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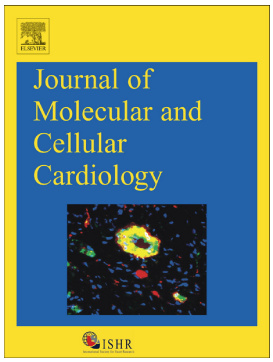

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Review article

The role of endothelial miRNAs in myocardial biology and disease

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Abstract

The myocardium is a highly structured pluricellular tissue which is governed by an intricate network of intercellular communication. Endothelial cells are the most abundant cell type in the myocardium and exert crucial roles in both healthy myocardium and during myocardial disease. In the last decade, microRNAs have emerged as new actors in the regulation of cellular function in almost every cell type. Here, we review recent evidence on the regulatory function of different microRNAs expressed in endothelial cells, also called endothelial microRNAs, in healthy and diseased myocardium. Endothelial microRNA emerged as modulators of angiogenesis in the myocardium, they are implicated in the paracrine role of endothelial cells in regulating cardiac contractility and homeostasis, and interfere in the crosstalk between endothelial cells and cardiomyocytes.

Key words: intercellular communication**,** endothelial cell, microRNA, extracellular vesicle, angiogenesis

1. Introduction

The myocardium is a highly structured tissue, composed of extracellular matrix and different cell types, which include cardiomyocytes, endothelial cells (ECs), fibroblasts, smooth muscle cells and immune cells. Although cardiomyocytes constitute the largest volume of the myocardium, they only represent around 30% of the total cell population [1]. In contrast, ECs are the most numerous cell type in the myocardium, constituting 43% of the total cell population and more than 60% of the non-cardiomyocytes [1]. This pluricellularity requires collaboration between cells to ensure appropriate cardiac function and adaptation, and this intercellular interaction is also important for processes like myocardial inflammation, extracellular matrix remodeling, and angiogenesis [2]. To facilitate collaboration between cells, an elaborate network of intercellular communication exists [3-5]. Communication between cells is mediated by direct cell-cell contact via cell junctions, by paracrine signaling, or by signaling via extracellular vesicles [6].

Recently, there is an increased interest in the role and underlying mechanisms of microRNAs (miRNAs) as epigenetic regulators in cardiovascular health and diseases [7, 8]. MiRNAs are small (~18-24nt), well-conserved single-stranded non-coding RNA molecules, that belong to the class of epigenetic regulators [9, 10]. MiRNAs are involved in several cellular processes in the myocardium by influencing translation of their target messenger RNAs (mRNAs) [11]. For example, miR-499 is related to cardiac myoblast proliferation and differentiation by targeting Transforming Growth Factor Beta Receptor 1 (TGFβR1) [12]. Likewise, in cardiomyopathies dysregulation of numerous miRNAs in multiple cardiac cell types – e.g. fibroblasts, ECs, pericytes, stem cells, and cardiomyocytes – has been demonstrated [13, 14]. For instance, cardiomyocyte-enriched miR-378, which modifies cardiac apoptosis and mitochondrial processes by modulating the levels of Insulin-like Growth Factor Receptor 1 (IGFR1) and ATP6 respectively [15, 16], shows cardiac upregulation in heart failure patients [17, 18]. Cells also release miRNAs in the circulation, a finding that can turn miRNAs into biomarkers of disease. Furthermore, because distant cells can internalize circulating miRNAs, synthetic miRNA agonists or antagonists hold a certain therapeutic promise. In this review, we will summarize the current knowledge on endothelial miRNAs as modulators of physiological and pathological angiogenesis, and as mediators of paracrine signaling in the myocardium. Additionally, we will discuss the contribution of miRNAs in EC-cardiomyocyte crosstalk in health and disease.

2. Endothelial cells regulate myocardial function

The endothelium, composed of a monolayer of ECs, constitutes the inner layer of all blood vessels present in the body [19]. ECs are highly interactive cells in the myocardium that influence neighboring cells – i.e. myocytes, fibroblasts, immune cells, and stem cells – through their sensing and effector functions [5]. Their sensing function enables an appropriate response upon detection of different stimuli – mechanical, hemodynamic, neurohormonal, or chemical – while their effector function enables communication with other cells via secretion of paracrine factors (Figure 1) [3, 5, 20]. In healthy conditions, the endothelium exerts various effects to maintain cardiac homeostasis; it promotes an anti-inflammatory and anti-coagulant state, it prevents smooth muscle cell proliferation and cardiomyocyte hypertrophy, and it regulates vascular tone. Other processes regulated by the endothelium include angiogenesis, cardiac repair, wound healing, and fibrosis [19, 21].

