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Review Article

Extracellular/circulating microRNAs and their potential role in cardiovascular disease

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Abstract: microRNAs (miRs, miRNAs) are small non-coding RNAs that regulate hundreds of gene expression. Numerous studies have demonstrated that miRNAs are not only found intracellularly, but also detectable outside cells, including various body fluids (i.e. serum, plasma, saliva, urine, breast milk, and tears). Interestingly, ~90% of extracellular miRNAs are packaged with proteins (i.e. Ago2, HDL, and other RNA-binding proteins) and ~10% are wrapped in small membranous particles (i.e. exosomes, microvesicles, and apoptotic bodies). It is believed that these extracellular miRNAs mediate cell-to-cell communication. Recent studies further indicated that the level and composition of these extracellular/circulating miRNAs correlated well with disease or injurious conditions. Uncovering the potential role of extracellular miRNAs in the heart is just emerging. This review will highlight recent exciting findings in the regulation of miRNA biogenesis and secretion, their functional roles in paracrine signaling, and the potential as non-invasive biomarkers for cardiovascular disease.

Keywords: microRNA, Extracellular, Biomarker, Paracrine, Intercellular communication

Introduction

microRNAs (miRNAs, miRs) are small (~18–24 nucleotides), highly conserved, non-coding RNAs that are ubiquitously expressed in all species [1, 2]. They usually act as endogenous repressors of target genes by either inhibiting translation and/or by promoting degradation of the mRNA, or alternatively by increasing translation [3,4]. It is well documented that an individual miRNA can influence hundreds of gene transcripts to coordinate complex programs of gene expression and thereby, affect global changes in the physiology of a cell [4-7]. Accordingly, miRNA has been implicated as key molecular players in virtually all the cellular processes, including cardiovascular development and pathophysiology [8,9]. Numerous studies have demonstrated that alterations in the spectrum of intracellular miRNAs are correlated with various cardiovascular conditions such as myocardial infarction, hypertrophy, cardiomyopathy, and arrhythmias [10-20]. These unique signatures of tissue miRNA expression may hold promise as novel diagnostic tools for cardiovascular disease. However, heart tissue is often difficult to collect making miRNA-based biomarkers troublesome and time-consuming. Fortunately, while the majority of miRNAs are found intracellularly, a number of miRNAs have been detected outside cells, including various body fluids (i.e. serum, plasma, saliva, urine, breast milk, and tears) from normal individuals [21-24]. Furthermore, alterations in the level and composition of these extracellular miRNAs have been well correlated with disease or injurious conditions [25-29], suggesting these extracellular miRNAs can be served as informative biomarkers to assess and monitor the body's pathophysiological status. In this review, we highlight recent exciting findings in the regulation of miRNA biogenesis and secretion, their potential as paracrine signaling and as non-invasive biomarkers for cardiovascular disease.

