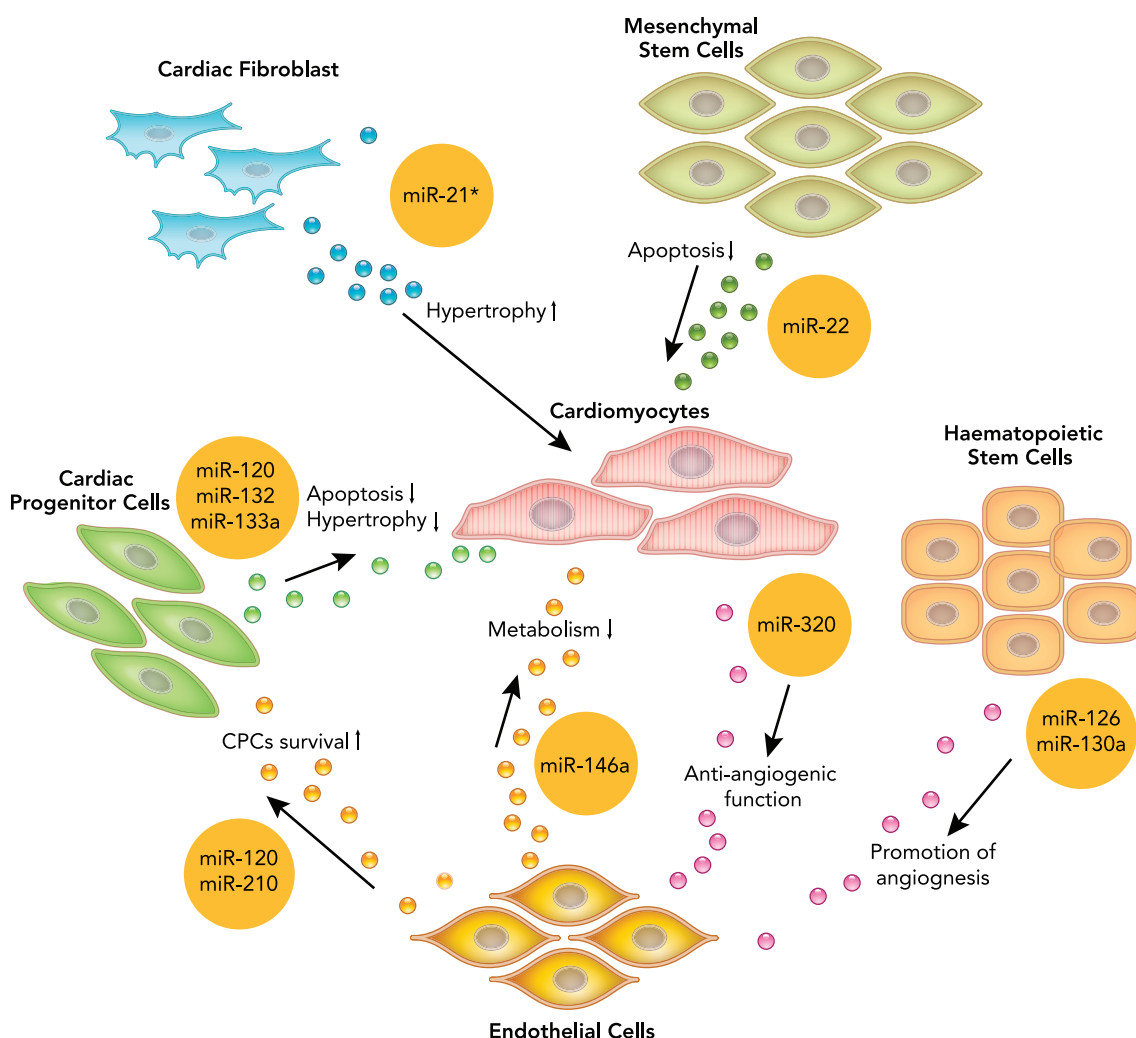


FIGURE 2. Exosomal miRNAs in cardiac cell-cell communication

A summary of exosomal release of miRNAs from different cell types in the heart and their functional effects.