A major player in endothelial function is the vasoactive molecule nitric oxide (NO), which is produced by endothelial NO synthase (eNOS) [21, 22]. Secretion of NO by ECs affects cardiac contractility as well. NO readily diffuses into neighboring cardiomyocytes, where the

NO-sGC-cGMP axis enables activation of protein kinase G (PKG), which impedes pathways involved in cardiac hypertrophy, and decreases cardiomyocyte stiffness [3].
Furthermore, disruption of mechanotransduction in ECs results in decreased endothelial NO production leading to cardiac fibrosis [23].
Therefore, ECs do not only affect cardiac function indirectly by improved myocardial perfusion and by regulating vascular permeability, but they also affect cardiac function directly, by releasing multiple paracrine signaling factors [3, 5].
Paracrine factors secreted by ECs, such as endothelin-1, C1q-tumor necrosis factor–related protein-9 (CTRP9), and neuregulin-1, can modulate multiple aspects of myocardial function and can interact either in synergy or in an inhibitory manner (Figure 2) [5, 24-27]. Consequently, endothelial dysfunction triggered by stressors is a common pathophysiological feature of different cardiovascular disorders, including heart failure, cardiomyopathies, and atherosclerosis [26, 28-30]. Hence, clinical measurement of endothelial function assists in cardiovascular risk stratification [31, 32].

Interestingly, paracrine signaling by cardiomyocytes affects endothelial function as well. For example, cardiomyocyte secretion of endothelin-1 and fibroblast growth factors mediate vascular tone, while cardiac expression of GATA4 induces vascular endothelial growth factor A (VEGF-A) expression, that stimulates endothelial angiogenic activity [33, 34]. Myocardial contractile dysfunction can contribute to endothelial dysfunction as cardiac output, shear stress, and vessel injury modulate NO bioavailability and endothelial integrity. For a more detailed description of EC-cardiomyocyte crosstalk (Figure 2), we refer the reader to excellent reviews [3, 26, 27].

3. MiRNAs regulate gene expression

Recently, miRNAs have been identified as important players in cardiovascular diseases [35]. MiRNAs are synthesized as precursor miRNAs and cleaved by Drosha and Dicer enzymes, turning them into mature miRNAs. One strand (~guide strand) is loaded on the RNA-induced silencing complex (RISC) with Argonaute protein for RNA-interference, while the other strand (~passenger strand) is degraded (Figure 3) [35].

MiRNAs modulate gene expression at the post-transcriptional level, either by inhibiting translation or by destabilizing mRNA. The final effect depends on a sequence-specific interaction with the 3'-untranslated region or 5'-untranslated region of the target mRNA, which can be a perfect match or show some mismatches [8, 36, 37]. As a consequence, a single miRNA can modulate multiple genes, while one gene can be influenced by multiple miRNAs. This enables a single miRNA to induce synergistic effects at multiple levels in signaling cascades [20]. It is estimated that around 60% of all protein-coding genes are modulated by endogenous miRNAs, which explains their strong impact on different physiological pathways [9, 38-40].
Currently, unraveling the functional spectrum of individual miRNAs has become an evolving field of research, with a focus on tissue-specific miRNA signatures [41].
Cardiomyocyte-specific deletion of Dicer resulted in dilated cardiomyopathy and heart failure, which illustrates the importance of miRNAs in the heart.
Of note, expression of miRNAs is tissue- and time-dependent [42-45], while also, intra- and inter-laboratory variation in detection of miRNAs is still an issue. Standardized, validated protocols are now emerging in an effort to reduce this variation [46, 47].

4. Endothelial miRNAs modulate angiogenesis in the myocardium

Angiogenesis is a complex process involving sprouting of new blood vessels from pre-existing ones. It is part of cardiac remodeling observed in the adult myocardium, particularly important for tissue homeostasis and for the reparative process after cardiac injury. Cardiac hypertrophy is a common feature of cardiac remodeling after sustained pressure overload. However, important differences exist between physiological, i.e. exercise-induced, and pathological cardiac remodeling, the latter characterized by a decrease in capillary density [28]. When angiogenesis does not match the hypertrophic response, relative ischemia will occur and contribute to myocardial dysfunction, including reduced contractility and impaired cardiac growth. The latter steers the progression from adaptive cardiac remodeling to heart failure [48], and is seen in multiple disorders [49-51]. Accordingly, strategies that increase angiogenesis after myocardial infarction have been shown to improve cardiac function *in vivo* [52, 53]. In reverse, angiogenesis is not only necessary to support cardiac hypertrophy in the normal heart, it might also activate cardiac hypertrophy, called angiogenesis-driven hypertrophy [54].

ECs are key players in angiogenesis: (i) their proliferating, migrating, and differentiating capacity is central in the process, and (ii) they modulate the balance of pro/anti-angiogenic proteins and growth factors [55]. MiRNAs take part in this process, since recent studies in endothelial-specific Dicer-null mice and *in vitro* Dicer-knockdown cells demonstrate altered expression of important angiogenic regulators, such as VEGF receptor (VEGFR), Tie-1, IL8, c-Kit, and eNOS (Figure 4) [20, 56].