Regulation of miRNA biogenesis

Promoter of miRNA gene

While some miRNAs are processed from introns

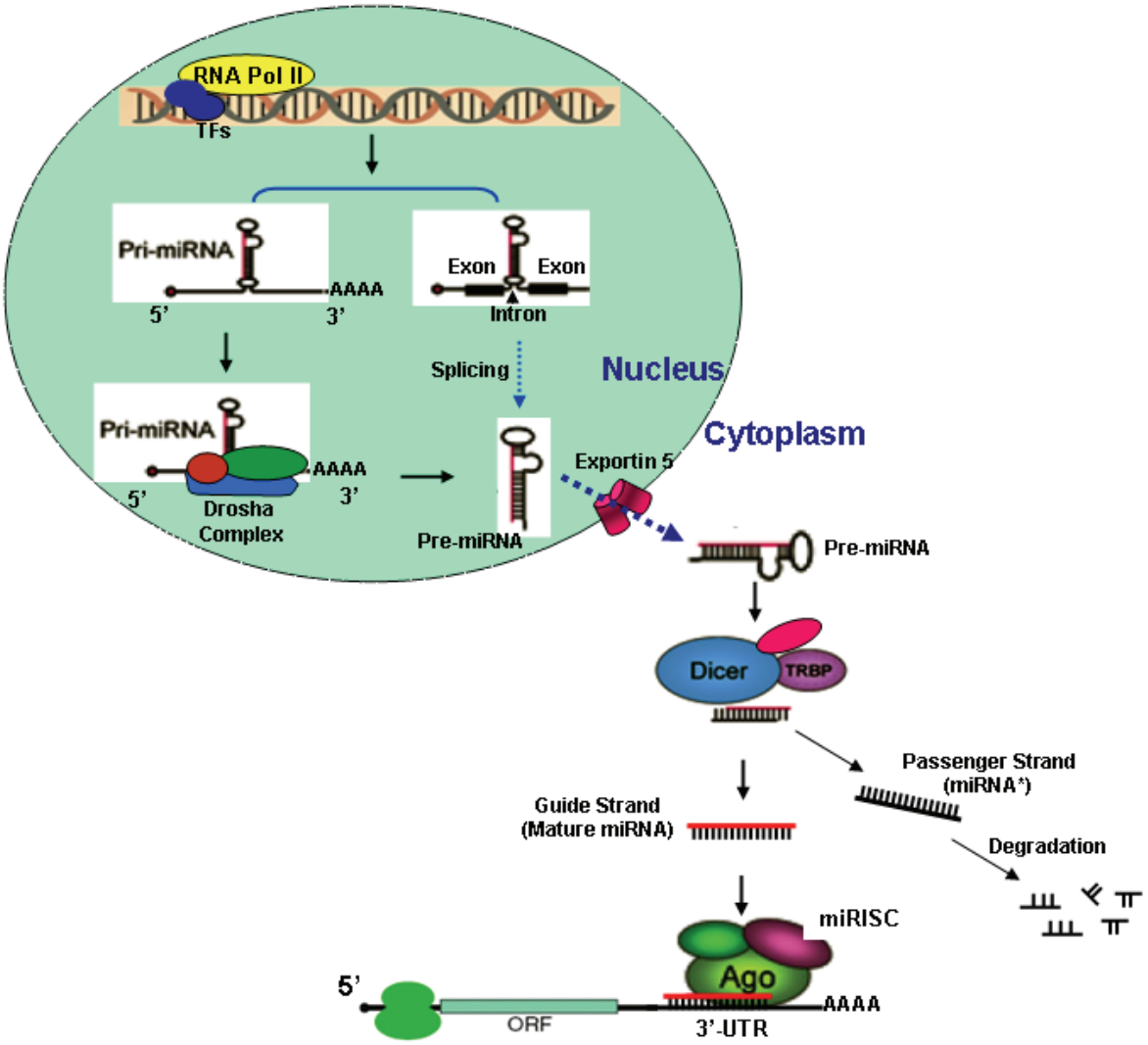

Figure 1. Schematic representation of microRNA (miRNA) biogenesis. miRNA is mainly transcribed by RNA polymerase II, followed by the Drosha complex cleaves off to generate the pre-miRNA that is exported to the cytoplasm by Exportin-5. Subsequent to Dicer cleavage, the miRNA duplex is unwound and the passenger strand degraded. The guide strand (mature miRNA) is then incorporated into the miRISC where gene silence can be accomplished via mRNA target cleavage, translational repression, or mRNA deadenylation.

of protein-coding gene transcripts, most miRNAs are transcribed from independent gene locus by RNA polymerase II transcripts and bear the 7-methylguanylate cap at the 5'-end and poly (A) tail at the 3'-end, which is similar to mRNAs of protein-coding genes (**Figure 1**) [30, 31]. Large-scale mapping of the promoters of 175 human miRNA genes through nucleosome positioning and chromatin immunoprecipitation-on-genomic DNA microarray chip (ChIP-on-chip) analysis indicate that the promoter regions of miRNA genes are highly similar to those of protein-coding genes which contain CpG islands, TATA box sequences, initiator elements and certain histone modifications [32, 33]. These features of miRNA promoters suggest that the biogenesis of miRNA can be controlled by transcription factors (TFs), enahncers, silencing elements and chromatin modifications. For example, we recently identified that the promoter of miR-144/451 contains GATA-binding motifs, and overexpression of GATA-4 increased the expression of the miR-144/451 cluster in cardiomyocytes [34]. Therefore, it is reasonable that the

levels of miRNAs can be dynamically regulated in the heart upon various pathophysiological conditions such as ischemia/reperfusion, pressure overload, growth factor stimulation, and exercise training.

