Circulating MicroRNAs
Biomarkers or Mediators of Cardiovascular Diseases?

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Abstract—MicroRNAs (miRs) are small, noncoding RNAs that posttranscriptionally control gene expression by inhibiting protein translation or inducing target mRNA destabilization. Besides their intracellular function, recent studies demonstrate that miRs can be exported or released by cells and circulate with the blood in a remarkably stable form. The discovery of circulating miRs opens up intriguing possibilities to use the circulating miR patterns as biomarker for cardiovascular diseases. Cardiac injury as it occurs after acute myocardial infarction increases the circulating levels of several myocardial-derived miRs (eg, miR-1, miR-133, miR-499, miR-208), whereas patients with coronary artery disease or diabetes showed reduced levels of endothelial-enriched miRs, such as miR-126. This review article summarizes the current clinical and experimental studies addressing the role of circulating miRs as a diagnostic or prognostic biomarker in cardiovascular disease. In addition, the mechanisms by which miRs are released and their putative function as long-distance communicators are discussed. (*Arterioscler Thromb Vasc Biol*. 2011;31:2383-2390.)

Key Words: acute coronary syndromes ■ diabetes mellitus ■ biomarker ■ microRNAs

MicroRNAs (miRs) are short, noncoding RNAs that are important for many aspects of homeostasis and disease.[1–3] miRs are generally considered to act as intracellular endogenous RNAs to control gene expression on a posttranslational level. However, recent studies have demonstrated that miRs can be detected in circulating blood and that these circulating miRs might therefore be useful as disease biomarkers, eg, for certain forms of cancer.[4–6] These findings have raised the question of whether circulating miRs may also play a role as diagnostic or prognostic biomarkers in cardiovascular disease.[7]

miRs as Biomarkers for Acute Myocardial Infarction

In the scenario of cardiovascular diseases, acute myocardial infarction (AMI) is potentially the easiest target to establish a potential role of circulating miRs. Myocardial infarction is characterized by the sudden induction of cardiomyocyte death, which results in the release of cardiac proteins such as the established biomarker troponin. Therefore, initial studies tested the hypothesis that AMI also induces the release of cardiomyocyte miRs. It is well known that some miRs are expressed in a cell type– and tissue-specific manner. Specifically, miR-208a is encoded by the α- myosin heavy chain gene and therefore is exclusively expressed in cardiac myocytes.[8] The levels of circulating miR-208 are barely detectable in the absence of injury but are significantly increased in experimental AMI models,[9,10] as well as in patients with AMI.[9] Corsten et al confirmed this finding and demonstrated that circulating levels of miR-208b are highly elevated by approximately 1600-fold in patients with AMI (n=32) as compared with patients with chest pain but normal angiograms.[11] In addition, miR-208b levels correlated with plasma troponin T levels, confirming the link to myocardial damage.[11] However, in other studies, miR-208a concentrations were not detectable or were very low in plasma samples obtained from AMI patients,[12,13] or only detectable in some of the AMI patients.[14] The discrepancy between the studies likely reflects the fact that circulating miR-208 concentrations are rather low and that more RNA is required to reliably measure serum or plasma miR-208 levels. In addition, miR-208 is difficult to measure by TaqMan polymerase chain reaction compared with other miRs, as demonstrated by comparing the threshold cycle values of different recombinant miRs.[15]

Alternatively, muscle-enriched miRs, such as miR-1, miR-133a/b, and miR-499, which are not exclusively expressed in cardiac myocytes but are also detected in skeletal muscle cells,[1] have been evaluated (Table 1). Several studies showed that miR-1 levels are increased in both experimental AMI models and in patients with AMI.[9,14,16,17] Consistently, serum levels of miR-133, which belongs to the same cluster and is cotranscribed with miR-1, was found to be elevated in humans after AMI.[9,11,12,14] Finally, a recent study measuring