Examples of anti-angiogenic miRNAs in the heart are miR-92a, miR-24, miR-26a, and miR-100, while pro-angiogenic activity is linked with angiomiRs like miR-126 and the miR-23~27 cluster. Some miRNAs, like miR-21, have a dual role: they can stimulate and inhibit angiogenesis depending on the cellular context. With respect to their angiogenesis modulating activity, miRNAs emerge as potential therapeutic targets in patients after an ischemic event or in patients suffering from myocardial capillary rarefaction, which is a hallmark of heart failure pathology [20].

In the next sections, we will discuss the actual knowledge of several endothelial miRNAs with a known role in myocardial angiogenesis.

4.1 MiRNAs with anti-angiogenic properties

4.1.1 MiR-92a

Shear stress regulates the expression of specific miRNAs, including the miR-17~92 cluster, which consists of miR-17, miR-18a, miR-19a/b, miR-20a, and miR-92a. This cluster has both pro- and anti-angiogenic capacities, but anti-angiogenic properties prevail for most members upon stimulation with VEGF-expression [57, 58]. Among them, miR-92a is abundantly present in ECs and elicits an anti-angiogenic effect in the myocardium. *In vitro* assays with ECs and *in vivo* mouse models of acute myocardial infarction demonstrate an interaction of miR-92a with MAP kinase kinase 4 (MKK4), Krüppel-like factor 4 (KLF4), and integrin subunits α5 (ITGA5). All these targets are involved in endothelial homeostasis and angiogenesis, and interfere with eNOS levels [59-63].

Interestingly, increased levels of miR-92a-containing microvesicles were detected in blood samples of patients with acute myocardial infarction and the same observation was found in murine hearts after myocardial infarction [59, 64]. Preclinical studies indicate that miR-92a inhibition could have therapeutic potential. For instance, intracoronary injection of miR-92a antagomir in pig and mouse models of acute myocardial infarction resulted in increased vessel growth, attenuation of cardiac remodeling and functional cardiac recovery [62, 65].

4.1.2 MiR-26a

MiR-26a is abundant in ECs [66], but is also expressed in other cell types, such as cardiomyocytes and vascular smooth muscle cells [67, 68].

Evidence provided by overexpression and knockdown studies in mice and zebrafish demonstrate that miR-26a impairs myocardial angiogenesis by targeting the bone morphogenetic protein (BMP) – SMAD1 – inhibitor of DNA binding 1 (Id1) axis [69]. Repression of SMAD1 expression leads to decreased levels of the Id1 transcription factor [70], and accordingly to the inhibition of growth and migration of ECs due to changes in the $p21^{WAF/CIP}$ /p27 cell cycle inhibitor [71-73]. Furthermore, *in vitro* studies show that miR-26a targets the Nogo B receptor (NgBR), thus, affects NgBR – NO – VEGF axis leading to attenuated VEGF-mediated angiogenesis [74].

MiR-26a also plays a role in pathological angiogenesis in the myocardium: endothelial expression of miR-26a was elevated in a mouse model of acute myocardial infarction, with peak expression in the infarct zone 1 hour after the ischemic event. In contrast, pro-angiogenic stimuli, e.g. VEGF, reduced endothelial miR-26a expression levels [69]. Consistent with these findings, administration of a synthetic miR-26a inhibitor in this murine model enhanced cardiac angiogenesis even in the early ischemic phase, and therefore reduced infarct size and ameliorated left ventricular cardiac function. These data indicate that miR-26 inhibition could be a potential therapy to enhance early angiogenesis in ischemic myocardium [69].

Interestingly, miR-26a plasma levels were increased in this murine model 45 minutes after an acute ischemic event and the same phenomenon was seen in patients with acute coronary syndrome [69, 75]. The fact that miR-26a is present in the circulation could indicate that it plays a role in intercellular communication. Another study focusing on serum-based diagnostic miRNAs, however, identified reduced miR-26a serum levels in patients with acute myocardial infarction [76].

The difference between those studies could be due to different processing of samples (i.e. serum vs plasma), different working procedures including different normalization methods (i.e. U5S vs spike in), different RNA isolation methods, different statistical analyses, cohort-associated discrepancies such as patient age, disease severity, comorbidities and group size, and a different time point of sampling after the ischemic event.

Taken together, more research is needed to clarify the role of miR-26a in cardiovascular disease.