Post-transcription of miRNAs

Long-primary transcripts of miRNA genes (pri-miRNAs), which are generally several thousand nucleotides, undergo two sequential cleavages to become the mature miRNAs of ~22 nt (**Figure 1**) [35-37]. The first step of miRNA processing is catalysed in the nucleus by the Drosha complex cleaving at the base of the stem to generate a ~70-100 nt hairpin (pre-miRNA) with a 2 nt overhang at the 3'-end [35]. The Drosha complex contains at least 20 proteins, and major components include: 1) Drosha, an RNase III enzyme, 2) DGCR8, a cofactor to promote the efficient cleavage of Drosha, and 3) the DEAD-box RNA helicases p68 (DDX5) and p72 (DDX17), which serve as a scaffold for the recruitment of other protein factors in the Drosha complex [36, 38-42].

Following the cleavage, pre-miRNA is exported from the necules to the cytoplasm, where the pre-miRNA is cleaved near the terminal loop by a Dicer RNase III enzyme [complexed with TAR RNA binding protein (TRBP) in mammals] to generate a ~22 nt double-strand miRNA (**Figure 1**) [43, 44]. One strand called active guide strand is then selected to incorporate into the RISC-loading complex (miRISC) and function as a mature miRNA, whereas the other strand called passenger strand (miRNA*) is degraded [43-45]. The key components of miRISC are proteins of the Argonaute (AGO) family which include eight members (*Ago*1-4 and *Piwi*1-4) in human genome [46]. Ago2 is the only one with RNA cleavage activity and is thought to play a critical role in miRNA-mediated mRNA silencing [47]. Ectopic expression of Ago proteins results in a dramatic increase in mature miRNAs [48,49]. In contrast, Ago2-null cells exhibit reduced levels of all mature miRNAs examined [48,49]. These results suggest that intracellular levels of mature miRNAs may be tightly controlled by the Ago protein.

Finally, when miRISCs bind to mRNAs, their expression can be repressed by several mechanisms: 1) inhibition of translation elongation, 2) co-translational protein degradation, 3) competition for the cap structure, 4) inhibition of ribosomal subunit joining, and 5) inhibition of mRNA circularization through deadenylation and decapping [1-4]. Interestingly, recent studies have found that multivesicular bodies (MVBs), specialized late-endosomal compartments, can regulate miRNA function [50,51]. Blocking MVB formation by depleting endosomal sorting complex required for transport (ESCRT) factors inhibits miRNA silencing, whereas blocking MVB turnover by inactivation of the Hermansky–Pudlak syndrome 4 (*HSP4*) gene stimulates repression by miRNAs [50,51]. In addition, several studies further indicated that components of the miRISC loading complex, namely Dicer and Ago2, existed in membranous fractions, and mammalian Ago2 has been characterized as a Golgi-associated or endoplasmic reticulum(ER)-associated protein [52-54]. Together, these results suggest that MVBs and Golgi-ER may represent major miRISC turnover pathways, and may facilitate miRNA degradation or secretion. Accordingly, it would not be surprising to identify the existence of miRNAs within small membranous particles or with the Ago2 protein in various body fluids, as reviewed below.