Table 1. Circulating miRs in Patients With Cardiac Disease

Clinical Study Cohort	Groups and Numbers of Patients Studied	Major Findings	Association/Correlation	RNA Isolation	miR Detection	Reference
Acute coronary syndromes						
AMI	33 pts with AMI	miR-208a, miR-1, miR-133a, miR-499 ↑	ROC curve: miR-208a shows highest sensitivity and specificity, comparable to TnI	Plasma, TRI Reagent BD, Spiking with cel-miR-39	qPCR TaqMan (ABI)	9
	33 pts with stable CAD/other cardiovascular disease					
	30 healthy controls					
AMI	33 pts with STEMI	miR-1, miR-133a, miR-133b, miR-499-5p ↑	Time course of upregulated miR associated with TnI	Vana PARIS isolation kit (Ambion, Austin, TX)	qPCR TaqMan (ABI) (normalized to miR-17)	12
	17 healthy controls					
AMI unstable angina	9 pts with STEMI	miR-499 ↑ in STEMI (within 48 h)	Correlation with CKMB	Vana PARIS Kit (Ambion) Spiking with small RNA	qPCR TaqMan (ABI)	13
	5 pts with unstable angina					
	10 healthy controls					
AMI	93 pts with AMI	miR-1 ↑	Association with QRS duration	mirVana PARIS (Ambion) protocol	qPCR	16
	66 healthy controls					
AMI	31 pts with AMI	miR-1 ↑	Association with CKMB levels	miR Isolation Kit (RNA Bioscience)	qPCR, primer ABI, Roche Light cycler	17
	20 healthy controls					
AMI	29 pts with ACS	miR-1, miR-133 ↑	Correlation with TnT	TRIzol LD (Invitrogen)	qPCR, TaqMan (ABI)	14
	42 pts without ACS					
AMI	32 pts with AMI	miR-208b, miR-133a, miR-499 ↑ miR-223 ↓	miR-208b and miR-499 correlate with TnT	mirVana PARIS kit (Ambion)	qPCR, MiScript primers (Qiagen), BR SYBR Green (Quanta Biosciences)	11
	36 pts with chest pain but normal angiogram					
AMI	20 pts with AMI	Whole blood: miR-1291, miR-663b ↑	miR-30c and miR-145 correlated with hsTnT	miRNeasy Mini Kit (Qiagen)	Geniom Biochip (Febit); qPCR: TaqMan (ABI)	19
	20 pts without AMI					
ACS	444 pts with ACS	In pts with ACS: miR-1, miR-133a/b, miR-208b ↑	miR-133a and miR-208b associated with risk of death	miRNeasy RNA isolation kit (Qiagen, Inc.)	qPCR, TaqMan (ABI)	21
ACS	7 pts non-CAD	In pts with ACS: miR-133, miR-499, miR-208 ↑	Transcoronary gradients document cardiac release of miR-133 and miR-499	miRNeasy RNA isolation kit (Qiagen, Inc.)	qPCR, TaqMan (ABI)	18
	31 pts stable CAD					
	19 pts ACS					
Heart failure						
HF	30 pts with heart failure	miR-423-5p ↑	ROC showing that miR-423-5p is a predictor of heart failure	mirVana PARIS kit (Ambion)	qPCR High Resolution Melting Master (Roche)	23
	20 pts with dyspnea (non-HF)					
	39 healthy controls					
HF	15 pts CHF (II and III)	miR-499 below detection limit of the PCR in CHF pts and healthy controls		mirVana PARIS kit (Ambion)	qPCR TaqMan (ABI)	13
	10 healthy controls					
HF	33 pts with ischemic HF	miR-126 ↓	miR-126 negatively correlated with age, BNP and NYHA class	mirVana PARIS kit (Ambion)	qPCR TaqMan (ABI)	22
	17 asymptomatic controls					
Acute HF	33 pts with acute HF	Acute Heart failure: miR-499 ↑		mirVana PARIS kit (Ambion)	qPCR, MiScript primers (Qiagen), BR SYBR Green (Quanta Biosciences)	11
	34 healthy controls					
Diastolic dysfunction	39 pts with diastolic dysfunction	No significant changes	Correlation of miR-133a and NT-proBNP	mirVana PARIS kit (Ambion)	qPCR, MiScript primers (Qiagen), BR SYBR Green (Quanta Biosciences)	11
	20 pts with hypertension 20 controls					
Viral myocarditis	14 pts with acute viral myocarditis	Acute viral myocarditis: miR-208, miR-499 ↑	Course of viral myocarditis	mirVana PARIS kit (Ambion)	qPCR, MiScript primers (Qiagen) BR SYBR Green (Quanta Biosciences)	11
	20 pts post-viral myocarditis					
	20 aged-matched controls					

miR indicates microRNA; pts, patients; ROC, receiver operating characteristic; TnI, cardiac troponin; STEMI, ST segment elevation myocardial infarction; qPCR, quantitative polymerase chain reaction; AMI, acute myocardial infarction; ACS, acute coronary syndrome; hsTnT, high-sensitivity cardiac troponin T; CAD, coronary artery disease; HF, heart failure; CHF, congestive heart failure; PCR, polymerase chain reaction; CKMB, creatine kinase muscle b; NT-proBNP, N-terminal prohormone of brain natriuretic peptide; NYHA, New York Heart Association.