4.1.3 MiR-100

MiR-100 is mainly expressed in ECs and vascular smooth muscle cells, where it exerts anti-angiogenic activity via targeting of mammalian target of rapamycin (mTOR) [77]. Evidence from *in vitro* studies shows a role for miR-100 in sprouting activity, proliferation, and tube formation in ECs. During hypoxic conditions, miR-100 downregulation was observed. Meanwhile, *in vivo* examination of miR-100 knockdown in the hind limb ischemia mouse

model demonstrated increased angiogenic activity and improved perfusion after ischemic events [77].

In line with these observations, a role for miR-100 in cardiac angiogenesis was described; higher miR-100 expression levels were identified in patients with heart failure, where it might contribute to capillary rarefaction in the myocardium [78].

4.2 MiRNAs with both pro- and anti-angiogenic properties

4.2.1 MiR-21

MiR-21 is expressed in most cells of the cardiac system, including cardiac ECs [56, 79, 80], and it exerts its beneficial cardiovascular effects by stimulating angiogenic activity. In a rat model of acute myocardial infarction by LAD ligation, Phosphatase and Tensin Homologue (Pten) was identified as a target of miR-21. Pten is an upstream regulator of the Phosphoinositide 3-kinases (PI3K)/Akt and MEK/ERK pathway; hence, administration of miR-21 mimics in this model led to increased VEGF expression resulting in reduced infarct size and lower endothelial injury [81]. Of note, the cardiac effects of miR-21 targeted therapy are not necessarily mediated by ECs. Cardiac fibroblast also express miR-21, and *in vivo* silencing of miR-21 in fibroblasts improved cardiac hypertrophy and fibrosis in a mouse model of pressure overload [82].

In contrast to cardiac ECs, investigation of this miRNA in angiogenic assays with human umbilical vein ECs revealed both pro- and anti-angiogenic capacities of miR-21. In this setting, miR-21 targeted both the pro-angiogenic Rho-GTPase and Rho-related GTP-binding protein RhoB, and also the anti-angiogenic Sprouty RTK Signaling Antagonist 1 (Spry1) [83]. Both *in vitro* observations and *in vivo* findings in a model of choroidal neovascularization demonstrate reduced angiogenic activity when miR-21 is overexpressed, indicating that anti-angiogenic properties prevail in these models [83]. A study confirming those targets in cardiac ECs is still missing.

In humans, upregulation of miR-21 is observed in the failing heart and is associated with cardiac hypertrophy [82].

In summary, these results indicate that endothelial miR-21 has an ambiguous role in angiogenesis, possibly due to tissue-related and spatiotemporal effects. Nevertheless, evidence highlights miR-21 mediated pro-angiogenic activity in myocardial ECs [84].

4.2.2 MiR-23~24~27 cluster

The miR-23~24~27 cluster is highly expressed in cardiac ECs and in strongly vascularized tissues [85]. This cluster consists of both pro-and anti-angiogenic miRNAs. Among them, miR-23 and miR-27 stimulate angiogenic activity through modulation of their anti-angiogenic targets, Spry2 and Sem6a, which preserve an active RAF/ERK pathway following VEGF stimulation. Consistently, knockdown of corresponding miRNAs leads to impaired vascular network formation in a model of choroidal neovascularization [86]. In reverse, administration of miR-27 mimics in a mouse model of cardiac ischemia by coronary artery ligation significantly improved cardiac function, illustrating an important role in myocardial function [87].

MiR-24 provides mostly anti-angiogenic effects and is upregulated in cardiac ECs during stress conditions, e.g. myocardial ischemia [85]. Its mode of action involves the induction of endothelial apoptosis on one hand and impairment of vascular development on the other hand. More specifically, miR-24 targets the transcriptional factors GATA2, P21 activated

kinase 4 (PAK4), and pro-angiogenic eNOS [88, 89]. Downstream of miR-24, multiple effects occur. First, miR-24 represses the vasoprotective and anti-apoptotic *HMOX1* gene, which affects neovascularization through VEGF and stromal cell-derived factor 1 (SDF-1) signaling. Second, miR-24 inhibits Sirtuin 1 (SIRT1), which influences negative regulators of angiogenesis [90]. Third, miR-24 reduces inactivation of the pro-apoptotic protein BAD [88]. Overexpression of miR-24 also increases production of reactive oxygen species (ROS) and increases sensitivity for DNA-damage [91, 92]. After myocardial ischemic events, levels of miR-24 were primarily upregulated in the peri-infarct endothelium [92]. In line with these observations, *in vitro* and *in vivo* studies using both miR-24 overexpression and knockdown in zebrafish models and cardiac ischemic mouse models, demonstrated a therapeutic benefit of inhibiting miR-24 after cardiac ischemic injury [89, 92]. Moreover, increased expression levels of both miR-23 and miR-24 were observed in murine cases of cardiac hypertrophy [93].