Regulation of miRNA release

While the majority of miRNAs are found intracellularly, a handful of miRNAs have been detected outside cells, including various body fluids [21-24]. Notably, extracellular miRNAs show a distinct expression profile among different types of body fluids [25-29], suggesting that extracellular miRNAs are not only passive released outside the necrotic or injuried cells. In addition, these extracellualr miRNAs are remarkably stable despite high extracellular RNase activity [21], indicating that extracellular miRNAs are likely packaged in some manner to protect them against RNase digestion. Actually, recent studies have demonstrated that extracellular miRNAs could be shielded from degradation by several mechanisms, including their packaging in microvesicles (MVs), exosomes, and apoptotic bodies as well as through the formation of protein-miRNA complexes (**Figure 2**) [55-62].

miRNAs have been identified in both exosomes and microvesicles derived from a variety of sources, including human and mouse mast cells , glioblastoma tumors, plasma, saliva and urine [58, 59, 63]. While both microvesicles and exosomes are known to be membrane-enclosed

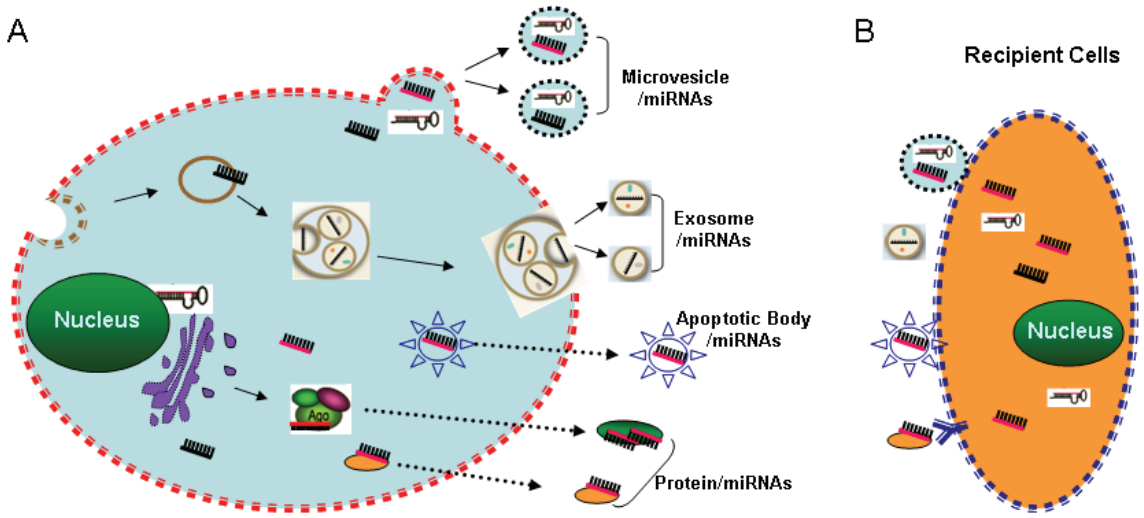

Figure 2. Cellular releases (A) and uptake (B) of miRNAs. (A) Extracellular miRNAs may be contained within vesicles, including microvesicles, exosomes, and apoptotic bodies, as well as within proteins such as Ago2, HDL, and other RNA-binding proteins. (B) Extracellular miRNAs may potentially interact with recipient cells via direct fusion, internalization, receptor-mediated interactions and other possible mechanisms.

vesicles, they are two distinct vesicle populations in their size, origin and release pathway (**Figure 2A**) [64-70]. Microvesicles (also referred to as microparticles or ectosomes) are plasma-membrane-derived particles that are released into the extracellular space by outward budding and fission of the plasma membrane. They appear heterogeneous in size, ranging from 100nm-1μm. Exosomes are formed intracellularly via endocytic invagination and are released into a structure known as a multivesicular body (MVB). The MVB then fuses with the plasma membrane, releasing its cargo of exosomes into the extracellular space [64-70]. Recent studies suggest that a variety of miRNAs are selectively incorporated into microvesicles and exosomes [71-73]. For example, Let-7 miRNA family is selectively secreted into the extracellular environment via exosomes in metastatic gastric cancer line which may facilitate to maintain their oncogenesis and metastasis [72]. Similar findings were also reported by Pigati et al. that released miRNAs by exosomes do not exactly reflect the abundance of miRNA in the cell of origin [73]. They observed that a significant level of miR-451 and miR-1246 was released from malignant mammary epithelial cells in which mammary epithelial cell-enriched miRs were retained [73]. Furthermore, a recent study by Wang et al. reported that several human cell lines (HepG2, A549, T98 and BSEA2B) actively release miRNAs which appears to be a short pulse lasting about 1 hour immediately after serum deprivation [55], suggesting that the secretion of miRNAs is responsive to stress conditions. Intriguingly, they observed that a significant fraction of extracellular miRNAs is rather packaged with RNA-binding proteins than within cell-derived vesicles, the microvesicles and exosomes [55]. These results indicate that there may be at least two pathways for the packaging and export miRNAs outside cells: small membranous particle-mediated and protein-mediated processes.