transcoronary gradients of circulating miRs confirmed the cardiac origin of miR-133 in the systemic circulation of patients with acute coronary syndromes.[18] Several additional studies showed that circulating levels of the myosin-related miR-499 are increased in patients after AMI.[11–13] By profiling techniques, 2 additional not previously characterized miRs, namely miR-1291 and miR-663b, were identified to be elevated in whole peripheral blood samples obtained from patients with AMI.[19] This study additionally showed that levels of the smooth muscle cell enriched miR-145 and miR-30c are

increased in patients with AMI and correlated with high-sensitivity troponin T serum levels in whole peripheral blood samples.[19] Of note, these data cannot be directly compared with the other studies using plasma/serum because whole-blood samples also contain circulating cells, which could significantly influence the miR expression pattern.

Although all of the cited studies suggest that these circulating muscle-/myocardium-derived miRs might be useful as potential diagnostic biomarkers for infarction, it is unclear which of the measured miRs is best suited and might represent a valuable superior alternative to already established markers of AMI, such as troponin. A few studies directly compared several miRs with established markers. In a rat model of AMI, miR-1, miR-133, and miR-208 were similarly increased after coronary artery ligation.[9] However, miR-208 was the only miR, which was specifically elevated after AMI but not in sham controls, whereas miR-1 and miR-133 were also elevated in the sham group,[9] confirming that miR-208 is exclusively released by myocardial injury but not by any muscle injury. Consistently, increased levels of circulating miR-1 and miR-133 were observed in patients with Duchenne muscular dystrophy,[20] demonstrating that muscle injury also induces the release of these miRs.

In 66 patients with chest pain, a side-by-side comparison of miR-1, miR-133a, miR-499, and miR-208a showed a superior receiver operating characteristic curve for the cardiac specific miR-208a, which was similar to the well-established marker troponin I, indicating a high sensitivity and specificity.[9] Likewise, the comparison of miR-208a, miR-499, miR-133, and miR-1 by Corsten et revealed that miR-208 and miR-499 were more profoundly regulated and showed a higher specificity and sensitivity (areas under the curve of 0.94 and 0.92, respectively) in comparison to miR-1 and miR-133, which were only modestly increased in patient with AMI in this study.[11]

Based on the small numbers of patients enrolled in these studies and given the fact that most of the studies only compared the levels of patients with AMI to matched controls and, therefore, are (potentially) rather hypothesis generating in nature, additional studies are mandatory to confirm the findings. Moreover, future studies should specifically evaluate whether any of the newly identified circulating miRs are competitive with the well-established and highly sensitive known markers of cardiac injury, such as high-sensitivity troponins. Furthermore, little is known about whether circulating miRs might serve as prognostic marker in patients with acute coronary syndromes. Recently, a first study measured all 6 muscle-enriched miRs (miR-1, miR-133a, miR-33b, miR-208a, miR-208b, and miR-499) in patients with acute coronary syndromes and determined their potential impact on prognosis. Patients with AMI showed significantly higher levels of miR-1, miR-133a, and miR-208b compared with patients with stable angina. In addition, the levels of the investigated miRs were closely related with high-sensitivity cardiac troponin T.[21] Univariate analysis revealed that miR-133a and miR-208b levels were significantly associated with the risk of death.[21] However, if adjusted for high-sensitivity cardiac troponin T, no independent prognostic significance could be determined.[21] Indeed, comparing circulating levels of miR-499 and miR-133 with high-sensitivity troponin

levels in the coronary sinus blood of patients with acute coronary syndrome revealed that there appears to be a threshold level for high-sensitivity troponin below which circulating miRs are not increased despite very minute elevations in high-sensitivity troponin.[18]