4.3 MiRNAs with pro-angiogenic properties, angiomiRs

4.3.1 MiR-126

MiR-126 is almost exclusively present in ECs [28, 94], but lower levels of miR-126 have also been described in other cell types including cardiomyocytes [95].
This master regulator of vascular function is responsive to hypoxia and regulates many aspects of EC function and physiology. Examples include cell survival, cell migration, cytoskeletal function, adhesion factor expression, and maintenance of vascular integrity [96, 97].

Regarding the process of angiogenesis, miR-126 regulates the response of ECs to angiogenic factors like VEGF, basic fibroblast growth factor (bFGF), and endothelial growth factor (EGF) by targeting negative regulators of the VEGF pathway, including the Sprouty-related EVH1 domain-containing 1 (*SPRED1*), regulator of G-protein signaling 16 (*RGS16),* delta-like 1 homolog *(Dlk1)* and phosphoinositol-3 kinase regulatory subunit 2 (*PIK3R2).* Meanwhile, miR-126 also directly targets SDF-1 [98], which is a chemokine that attracts endothelial progenitor cells [96, 99, 100].

In vivo evidence by knockdown experiments in zebrafish and mice showed that loss of miR-126 results in compromised vascular integrity during embryonic development with a deficient response to angiogenic stimuli and impaired cardiac neovascularization, often accompanied by myocardial ischemia [96, 100]. Likewise, evidence was provided *in vivo* illustrating a supporting role for miR-126 in ischemia/reperfusion-induced angiogenesis [101].

Overall, miR-126 is not necessary for endothelial lineage commitment, but does seem to play an important role in maintaining vascular integrity [96].

4.3.2 MiR-210

MiR-210 is upregulated by hypoxia, mediated through hypoxia-inducible factor 1α (HIF-1α); its upregulation increases angiogenesis in ECs by repression of its targets ephrin-A3, caspase 8-associated protein 2, Notch, and Polypyrimidine Tract Binding Protein 1 (Ptpb1), leading to increased expression of pro-angiogenic factors [102, 103]. Overexpression of miR-210 in rodent animal models of myocardial ischemia rescued cardiac function by increasing capillary density and reducing apoptosis [104, 105].

4.3.3 MiR-132

Endothelial miR-132 is an early activator of quiescent ECs and is induced by the expression of angiogenic growth factors during developmental and pathological conditions. The effects of miR-132 are related to its downregulating activity of p120RasGAP, a negative modulator of the RAS signaling pathway, and thus stimulates EC proliferation and migration [106, 107]. Interestingly, *in vivo* transplantation of miR-132- containing exosomes to ischemic hearts in an acute myocardial infarction mouse model increased neovascularization in the peri-infarct zone, while cardiac function was maintained [108].

More studies of this interesting miRNA are needed, because its growth factor-inducible character indicates that it participates only in angiogenesis if a set threshold of growth-stimulation is reached [109].

5. Endothelial miRNAs modulate paracrine signaling in the cardiovascular system

Paracrine signaling defines a mode of intercellular communication by secretion of signaling molecules to neighboring cells. The previous section showed that expression of paracrine factors by ECs can be modulated by specific endothelial miRNAs in the myocardium, for example: (i) miR-126 stimulates SDF-1 expression in cardiac tissue, while (ii) miR-24 attenuates cardiac NO production by targeting eNOS. These examples show that endothelial miRNAs with a clear role in cardiac angiogenesis can influence endothelial function in multiple ways.

Nevertheless, the spectrum of endothelial miRNAs that interfere with paracrine signaling in the cardiovascular system is much broader. For instance, a wide range of inflammatory factors like NFκβ and its target genes IL6, IL8, and tumor necrosis factor alpha (TNFα) have been shown to be regulated by endothelial miR-146a in cardiac tissue [110-112].

Moreover, there is evidence demonstrating that miR-125a/b modulates endothelin-1 expression in vascular ECs. Endothelin-1 is a potent vasoconstrictive peptide involved in endothelial function, which shows very low baseline levels in normal physiological conditions. Howbeit, a major role has also been described during pathophysiological conditions, like heart failure, hypertension, and atherosclerosis [113, 114]. In vitro approaches, including luciferase reporter assays, overexpression cell models, and western blotting, indicate a direct interaction between miR-125a/b and the 3' untranslated region of prepro-endothelin-1 mRNA. In accordance with this, upregulation of endothelin-1 was detected in aortic tissue upon administration of miR-125a/b inhibitors to stroke-prone spontaneously hypertensive rats and in pericardial fluid of patients with acute coronary syndrome [115, 116].

Another important paracrine factor for vascular pathology, which is partly regulated in a miRNA-dependent manner, is neuregulin-1. Neuregulin-1 provides pro-survival and anti-apoptotic effects in vasculature [117]. It has been demonstrated that circular neuregulin-1 RNA can function as miRNA sponge for miR-193b, which leads to reduced protein expression of neuregulin-1. Upregulation of this RNA/miRNA axis has been described upon angiotensin II expression to modulate expression levels of neuregulin-1 protein [118].