which is involved in mediating Toll-like receptor 4-induced cardiomyocyte apoptosis. Cardiomyocyte-derived exosomes were also shown to carry tumor necrosis factor- α (84), HSP20 (86), and several nucleic acids. These findings support the concept that exosomes could mediate the cross-talk between cardiomyocytes and other cardiac cell types within the heart. More recent studies reported miRNA content in exosomes generated by cardiomyocytes. As examples, Wang et al. (79) recently reported that functional miRNAs delivered through exosomes released from cardiomyocytes are crucial for the development of diabetes mellitus-induced myocardial vascular deficiency. This study provides evidence that cardiomyocytes from Type 2 diabetic Goto-Kakizaki (GK) rats can negatively regulate endothelial cell proliferation and migration by means of specific miRNAs transferred thorough exosomes. The authors showed that exosomes derived from diabetic cardiomyocytes contain higher levels of miR-320. Interestingly, this miRNA secreted by cardiomyocytes into exosomes can be transferred to endothelial cells, which leads to a reduction in the expression levels of specific targets such as heat-shock protein 20 (HSP20), insulin-like growth factor 1 (IGF-1), and transcription factor ETS2. Altogether, this study suggests that cardiomyocytes could exert an anti-angiogenic effect through the release of miR-320-enriched exosomes in diabetes.

Despite the still incomplete understanding of all mechanisms, several groups have independently demonstrated that stem cells can exert a beneficial effect on cardiac function (53, 17, 16, 56, 23). However, it is increasingly evident that the role of adult stem cells in cardiac tissue repair is related, at least in part, to the release of a variety of paracrine factors (22, 67, 14). More recently, exosomes derived from different types of stem cells displayed therapeutic effects in heart disease models. Specific sets of miRNAs were found in exosomes released from hematopoietic stem cells (HSCs), cardiac progenitor cells (CPCs), or ESC-derived mesenchymal stem cells (MSCs). Among the others, MSCs are particularly interesting because their ability to differentiate into cardiovascular cells together with their paracrine action makes them a promising source for heart repair.

A recent study reported the release of exosomes with a specific pattern of miRNAs from ischemic-preconditioned MSCs (18). Briefly, the authors performed a microarray profile analysis of miRNAs in exosomes released from MSCs subjected to ischemic preconditioning and showing that levels of miR-22, miR-21, miR-210, miR-199a-3p, and miR-24 were significantly modulated in response to treatment. Using a co-culture system, the authors further demonstrated that exosomes derived

from ischemic-preconditioned MSCs can fuse with cardiomyocytes, releasing their miRNA content and resulting in protection from ischemic injury. Indeed, *in vivo* injection of exosomes released from ischemic-preconditioned MSCs resulted in significant prevention of the reduction of cardiac fibrosis and apoptosis in response to myocardium ischemia in a mouse model.

A pool of tissue-specific resident cardiac stem cells, known as cardiac progenitor cells (CPCs), was identified in the adult human myocardium.

Barile et al. (4) recently showed that culture medium from these CPC cells can protect HL-1 cardiomyocytes from starvation-induced apoptosis and induce tube formation in HUVECs. In particular, they showed that CPC-derived exosomes were enriched with several cardioprotective and proangiogenic miRNAs, such as miR-210, miR-132, and miR-146a-3p, compared with exosomes secreted by normal human dermal fibroblasts. Among the others, they found that miR-210 reduced cardiomyocyte apoptosis, inhibiting expression of ephrin A3 and PTP1 (its known targets). Moreover, the authors showed that the proangiogenic effect of CPC-conditioned medium is, at least in part, mediated by miR-132. Interestingly, the intracellular concentration of miR-210 and miR-132 was markedly increased by exposure to CPC-derived exosomes. Moreover, the same beneficial effects were confirmed in a rat model of myocardial infarction; the author found that treatment with CPC-derived exosomes improved recovery of cardiac function (4).

Similarly, CPC-derived exosomes carrying miR-133a were able to reduce cardiac hypertrophy and cardiomyocyte apoptosis in murine models of acute myocardial infarction (37).