One advantage that miRs can offer compared with already established biomarkers might be their early release after myocardial injury. Two experimental studies addressed the kinetics of circulating miRs in comparison to the established markers of AMI such as troponin. Muscle-derived miRs were shown to be elevated as early as 1 hour after induction of coronary artery ligation in rats[9] and miR-499-5p was already significantly 1.7-fold increased after 15 minutes of permanent coronary artery ligation in mice.[12] In humans, miR-1 and miR-133a/b were elevated as early as 156 minutes after onset of symptoms and declined thereafter, whereas miR-499 further increased achieving maximal levels approximately 9 hours after symptom onset.[12] Because of the variability of symptom onset after AMI in patients, however, these data have to be taken with caution and further studies are required eg, in patients with iatrogenic induction of myocardial damage, such as transcoronary ablation of septal hypertrophy, to monitor the kinetics of miR release in comparison to troponins. Conceptually, it is intriguing to speculate that specific miRs might be released very early after cellular injury within microvesicles or shed membrane blebs, which are characteristic features of the initiating processes of cellular stress responses or apoptosis.

miRs as Biomarkers for Chronic Heart Failure

The measurement of circulating cardiac miRs in patients with heart failure might be more challenging because the amount of ongoing cardiac myocyte injury is rather low compared with the acutely infarcted heart. However, it is tempting to speculate whether in comparison to AMI, where established markers such as troponins are already available and hard to compete with, in chronic heart failure additional information regarding the pathophysiological cause of heart failure (eg, myocarditis) might be gained and would potentially eliminate the need for biopsies.

Muscle-derived miRs were measured in several different heart failure populations. In patients with Takotsubo cardiomyopathy, serum levels of miR-1 and miR-133a were significantly increased even in the absence of any elevation of serum creatine phosphokinase or cardiac troponin,[14] suggesting that activation of cardiomyocytes or very-low-grade cardiac injury might be sufficient to release miRs even before troponin can be detected. In the acute phase of viral myocarditis, miR-208b and miR-499 were significantly elevated, although the absolute increase was not as pronounced as in AMI patients.[11] The elevated levels of plasma miR-208 and miR-499 declined in post-viral myocarditis patients, indicating that elevated levels of these miRs may reflect myocardial damage rather than inflammation.[11]

In patients with acute heart failure, only miR-499 was significantly elevated (2-fold), whereas miR-208b and miR-133 showed a trend toward increased levels without reaching statistical significance.[11] In patients with diastolic dysfunction, no significant changes in miR-1, miR-133, and miR-208 were

observed.[11] Despite the lack of significant differences between the groups, miR-133 levels were significantly positively correlated with N-terminal prohormone of brain natriuretic peptide, a prognostically relevant biomarker in patients with heart failure.[11] However, Adachi et al[13] and Fukushima et al[22] were unable to detect circulating miR-499 in patients with New York Heart Association class II and III, and no change in miR-1 and miR-208 levels was seen in patients with dyspnea and heart failure.[23] The failure to detect major changes in muscle-related miRs in chronic heart failure or pathological hypertrophy is consistent with an experimental study showing that—in contrast to AMI—cardiac hypertrophy induced by a high-salt diet in salt-sensitive Dahl rats did not elevate circulating levels of miR-208 levels.[10] However, as discussed above, it remains to be determined whether optimized assays may be helpful to more reliably measure the low levels of muscle-enriched (myocardial) myo-miRs to determine low-grade cardiac injury or activation. Moreover, it should be kept in mind that low output heart failure may contribute to systemic hypoxia with subsequent release of miRs from the skeletal muscle.

In addition to the muscle-derived miRs, profiling of plasma samples from patients with heart failure revealed that miR-423-5p and several additional miRs (eg, miR-18b*, miR-129-5p) might be a potentially promising marker.[23] In particular, miR-423-5p was found to be able to discriminate the origin of dyspnea, differentiating heart failure from non–heart-failure–related dyspnea and controls in a validation cohort. In this study, receiver operating characteristic curve analysis showed that miR-423-5p may be a useful diagnostic predictor of heart failure with promising sensitivity and specificity (area under the curve 0.91).[23]

Interestingly, a recent study reported that patients with ischemic heart failure showed lower circulating levels of the endothelial-enriched miR-126,[22] which is essential for ischemia-induced angiogenesis and controls endothelial cell function.[24,25] miR-126 levels were negatively correlated with BNP serum levels, and clinical improvement as demonstrated by changes in New York Heart Association classification was associated with an increased level of miR-126.[22] Although several studies linked a worsening of endothelial function and angiogenesis to heart failure,[26] the biological significance of reduced miR-126 levels remains to be determined.