Yet, modulation of paracrine factors by miRNAs is not restricted to direct repression of gene expression, but is present at the receptor level as well. VEGFR2 and fibroblast growth factor receptor 2 (FGFR2) levels, for instance, are reduced by endothelial miR-16 and miR-424 [119], however, evidence related to activity in the cardiac system for the latter is still lacking.

In summary, endothelial miRNAs can fine-tune paracrine signaling of factors in the cardiovascular system, either in a direct or indirect way.

6. Circulating miRNAs in myocardial biology

MiRNAs are not only present intracellularly to induce local effects and modulate paracrine signaling as described in previous sections, but they are also secreted in the extracellular environment including the circulation [120].

MiRNA secretion from different cell types is involved in regulation of myocardial biology and is promoted by various stimuli, including hypoxia, shear stress, oxidative stress, and cell damage [99, 121]. Generally, secretion of miRNAs in the circulation occurs in different ways: (i) shedding of miRNA-enriched extracellular vesicles, (ii) miRNAs bound to high-density lipoprotein complexes and RNA-binding protein complexes, and (iii) leakage of miRNAs from apoptotic/necrotic cells [122-124]. Those circulating miRNAs can be internalized by target cells at remote locations, locally inducing alterations in gene expression. This phenomenon reflects a role of miRNAs in *inter*cellular signaling besides their role in *intra*cellular regulation of gene expression [125]. Thus, in addition to classic forms of communication in the myocardium (e.g. paracrine signaling of soluble molecules, endocrine signaling, and cell-cell junctions), circulating miRNAs can be regarded as a distinct mode of long-distance intercellular communication for modulation of myocardial processes, including angiogenesis and cardiac metabolism [126-128].

Another interesting feature of circulating miRNAs is related to their small size and the compartmentalization into different complexes that protect against degradation. This makes them very stable in the extracellular compartment, hence, enables the measurement of circulating miRNAs in the peripheral blood [129]. Because miRNAs are highly sensitive to biological changes such as alterations in myocardial biology, circulating miRNA levels can respond to an ongoing pathological process in the body, for instance myocardial injury, and thus serve as potential biomarkers for cardiovascular disease [125, 130-132]. Research has demonstrated that increased levels of circulating miR-17, miR-126 and miR-143 provide a potential diagnostic value for acute myocardial events, reflecting endothelial dysfunction of the myocardium [133]. Furthermore, a few studies focusing on miRNA-based biomarkers for cardiovascular diseases have already entered the clinical trial phases [134, 135]. Yet, for a more comprehensive overview, we refer the reader to a more detailed review [35].

Notwithstanding extensive research in this field, many issues remain to be resolved. For example, the origin of circulating miRNAs, and the mechanisms for selective loading of specific miRNAs in these secreted vesicles are largely unexplained. Several mechanisms for the latter have been suggested: (i) neural sphingomyelinase 2 (nSMase2)-dependent sorting [136], (ii) heterogeneous nuclear ribonucleoproteins (hRNPs)-dependent sorting [137], and (iii) miRNA-induced silencing complex (miRISC)-dependent sorting [138]. For a detailed review on this topic, we refer the reader to [138].

7. Endothelial miRNAs in extracellular vesicles act as paracrine signals

Several studies have shown that miRNAs are stable in the circulation, where they take part in long-distance intercellular communication as described in previous section. Additionally, it

has been demonstrated that circulating miRNAs can serve as paracrine factors between different cells *in vivo*. This observation could be partly explained by the complexation with extracellular vesicles or the binding to RNA-binding proteins, as they provide a higher stability in body fluids and enable intercellular transport [28, 122, 123].

Extracellular vesicles define a group of small membrane-enclosed vesicles that are secreted by multiple cell types in a stimuli-induced or constitutive manner. In most cases, they manage the transfer of biological material to cells at remote locations [139, 140]. Different types of extracellular vesicles exist, with microvesicles, exosomes and apoptotic bodies being the most studied ones.

Microvesicles constitute a group of vesicles that are formed by direct (outward) membrane budding and are delineated by a cellular membrane with a size of 100-1000nm. Their biological content is dependent on the membrane of origin and includes proteins, DNA, but also miRNAs [95, 140-142]. Internalization via endocytosis of these microvesicles by neighboring cells as well as cells at remote locations represents a way of intercellular communication.