Indeed, using differential centrifugation and size-exclusion chromatography as orthogonal approaches to characterize circulating miRNA complexes in human plasma and serum, Arroyo et al. excitingly observed that vesicle-associated plasma miRNAs represented the minority, whereas potentially 90% of miRNAs in the circulation are present in a non-membrane-bound form which was further identified as Ago2-miRNA complex [61]. They speculate that the vesicle-associated versus Ago2 complex-associated miRNAs originate from different cell types and reflect cell type-specific miRNA expression and/or release mechanisms. Supportively, an early study by Chang et al. also showed that the liver-specific miRNA, miR-122, was detected only in the protein-associated frac-

tions, suggesting that hepatocytes may release miR-122 through a protein carrier pathway [74]. Consistently, Turchinovich et al. found most of the extracellular miRNAs in human blood and cultured cells are bound to the Ago2 protein, independent from exosomes [60]. In addition, Vickers et al. [62] reported that purified fractions of high-density lipoprotein (HDL) from healthy human plasma contained a number of miRNAs with the most abundant hsa-miR-135a*, hsa-miR-188-5p and hsa-miR-877. Furthermore, Kosaka et al. [71] showed that the release of miRNAs is controlled by neutral sphingomyelinase 2 (nSMase2) and through ceramide-dependent secretory machinery. Taken together, these findings indicate that the existence of miRNA export system could selectively export miRNAs which are packaged with small membranous particles or proteins. Nonetheless, whether miRNAs are actively secreted outside cardiomyocytes remains obscure. Therefore, future studies will be greatly needed to clarify whether miRNAs are selectively released from cardiomyocytes upon various pathophysiological stress conditions.

Extracellular miRNAs function as paracrine signaling

Numerous studies have demonstrated that exosomes, microvesicles, apoptotic bodies and the complex of protein/RNA that are released from many cell types can transfer proteins, mRNAs and miRNAs to neighboring or distant cells to modulate angiogenesis, cell proliferation/death, tumor cell invasion, and cell-to-cell communication (**Figure 2**) [55-80]. Hence, the extracellular miRNAs wrapped in these small membranous particles or packaged with proteins may function as paracrine signaling to influence the recipient cell phenotypes.

Microvesicle/exosome-mediated miRNA transfer

Recently, Akao et al. [59] showed that chemically modified miR-143 entrapped by microvesicles (MVs) was significantly secreted from miR-143-transfected human monocytic leukemia THP-1 cells during incubation in serum-free medium. Importantly, nude mice intravenously injected with these miR-143-contained MVs revealed a significant increase in the levels of miR-143 in the serum and kidney. Similar observations were also reported that miR-150 secreted in MVs from human blood cells or cultured THP-1 cells can be taken up by HMEC-1 microvascular endothelial cells and consequently, regulate the expression of c-Myb, a *bona fide* target of miR-150 [76]. Additionally, Yuan et al. [58] reported that MVs from embryonic stem cells contain abundant miRNAs, which can be transferred to mouse embryonic fibroblasts *in vitro*. Furthermore, Pegtel et al. [78] have shown that miRNAs released in exosomes by Epstein–Barr virus (EBV)-infected cells can be taken up by peripheral blood mononuclear cells and can suppress confirmed EBV target genes. Together, these findings strongly support that at least some exported miRNAs are used for cell-to-cell communication, although more studies are needed to determine how miRNAs are specifically targeted for secretion, recognized for uptake, and what information can be transmitted via this process.