In line with these studies, Gray and colleagues (27) showed that CPCs secrete pro-regenerative exosomes in response to hypoxia. Treatment with exosomes secreted from CDCs under hypoxia significantly enhanced tube formation by cardiac endothelial cells and reduced the expression of fibrosis-associated genes in TGF- β -stimulated fibroblasts. Furthermore, exosomes from hypoxic CPCs improved cardiac function and reduced fibrosis in a model of ischemic injury in mice, which confirms the pathophysiological relevance of this mechanism. Using an miRNA array, the authors found a significant increase in the level of a subset of 11 miRNAs in exosomes secreted from CPCs. A principal component analysis (PCA) of the selected miRNAs identified four unique miRNA clusters, which seem to be correlated with a biological effect resulting in therapeutic benefits. The miRNA signature, if validated in other prospective studies, could have important implications for cell therapy.

Table 1. Design considerations for cardiovascular tissue engineering

Disregulated miRNAs	Disease Type	Study Design	Reference
miR-192, -194, -34a	HF	65 healthy subjects; 21 patients post-AMI	46
miR-1, -133a	ACS	42 patients non-ACS; 29 patients with ACS	41
miR-146a	PPCM	18 postpartum controls; 30 patients with dilated CM; 38 patients with acute PPCM	29
miR-423-5p, -320, -22, -92b	HF	30 controls; 30 patients with chronic HF	26

HF, heart failure; ACS, acute coronary syndrome; PPCM, peripartum cardiomyopathy; AMI, acute myocardial infarction; CM, cardiomyopathy.

It has been observed that the transplantation of CD34+ peripheral blood-derived hematopoietic stem cells improved cardiac function in both animal models and human patients. CD34+ cells were also shown to promote therapeutic angiogenesis in animal models of myocardial ischemia. The exact mechanism by which CD34+ cells improve therapeutic angiogenesis is not completely understood, although evidence supports paracrine secretion of different proangiogenic factors. Recently, Sahoo and colleagues (59) investigated the relationship between CD34+ cells and vascular angiogenesis. They reported that CD34+ cells release exosomes that are able to stimulate angiogenic activity in isolated endothelial cells and in murine models of vessel growth. Moreover, CD34+ cell-derived exosomes contain significant levels of pro-angiogenic miR-126 and miR-130a. Thus the exosome-mediated transfer of specific miRNAs to endothelial cells could be responsible for the observed benefits of CD34+ cells on angiogenesis.

Collectively, these data indicate a direct involvement of exosomal miRNAs in a beneficial effect on the cardiac function of stem cell-based therapies.

Circulating Exosomal miRNAs as Biomarkers of Heart Disease

Circulating, extracellular miRNAs hold great promise as a new class of diagnostic or prognostic biomarkers for predicting cardiovascular diseases such as acute myocardial infarction (AMI), chronic heart failure (CHF), and coronary artery disease (CAD).

Circulating cell-free miRNAs have been found to be remarkably stable in body fluids. In fact, different groups have independently reported that miRNAs are resistant to circulating RNase activity. In addition, they can undergo extended storage, multiple freeze-thaw cycles, and extreme pH (49, 72, 80). Several possible mechanisms have been suggested to explain the surprising stability of circulating miRNAs. Among the other mechanisms, binding to transport proteins (1) or high-density lipoprotein (76) was experimentally confirmed. An-

other intriguing mechanism is miRNA packaging into extracellular vesicles such as exosomes, apoptotic bodies, or microvesicles. The current review is focused on circulating exosomal miRNAs. Table 1 reports an overview of circulating exosomal miRNAs that are dysregulated in cardiovascular disease.

Increasing evidence shows that circulating miRNAs can be used as potential diagnostic biomarkers for myocardial infarction (8, 32, 42, 85). This hypothesis was strengthened by reports that miRNAs are released from the heart into the circulation system upon myocardial injury. In fact, De Rosa and colleagues (13) measured circulating levels of miRNAs from plasma samples simultaneously obtained from the aorta and the coronary venous sinus in 7 control subjects, 31 patients with stable CAD, and 19 patients with troponin-positive acute coronary syndromes (ACS). A significant increase in circulating levels of muscle-enriched miRNAs (miR-499, miR-133a, and miR-208) across the coronary circulation was observed in troponin-positive ACS patients compared with patients with stable CAD, which suggests that these miRNAs are released into the coronary circulation during myocardial injury. Other studies independently (9, 11, 21, 78) confirmed that cardiac-enriched miR-133, miR-208, and miR-499 are elevated in the blood of patients with acute MI, which supports their use as blood-borne biomarkers.