Exosomes are the smallest type of extracellular vesicles, with dimensions of less than 100nm [143]. These double-membraned vesicles arise from the fusion of multivesicular bodies located in early endosomes with the plasma membrane, and thus have an endocytic origin. In contrast to microvesicles, exosomes are only derived from viable cells and require Alix protein and Rab27a/b for docking to the plasma membrane. Exosomes are unique to the cell of origin and possess a specific subset of surface tetraspanin proteins [139, 140, 144, 145]. The specific content of the vesicles is strongly dependent on external stimuli [6]. Following secretion, exosomes are internalized via endocytosis after binding to receptors present at the recipient cell (reviewed in [139]).

Apoptotic bodies on the other hand represent larger particles (0.5-5µm) that are shedded during the apoptotic process. In contrast to previous vesicle types, organelles are present within the apoptotic body and the only acceptor cells for clearance by phagocytosis are macrophages. Opposite to the general consideration that apoptotic bodies were only important for the clearance of cell debris, a role was demonstrated in intercellular exchange of genetic information [146].

Recent evidence demonstrates an important role for extracellular vesicle-mediated miRNA transport as an alternative way of cell-to-cell communication. For instance, miR-126 is detected in circulating apoptotic bodies and in microvesicles derived from ECs [99]. When secreted from ECs, miR-126 has anti-atherosclerotic effects, besides its pro-angiogenic activities [99]; injection of apoptotic bodies containing miR-126 in atherosclerotic-prone ApoE null mice reduces progression of atherosclerosis [99]. This study was the first to show that circulation of miRNAs in microvesicles can affect ECs and VSMCs at remote locations [99, 147]. It was also shown that miR-126 induces SDF-1 expression and inhibits the expression of vascular cell adhesion molecule 1 (VCAM-1) in ECs [99]. SDF-1 is a chemokine that attracts endothelial progenitor cells and has cardioprotective properties, while inhibition of VCAM-1 restricts the entrance of inflammatory cells [52, 53, 148].

In humans, reduced miR-126 levels were detected in plasma of patients with atherosclerotic coronary artery disease [149]. Interestingly, plasma levels of circulating members of the miR-17~92 cluster behaved in a similar way [149]. Besides, a decline of miR-126 enriched vesicles has been demonstrated in patients with type 2 diabetes, and is linked to the

occurrence of endothelial dysfunction and peripheral artery disease [150]. In contrast, patients with acute coronary syndrome show higher levels of circulating miR-126, presumably related to high rates of apoptotic body release from ECs [151].

In addition to miR-126, miR-222 in EC-derived microvesicles also provides anti-inflammatory effects in atherosclerosis. Systemic injection of miR-222-enriched microvesicles in ApoE-null mice results in reduced endothelial expression of ICAM-1, with subsequent reduced infiltration of macrophages in atherosclerotic plaques. Interestingly, miR-222-containing microvesicles are lowered in atherosclerotic plaques [152].

Similar to microvesicles, exosomes play a role in intercellular communication and cardiac homeostasis by transfer of proteins, mRNAs, and miRNAs [122, 153-155]. ECs are able to secrete or capture exosomes, while exosomal transfer of miRNAs influences endothelial function. For example, *in vitro* and *in vivo* evidence illustrates that secretion of miR-214-rich exosomes by ECs supports angiogenesis and suppresses senescence in endothelial recipient cells by repression of ataxia telangiectasia mutated (ATM) [156]. Upregulation of miR-214 in cardiac tissue was detected in human subjects of cardiomyocyte hypertrophy and heart failure [93].

Exosome-mediated communication is not restricted to ECs as receptor cells, as EC-secreted exosomes can also target other cell types, including vascular smooth muscle cells. For instance, it has been shown that laminar shear stress induces endothelial upregulation of the miR-143/145 cluster in a krüppel-like factor 2 (KLF2)-dependent manner, whereupon miRNA transfer occurs via exosomes to vascular smooth muscle cells to control their phenotype [157]. This *in vivo* study illustrates that the transcription factor KLF2 mediates an anti-atherosclerotic effect through exosome-miRNA signaling from ECs to vascular smooth muscle cells.

Altogether, these studies indicate that endothelial miRNAs, apart from their intracellular effects and modulation of paracrine signaling of growth factors and cytokines, could function as direct paracrine signaling factors [28]. Noteworthy, altered miRNA levels are already detectable years before clinical manifestation of cardiovascular disease, which highlights the potential use of (circulating) miRNAs as biomarkers [120].

8. MiRNAs in endothelial cell-cardiomyocyte crosstalk

Communication between ECs and cardiomyocytes is crucial to maintain normal cardiac function. EC-cardiomyocyte crosstalk involves multiple signaling molecules, including VEGF, neuregulin-1 and NO, but also miRNAs. MiRNA transfer between ECs and cardiomyocytes involves extracellular vesicles or shuttling via high-density lipoproteins [158]. MiRNAs can also be involved indirectly in EC-cardiomyocyte communication. For instance, endothelial-derived NO-production stimulates miR-182 expression in cardiomyocytes inducing a hypertrophic response, also referred to as angiogenesis-induced cardiac hypertrophy [159]. To date, our understanding of miRNAs in EC-cardiomyocyte crosstalk is still limited, but research on this topic is expanding.