miRs as Biomarkers for Cardiovascular Disease and Diabetes

Activation of the endothelium is believed to play a key role in the development of atherosclerosis and vascular complications associated with diabetes. Although the measurement of endothelial function by determining forearm blood flow allows for a noninvasive identification of patients at risk for coronary artery disease,[27] the measurement is time consuming and variable, and it is difficult to determine the risk in the individual patient. Therefore, the availability of miRs that are regulated and released in response to endothelial activation or injury, might provide important insights and might be used to identify patients at risk for coronary artery disease (CAD) (Table 2).

Interestingly, levels of circulating vascular miRs were shown to be reduced in patients with stable CAD.[15] Specifi-

cally, the endothelial enriched miR-126, members of the miR-17-92a cluster, as well as the antiatherosclerotic smooth muscle–enriched miR-145 were significantly lower in patients with stable CAD compared with healthy controls, whereas the muscle-derived miR-133a and miR-208a showed the opposite trend.[15] The levels of the member of the miR-17-92 cluster, miR-92a, in whole-blood samples increased during exercise training in patients with CAD undergoing coronary bypass surgery.[28] Unfortunately, the study addressing the regulation of miR-92a during exercise training did not include a control group without coronary artery bypass graft, and there was no side-by-side comparison with healthy controls.[28] Moreover, the biological implications of reduced levels of the circulating miR-17-92a cluster in patients with CAD is unclear, because recent data suggest that miR-92a has a proatherosclerotic function and impairs endothelial cells.[29,30]

In patients with peripheral arterial disease, specifically atherosclerosis obliterans, serum samples showed an increase in miR-21, miR-130a, miR-27b, and miR-210, whereas miR-221 and miR-222 were decreased.[31] Interestingly, the dysregulation of circulating miRs predominantly mimicked the regulation of the miRs in tissue samples of the intima obtained from the same patients.[31] The increase in miR-130a and miR-27b levels correlated with disease severity.[31] Because both miR-130a[32] and miR-27b[33] are highly expressed in vascular cells and are involved in the regulation of angiogenesis, it might be interesting to further address a potential biological role of these miRs in atherosclerosis obliterans.

In diabetic patients, the so far largest study analyzing miR serum samples in 822 individuals revealed that miR-126 is most significantly downregulated and that reduced levels of miR-126 are associated with the risk for diabetes.[34] These findings are consistent with the vasculoprotective function of miR-126.[24,25,35] Moreover, the downregulation of miR-126 levels was additionally confirmed in microparticles derived from glucose-exposed endothelial cells in vitro and in diabetic mice models in vivo.[34]

A profound downregulation of other miRs (miR-20b, miR-21, miR-24, miR-15a miR-191, miR-197, miR-223, miR-320, and miR-486) was observed in prevalent diabetes, whereas miR-28-3p was increased.[34] The authors propose that a combination of 5 miRs (miR-15a, miR-126, miR-320, miR-223, miR-28-3p) is necessary and sufficient for a nonredundant classification. Interestingly, a dysregulation of these 5 miRs identified 52% of normoglycemic subjects to develop diabetes over the 10 years of follow-up.[34]

In patients with newly diagnosed type II diabetes, Kong et al showed that serum levels of miR-9, miR-29a, miR-30d, miR-34a, miR-146, miR-124, and miR-375 were significantly higher compared with levels in individuals with normal glucose tolerance.[36] Among these tested miRs, the proapoptotic miR-34a showed the highest canonical discriminant function coefficient.[36]

Recently, miR-503 was shown to be significantly regulated in serum of diabetic patients.[37] In fact, increased circulating levels of miR-503 were reported to reflect the increased expression in calf muscle biopsies obtained from diabetic patients. This study also demonstrated that inhibition of