In a small cohort study conducted by Matsumoto et al. (46), specific exosomal miRNAs were identified as potential predictors of heart failure (HF) after acute myocardial infarction (AMI). Using a TaqMan Array Human MicroRNA, the authors examined a panel of 377 miRNAs in registry patients who developed HF and then confirmed their findings in a validation cohort of 21 patients. The authors demonstrated that the serum levels of p53-responsive miRNAs (miR-192, miR-194, and miR-34a) were significantly upregulated in AMI patients who developed a clinical picture of HF during the follow up. Interestingly, Matsumoto et al. found that miR-192, miR-194, and miR-34a were predom-

inantly present in the exosomal fraction of serum samples.

Kuwabara et al. (41) investigated the levels of miR-1 and miR-133a in serum samples from 29 patients with acute coronary syndrome (ACS), and 42 healthy controls were also analyzed. The authors found that miR-133a and miR-1 were over-expressed in ACS patients compared with the control group. In addition, Kuwabara et al. observed that miR-133a is present in exosomes released by cardiac H9C2 cells after treatment with the calcium ionophore A23187 (41). Accordingly, the authors suggested that miR-133a could be released into the blood circulation by active secretion in exosomes. As described above, a recent report (29) identified miR-146a as a potential therapeutic target of peripartum cardiomyopathy (PPCM). In this study, Halkein et al. also investigated whether exosomal miR-146a could serve as a blood biomarker to identify PPCM.

The study reported that circulating levels of exosomal miR-146a were significantly increased in plasma from patients with acute PPCM compared with healthy postpartum women and patients with dilated cardiomyopathy. Subsequently, Halkein and colleagues (29) showed a reduction of miR-146 loading into secreted exosomes in PPCM patients after standard therapy for heart failure. In total, this study confirmed that circulating exosomal miR-146a might be a useful biomarker in the diagnosis of patients with peripartum heart failure.

To investigate the role of serum miRNA in predicting the prognosis of heart failure, Goren et al. (26) performed a microarray profile analysis of 370 miRNAs, followed by a quantitative PCR validation, in serum samples from a small set of healthy and heart failure patients. The authors showed that four miRNAs (miR-423-5p, miR-320a, miR-22, and miR-92b) are detectable and significantly modulated in the circulation of heart failure patients. Interestingly, systemic levels of these selected miRNAs were quantitatively related to several clinical and prognostic heart failure parameters. The combined measurement of four serum miRNAs (cumulative miRNA score) was able to identify patients with heart failure with a larger accuracy than any single miRNA alone. Finally, Goren and colleagues performed analyses of miRNA expression levels in purified exosomal and non-exosomal fractions from serum of 10 HF patients and 10 controls, and demonstrated that there were not significant differences in miR-423-5p, miR-320a, and miR-22 levels between the HF group and the control group in the exosomal fraction and unfractionated serum. However, the difference was reduced in the exosomal levels of miR-92b in the heart failure group.

Limitations and Perspective

Despite several studies that are very encouraging on the therapeutic application of exosomal miRNAs in heart disease, many basic questions remain unanswered.

A potential role in heart pathophysiology has been attributed to exosomes, which may participate as mediators of intercellular communication by delivering proteins and various types of RNA molecules to recipient cells. It has been documented that cargo contained within exosomes could be released into the extracellular space, with functional consequences for surrounding cells. The mediators of these functional effects are mostly several protein types. Although the results obtained are very promising, the pathophysiological relevance of extracellular miRNAs and their potential off-target effect has not yet been established. In addition, the underlying mechanisms of sorting and secretion of exosomal miRNAs from cardiac cells have not been well characterized.

Furthermore, circulating miRNAs are vehiculated by different carriers, but the consequences of their differential association with lipids/vesicles/proteins for their stability and their potential use as biomarkers are largely unexplored. In fact, relatively few studies have attempted to distinguish between the different aspects of transport of miRNAs in biological fluids. Thus the biochemical composition of the circulating miRNAs requires further investigation to translate miRNA-based biomarkers into the clinical setting.

Conclusions

Exosomal miRNAs play an important role in heart disease development and progression through the modulation of intercellular communication in different cardiac cell types. There is significant potential for future clinical applications, including the use of exosomal miRNAs as potential novel biomarkers for the diagnosis and prognosis of different heart diseases or for applications in stem cell-based therapies. ■

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