Apoptotic body-mediated miRNA transfer

It has been recently reported that endothelial cell (EC)-derived apoptotic bodies are generated during atherosclerosis and contained mainly miR-126 as well as other minor miRNAs [56]. Incubation of these apoptotic bodies with human umbilical vein ECs (HUVECs) resulted in transfer of miR-126 into recipient cells and production of the anti-inflammatory chemokine, CXCR4, through inhibition of RGS16, a known target of miR-126 [56]. In addition, these apoptotic bodies induce endothelial progenitor cell proliferation and differentiation *in vitro* [79]. In *Apoe*$^{-/-}$ mice fed with a high-fat diet (HFD), intravenous injection of apoptotic bodies prepared from HUVECs increased the numbers of endothelial progenitor cells in the circulation and incorporation of endothelial progenitor cells into aortic root plaques, which reduced the size, as well as the number of macrophages and apoptosis in atherosclerotic lesions [80]. Consistently, when *Apoe*$^{-/-}$ mice fed a high-fat diet were treated with miR-126–containing EC apoptotic bodies from patients with atherosclerosis, lesion size and macrophage content were reduced in a CXCR4-dependent manner [81]. These observations confirm that EC-derived apoptotic bodies may serve as carriers to deliver miR-126 to humans with atherosclerosis or other vascular diseases that are associated with EC dysfunction.

Protein-based miRNA delivery

As reviewed above, several studies have shown that a number of extracellular miRNAs are bound to proteins such as nucleophosmin 1 (NPM1), Ago2, and HDL [55, 61, 62]. However, whether there is a protein carrier-based RNA communication mechanism in mammalians remains obscure. Recently, Vivkers et al. [62] incorporated exogenous miR-375 with native HDL, and then added to cultured hepatocytes (Huh7). They observed that, in cells treated with HDL/miR-373 complexes, intracellular levels of miR-375 were increased by 11.8-fold. Similarly, cellular delivery of HDL incorporated with miR-223 dramatically increased intracellular miR-223 levels by 250-fold, leading to 53% reduction of the RhoB protein, a *bona fide* target of miR-223 [62]. To further examine whether endogenous levels of miRNAs transported on HDL are sufficient to directly alter gene expression in recipient cells, Vivkers et al. isolated HDL from familial hypercholesterolemia which contains abundant levels of hsa-miR-105, and HDL from normal subjects which lacks hsa-miR-105. Treatment of hepatocytes (Huh7) with normal (healthy) HDL failed to increase intracellular levels of endogenous hsa-miR-105 [62]. In contrast, treatment with familial hypercholesterolemia HDL resulted in a significant increase in intracellular hsa-miR-105 levels which consequently, induced dramatic alterations of 217 gene expressions in Huh7 cells, compared with normal HDL-treated cells [62]. Importantly, 60% of the significantly downregulated mRNAs were putative targets of hsa-miR-105 [62]. Collectively, these results indicate that native HDL can efficiently deliver miRNA to recipient cells with functional targeting capabilities, contributing to altered gene expression.

Circulating miRNAs in cardiovascular disease

The identification of distinct circulating miRNA profiles in patients may impact the development of specific miRNAs as biomarkers. The first demonstration of a link between circulating miRNAs and disease came from cancer studies published in early 2008 [21,82-84]. In these studies, several miRNAs were detected specifically in the sera of human patients with each tumor type. Recent studies have also profiled circulating miRNAs from patients with cardiovascular disease, and several specific circulating miRNAs have been used to distinguish between heart disease/injury and healthy subjects, suggesting circulating miRNAs may serve as novel biomarkers in cardiovascular diseases [28,29,85-94]. It is important to note here that the use of circulating miRNAs as potential biomarkers in clinical scenarios depends on the sensitivity of methods used to detect them, source material (whole blood, serum, plasma or purified exosomes) to extract total RNA, and the accurate normalization. Currently, several endogenous circulating miRNAs (i.e. miR-17-5p, miR-1249, miR-454, U6, 5SrRNA, and RNU6b) and spiked-in miRNAs (i.e. synthetic *C. elegans* miR-39, -54, and -238) have been used for normalization of circulating miRNAs in cardiovascular disease [28,29,85-94].