Table 2. Circulating miRs in Patients With Vascular Disease

Clinical Study Cohort	Groups and Numbers of Patients Studied	Major Findings	Association/ Correlation	RNA Isolation	miR Detection	Reference
Stable CAD	31 pts with CAD 14 non-CAD pts	miR-126, miR-17, miR-92a, miR-155, miR-145 ↓	Association with sex, age, diabetes	TRIzol-based miRNA isolation protocol	qPCR TaqMan (ABI)	15
CAD	12 healthy controls 12 pts with CAD/CABG 10 pts with CAD/CABG undergoing rehabilitation	Whole blood: in CAD/CABG: miR-140-3P Rehabiliation: miR-92a/b ↑	Correlation of miR expression with regulation of miR target genes	PAXgene blood RNA kit (PreAnalytiX)	Illumina Arrays qPCR, TaqMan (ABI)	28
Peripheral artery disease	104 pts with atherosclerosis obliterans 105 age-matched controls	Intima samples: miR-21, miR-130a, miR-27b, miR-210 ↑ miR-221 and miR-222 ↓ Serum: miR-21, miR-130a, miR-27b, miR-210 ↑	Serum: miR-130a and miR-27b are positively correlated with Fontaine stages	mirVana miRNA isolation kit (Ambion)	SYBR Green real-time PCR	31
Diabetes	80 pts with diabetes 80 age-/sex-matched controls Confirmation: 822 individuals (Bruneck cohort)	miR-20b, miR-21, miR-24, miR-15a, miR-191, miR-197, miR-223, miR-320, miR-486, miR-126 ↓	Multivariate analysis showing that miR-126 was significantly reduced in diabetes (n=822)	miRNeasy kit (Qiagen)	TaqMan arrays Confirmation: qPCR, TaqMan (ABI)	34
Diabetes	18 pts newly diagnosed (T2D) 19 pts prediabetes impaired glucose tolerance 19 pts with normal glucose tolerance	In T2D compared to normal glucose tolerance: miR-9, miR-29a, miR-30d, miR-34, miR-146, miR-124, miR-375 ↑	miR-34 showed the highest canonical discriminant function coefficient	mirVana isolation kit (Ambion)	qPCR, TaqMan (ABI)	36
Diabetes	11 pts with diabetes 11 pts controls	Calf biopsies and plasma: miR-503 ↑		TRIzol (Invitrogen)	qPCR, TaqMan (ABI)	37
Hypertension	127 pts with hypertension 67 control subjects	let-7e, hcmv-miR-ULL112 ↑ miR-296–5p ↓		RNeasy mini kit (Qiagen)	miRCurry LNA Array qPCR, TaqMan (ABI)	38

miR indicates microRNA; CAD, coronary artery disease; qPCR, quantitative polymerase chain reaction; pts, patients; CABG, coronary artery bypass graft; PCR, polymerase chain reaction; T2D, type II diabetes.

miR-503 improves neovascularization in diabetic mice, suggesting that miR-503 might serve not only as a diagnostic tool but also as putative therapeutic target in diabetic patients.[37]

Profiling of plasma miRs in subjects with hypertension revealed 27 differentially expressed miRs, out of which 2 (namely let-7e and a human cytomegalovirus encoded miR hcmv-miR-UL112) were confirmed to be upregulated, whereas miR-296-5p was downregulated in a larger validation cohort.[38]

Of note, some additional studies might be relevant that determined the expression of miRs in whole-blood samples (which contain all blood-derived cells) or isolated circulating cells instead of plasma or serum. In RNA isolated from whole blood, patients undergoing coronary artery bypass surgery showed significantly reduced levels of miR-140-3p and miR-182.[28] In peripheral blood mononuclear cells, miR-146 levels were shown to be significantly higher in patients with CAD[39] and with rheumatoid arthritis,[40] supporting a role for vascular inflammation. Treatment of the CAD patients with statins (atorvastatin) and angiotensin II receptor blocker (telmisartan) decreased the miR-146 levels.[39] Because miR-146 levels are known to be regulated by the nuclear factor-κB pathway,[41] the reduction in miR-146 expression in peripheral blood mononuclear cells likely reflects the well-known antiin-flammatory effects of these drugs. In another study, analysis of miRs in circulating blood-derived endothelial progenitor cells revealed that miR-221/miR-222 levels were increased in patients with CAD, but reduced in those patients on statin therapy.[42] Because miR-221/222 are known to indirectly repress endothelial NO synthase,[43] which is required for endothelial progenitor cell function,[44] the increased expression of these miRs in patient-derived cells may contribute to the well-established reduced number and functional activity of endothelial progenitor cells in patients with CAD.[42,45]

Mechanisms of miR Release and Stabilization

Apart from their usefulness as potential disease biomarkers, circulating miRs might also have biological functions, eg, acting as long-distance signals, as is known from plants.[46] To exhibit any biological function, however, circulating miRs must be protected against degradation and likely need to be actively taken up, because the concentrations of circulating miRs are very low. Along this line, the understanding of the mechanisms underlying miR release and protection is of utmost importance to provide insights into their potential biological properties.