Ischemic heart disease

It is well known that some miRNAs are expressed in a cell type- and tissue-specific manner. Hence, Ji et al. used an isoproterenol-induced myocardial-injury rat model to confirm whether cardiac-specific miRNAs leak from injured cardiomyocytes into the circulating blood [85]. MiRNA array analysis reveals that miR-208 is exclusively expressed in cardiac myocytes. Consistently, the plasma concentration of miR-208, albeit undetectable at baseline, is significantly increased during the next 3-12h after administration of isoproterenol, which shows a good correlation with the plasma levels of cardiac troponin I (cTnI), a classic marker of myocardial injury [85]. Importantly, circulating levels of miR-208 are not increased in a renal-infarction rat model or a cardiac-hypertrophy model, indicating a high sensitivity and specificity [85]. However, in a recent follow-up study, the same group found that expression levels of miR-208a and miR-208b were very low in human hearts, and miR-499 is expressed almost exclusively [86]. When analysis of circulating miRNAs in 9 patients with acute myocardial infarction (AMI), 5 patients with unstable angina pectoris, 15 individuals with congestive heart failure (CHF) and 10 healthy subjects, miR-499 was only detected in AMI patients (not in controls or those with CHF) and levels returned to normal by the time of hospital discharge [86]. These results indicate that miR-499, a cardiac-specific miRNA, may serve as a biomarker of myocardial infarction in humans.

Nevertheless, the study by Wang et al. [87] in evaluation of plasma miRNAs from 33 AMI patients and 30 healthy people, showed that miR-

208a was easily detected in 90.9% AMI patients and in 100% AMI patients within 4 h of the onset of symptoms when the cTnI was still detected below the cut-off value. These findings suggest that miR-208a may serve as a more sensitive earlier marker of AMI, because the level of cTnI in blood usually begins to be detectable after 4-8 h of myocardial injury. Inconsistently, a recent study by Corsten et al. [88] observed a robust elevation of plasma miR-208b and miR-499 levels by 1600-fold and 100 -fold, respectively, in 32 AMI patients, compared to controls. Both miR-208b and miR-499 correlated significantly to serum levels of troponin T and creatine phosphokinase (CPK), two markers of cardiac injury [88].

In addition, Ai et al. [89] measured circulating levels of miR-1, a muscle-enriched miRNA, in a cohort of 93 patients with AMI. They found that miR-1 was significantly elevated in the blood of AMI patients compared to non-AMI controls, and increased miR-1 levels were well correlated with abnormal QRS widening in patients with AMI, whereas no correlation was found with ST-segment alterations or levels of cTnI or creatine kinase (CK)-MB [89]. Consistently, other studies also showed the circulating miR-1 to be increased in AMI, but in contrast to Ai et al., these studies demonstrated a positive correlation of miR-1 levels with CK-MB levels [90], whereas D'Alessandra et al. [91] showed that miR-1 upregulation in blood from AMI patients correlated with cTnI levels.

Taken together, despite these discrepancies, it is well accepted that circulating myocardial-derived miRNAs might be useful as potential biomarkers for ischemic heart disease.

Heart failure

Recently, several studies have indicated that patients with heart failure exhibit a different plasma miRNA profile. Using microarrays to determine the alternations of plasma miRNAs from 12 CHF patients and 12 healthy controls, Tijsen et al. [92] reported that 108 miRNAs were differentially expressed in CHF patients. Among these dysregulated miRNAs, the best16 candidates were further analyzed by RT-qPCR and miR-423-5p was specifically enriched in the blood of heart failure cases [92]. Importantly, circulating miR-423-5p correlated with N-terminal prohormone brain natriuretic peptide levels (NT-proBNP) and ejection fraction, suggesting that miR-423-5p appears to be a good predictor of CHF diagnosis [92]. Interestingly, miR-1 and miR-208, which are predictive of AMI, were not increased in CHF, suggesting a specific miRNA response in blood elicited by different pathologic conditions [92]. While miR-423-5p has been reported in array studies to be upregulated in human failing myocardium [16], it remains elusive whether increased circulating miR -423-5p is derived from the failing heart or whether there are other mechanisms contributing to the elevation of circulating miR-423-5p. Another study by Corsten et al. [88] found a significant increase in circulating miR-499 levels in patients with acute heart failure (AHF), whereas no alterations of miR-499 were found in diastolic heart failure. Notably, the liver-specific miR-122, which is associated with hepatic damage, was significantly increased in the blood from AHF patients, possibly reflecting hepatic venous congestion [88].

Coronary artery disease

Coronary artery disease (CAD) is still the leading cause of death worldwide. Endothelial activation is considered a first step in the development of atherosclerotic lesion, followed by invasion of proinflammatory cells and proliferation and de-differentiation of smooth muscle cells [93, 94]. Several risk factors, such as hypercholesterolemia, are known to promote atherosclerosis, and various biomarkers have been shown to identify patients at risk for CAD [93, 94]. Recent studies indicate that several miRNAs are dysregulated in isolated peripheral blood mononuclear cells from CAD patients [95]. Fichtlscherer et al. [29] further determined the circulating miRNA profiles in plasma of 8 CAD patients and 8 healthy volunteers by a Geniom Biochip. The array results indicate that most dysregulated circulating miRNAs are known to be expressed in the vascular wall, particularly in endothelial cells [29]. Among which, miR-126, the miR-17/92 cluster (miR-17, -20a, and -92a), miR-130a, miR-221, miR-21 and members of the let -7 family were significantly reduced [29]. Cardiac miRNAs, except that miR-208b was increased, were detected at lower levels and were not altered in the blood from CAD patients compared with healthy subjects. To prospectively validate these data, they measured levels of miR-126, -17, -92a, -145 and miR-155 in plasma of 36 CAD patients and 17 healthy con-

trols by quantitative RT-PCR, and consistently observed that these miRs were significantly decreased [29]. Correlation studies revealed that vascular and inflammation-linked miRNAs were altered by vasculoprotective therapies with inhibitors of the renin-angiotensin system (i.e. aspirin and statins). Patients suffering from prevalent diabetes displayed significantly reduction in circulating levels of miR-20b, miR-21, miR-24, miR-15a, miR-126, miR-191, miR-197, miR-223, miR-320, and miR-486 but a modest increase of miR-28–3p [29]. The reduced concentration of circulating vascular miRNAs detected in patients with CAD may be surprising, because one would expect that endothelial activation, as it occurs in patients with CAD, induces the release of exosomes, microvesicles, and apoptotic bodies, thereby elevating the levels of miRNAs. Given recent experimental studies demonstrating that miRNA packaged in exosomes, microvesicles or apoptotic bodies can be delivered to atherosclerotic lesion, one may speculate that the reduction of circulating miRNAs detected in patients with CAD might be caused by an uptake of circulating miRNAs into atherosclerotic lesions.

Conclusions and future directions

Over the last several years the presence of extracellular/circulating miRNAs has been detected in a variety of conditions. These miRNAs are extremely stable, often found in association with small membranous particles and mostly with RNA-binding proteins (Ago2, HDL, etc). The levels of circulating/extracellular miRNAs may be affected by multiple steps or factors such as intrinsic/extrinsic stress stimuli to modulate miRNA biogenesis and secretion. Nevertheless, circulating/extracellular miRNAs clearly have many requisite features of ideal markers, including: 1) stable in various bodily fluids, 2) conserved sequence among species, 3) specific to tissue and disease state, and 4) easily measurement by RT-PCR, suggesting that they may represent novel informative biomarkers for a range of diseases. Currently, the utmost challenges in the development of miRNA-based biomarker are how to set up a standardized process for sample preparation and a more accurate method to assess the quality and quantity of miRNA. For example, it is still difficult to determine appropriate suitable endogenous controls because the expression profile of circulating miRNAs may change depending on the patient's cardiovascular disease state and medication. In addition, future studies will also be needed to determine the potential role of circulating/extracellular miRNAs as paracrine signaling molecules in cardiovascular diseases.

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Conflict of interest disclosures

None

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