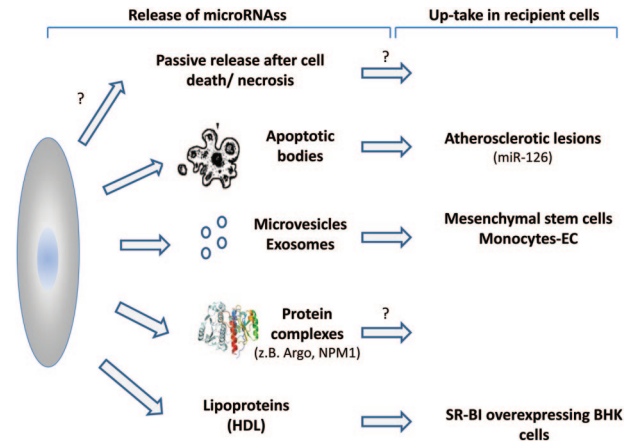miRs are detected in serum or plasma in a remarkably stable form, that can withstand repetitive freezing and thaw-

Figure. Mechanisms protecting microRNAs from degradation. EC indicates endothelial cells; HDL, high-density lipoprotein; NPM-1, nucleophosmin-1; SRB1, scavenger receptor class B member 1; BHK, baby hamster kidney.

ing cycles. In addition, circulating miRs are resistant against RNase-mediated degradation.[4,47] Increasing evidence suggest that several different mechanisms exist that protect miRs from degradation (Figure). One possible mechanism includes the storage of miRs in lipid vesicles. Several types of small lipid vesicles that are released by cells are described: microvesicles/microparticles are shed from the cell membrane from almost all cell types under physiological and pathological conditions and are relatively large (100 nm to 1 μm). In contrast, exosomes are smaller membrane fragments (30–100 nm) deriving from the endosomal compartment.[48] In addition, apoptotic bodies, which are larger (up to 4 μm), are released when cells are undergoing apoptotic cell death.[48]

Several groups have independently reported that mRNAs and miRs are actively secreted by a variety of cells in vitro either in exosomes[14,49,50] or in microvesicles.[5,34,51–53] miRs are also detectable in blood-borne microvesicles/microparticles isolated in humans.[34,54] miRs measured in microvesicles or exosomes are typically characterized by the resistance to RNase-dependent degradation under basal conditions, whereas the destruction of the lipid bilayers by detergents makes the miRs accessible to RNase-dependent degradation.[52]

The proposed mechanisms, which account for the release of miR-containing microvesicles, remains to be elucidated. In vitro studies using cultured cardiomyocytes recently suggested that miR-133 is exclusively released in exosomes after stimulation by the calcium ionophor A23.[14] In addition, it has been proposed that a ceramide-dependent pathway (by neutral sphingomyelinase 2) controls the intercellular transfer of miRs via exosomes.[50]

Besides microvesicles, miRs might also be exported in protein complexes protecting the miRs from being degraded. The coexistence of protein- and vesicle-bound miRs was elegantly documented by fractionation of human plasma samples by size exclusion chromatography and the demonstration that specific miRs were enriched in the fraction corresponding to the size of microvesicles (eg, let-7a), whereas others were detected in the protein fraction (eg, miR-92a).[55] Although little is known regarding the export mechanisms, protein-bound miRs were detected both in cell culture supernatants and in plasma samples. Nucleophosmin, a nuclear protein implicated in the nuclear export of the ribosome, was detected by a proteomic screen in cell culture supernatants.[51] Subsequent biochemical experiments demonstrated that nucleophosmin is bound to some but not all miRs and protects them from RNase-dependent degradation.[51] A second protein that was shown to be associated with circulating miRs is Argonaute 2. Thus, Argonaute 2 immunoprecipitates of human plasma contained some specific miRs (eg, miR-92a), as determined by polymerase chain reaction.[55] Thus, alterations in the levels of circulating miRs might also be secondary to differing levels of circulating protein complexes protecting the miRs from being degraded.

Very recently, lipid proteins were proposed to bind to and transfer miRs. In fact, high-density lipoprotein (HDL) isolated from human plasma contained small RNAs and miRs.[56] Some miRs (eg, miR-223, miR-105, miR-106a) were enriched in HDL isolated from patients with familial hypercholesterolemia.[56] Therefore, it will be important to investigate whether alterations in HDL serum levels might be accompanied by changes in the carrier function of HDL for selected miRs.

Biological Functions of Circulating miRs?

Besides using circulating miRs as biomarkers, one may speculate that circulating miRs control gene expression in an intracellular manner. In principle, extracellular RNA can be taken up by cells, as shown for RNA, which had been incorporated into microvesicles.[5,49] Additionally, several studies suggest that secreted microvesicles containing miRs can transfer the miRs to recipient cells and regulate target gene expression. Thus, microvesicles containing secreted monocytic miR-150, which are increased in the plasma of patients with atherosclerosis, were shown to be taken up by recipient endothelial cells and regulate migration and expression of the miR-150 target gene c-Myb.[52] Likewise, microvesicles derived from adult human bone marrow shuttle selected pattern of miRs to epithelial cells.[53] Others showed that apoptotic bodies, which are shed of endothelial cells and contain high levels of miR-126, regulate target gene expression in cocultured endothelial cells in vitro.[35] Moreover, the delivery of the miR-126 enriched apoptotic bodies reduced atherosclerotic lesion formation in apolipoprotein E−/− mice in vivo.[35]

Also, HDL-bound miRs have been shown to be transferred to cultured hepatocytes. For these experiments, native HDL was incubated in vitro with recombinant miRs and added to hepatocyte or baby hamster kidney cells. Moreover, HDL isolated from subjects with familial hypercholesterolemia, which shows very high concentrations of miR-105, induced an increased expression of miR-105 in recipient cells.[56]

Although most of these studies carefully document that either microvesicles or lipids influence the expression of miRs in recipient cells and that the increased levels of the miR is sufficient to affect target gene expression, one should keep in mind that both microvesicles and lipids are well known to influence cellular activities also by non-miR-related pathways.[57,58] Therefore, it needs to be ruled out that the effect is not mediated by cellular activation of the recipient cell, which may result in the change of endogenous miR

expression. Careful controls with miRs that are not endogenously expressed might be important to show that the concentration of the transferred miR is sufficient to directly mediate effects in the target cell.

Open Questions

The most important question is whether circulating miRs are indeed clinically useful as meaningful biomarkers. As discussed above, the publications so far support this concept. However, most available data derive from rather small numbers of patients, who are mostly compared with healthy controls. Furthermore, the prognostic impact of miRs has been examined in only 2 studies so far. At this point, larger prospective studies are mandatory to elucidate the contribution of circulating miRs on top of established risk assessment strategies (various scores and validated biomarkers).

Most importantly, the technology used to detect miRs requires optimization. Although several RNA isolation protocols with appropriate recovery rates are available, normalization remains the major challenge. Whereas spiking of the plasma/serum samples with recombinant nonhuman miRs (eg, *Caenorhabditis elegans* miRs) helps to normalize for efficiency of RNA isolation,[15] no housekeeping miR/small RNA has yet been established. Some studies used miR-17 to normalize the samples[12]; however, others have shown that miR-17 is increased in patients with acute coronary syndromes[14] and that the miR-17-92 cluster is known to be regulated by ischemia and inflammatory cytokines,[30,59] thus raising questions about the usefulness of this miR for normalization. Of note, serum protein biomarkers measured by ELISA are also usually not normalized. However, in contrast to real-time polymerase chain reaction, which varies from day to day even when using recombinant miRs as standard curves, ELISAs are well established and have little day-to-day variation. Finally, RNA isolation from plasma and subsequent quantification by real-time polymerase chain reaction, as done currently, is quite time consuming. For this reason, quickly available results, ideally in form of a bedside test, need to be developed to allow broad utilization of miR as biomarker.

As discussed above, circulating miRs might be relevant beyond their use as biomarker, and the ongoing studies will help to define whether miRs can give more detailed insights into the pathophysiology of cardiovascular disease. Given that miRs are also potential therapeutic targets and that various experimental and first clinical trials showed that miR inhibitors can be safely used in vivo, the detection of circulating miRs might be also helpful to monitor the efficiency of a specific therapy. Finally, the concept that miRs can be actively secreted raises interesting biological questions that may be relevant for cardiovascular diseases.

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None.

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