In this section, we will focus on the phenomenon of miRNA-based intercellular communication within EC-cardiomyocyte crosstalk (Figure 5).

8.1 Endothelial miRNAs can be transferred to cardiomyocytes by cell-cell contact

Recently, it has been demonstrated that ECs can modulate intracellular processes in cardiomyocytes by transfer of endothelial miRNAs (Figure 5). The process of angiogenesis, for instance, is key in cardiac remodeling and maintenance of cardiac function, and is partly controlled by EC-cardiomyocyte crosstalk with involvement of endothelial miRNA transfer. For example, trafficking of pro-angiogenic miR-210 and miR-126-enriched exosomes derived from hypoxic cardiac ECs to cardiac progenitor cells increases the resistance of the latter to hypoxic stress through regulation of angiogenic and pro-survival pathways, such as the PI3K/Akt pathway. Therefore, they provide a transferable cardioprotection during ischemic events [127, 160].

In a similar way, it has been shown *in vivo* that exercise training provides a cardiovascular protective effect, which is partly mediated by endothelial miRNAs involved in cardiomyocyte modulation. A study in patients and murine models found that exercise-induced laminar shear stress induces an upregulation of miR-342-5p in ECs [161]. Secretion of those miR-342-5p-rich exosomes and subsequent absorption by cardiomyocytes locally decreases apoptotic events in the latter following ischemic events by targeting the pro-apoptotic caspase 9 and Jnk2, and by promoting survival signaling by reducing Ppm1f expression. In this way, these endothelial-derived exosomes protect cardiomyocytes from ischemic injury [161].

MiRNA-mediated EC-cardiomyocyte crosstalk also participates in pathological conditions, such as peripartum cardiomyopathy. Peripartum cardiomyopathy is defined as pregnancy-associated heart failure and has a high mortality rate [162]. There seems to be a dual role for miR-146a in peripartum cardiomyopathy. On one hand, the 16kDa N-terminal Prolactin fragment (16K PRL), which is considered one of the initiating factors of peripartum cardiomyopathy, stimulates endothelial upregulation of miR-146a resulting in disturbed angiogenesis by direct targeting of NRAS signaling [128].

On the other hand, 16K PRL promotes the release of endothelial miR-146a loaded exosomes. *In vitro* evidence from cardiomyocyte culture and *in vivo* injection of miR-146a-enriched endothelial exosomes in murine models illustrates efficient uptake by cardiomyocytes, leading to decreased cardiac metabolic and contractile activity through targeting Notch1, Erbb4 and interleukin 1 receptor-associated kinase 1 (Irak1) [128].

Both modes of actions add to the development of cardiac dysfunction. Consistently, blocking miR-146a in a peripartum mouse model results in an attenuated cardiomyopathy phenotype [128].

Altogether, these findings indicate the added value of endothelial miRNAs as paracrine factors in EC-cardiomyocyte crosstalk in both health and disease. This additional layer of regulation opens a new window of potential therapeutic targets and when studied, will provide more insights in physiological and pathophysiological mechanisms in the cardiovascular system.

8.2 Extracellular vesicles secreted by cardiomyocytes regulate endothelial function

ECs not only modulate cardiomyocyte function by secreting extracellular vesicles, but the reverse phenomenon takes place as well.

In 2007, the first study was published showing that extracellular vesicle secretion by cardiomyocytes can modulate EC function upon hypoxia (Figure 5) [6]. Angiogenesis is one of the parameters controlled by cardiomyocyte-EC crosstalk. For instance, a study showed that glucose starvation in cardiomyocytes *in vitro* induces the secretion of exosomes that are trafficked to ECs in order to stimulate an angiogenic response [95]. These secreted microvesicles contain angiogenic proteins but also angiogenic miRNAs, including miR-17, miR-19a, miR-19b, miR-20a, miR-30c, and miR-126 [95, 163, 164].
Likewise, cardioprotective miRNAs, i.e. miR-210 and miR-132, are shipped from cardiomyocytes undergoing an ischemic event to remote cells to locally activate protective mechanisms against ischemic stress. The phenomenon of transferable cardioprotection improves cardiac and vascular function in rat models of myocardial infarction and might be amenable for therapeutic use [165].
In contrast, an anti-angiogenic response is observed when ECs are incubated with cardiomyocyte-derived exosomes from Goto-Kakizaki rats, which is a commonly used model of type 2 diabetes. The response is attributed to higher levels of miR-320 in exosomes derived from diabetic cardiomyocytes. MiR-320 exerts these anti-angiogenic effects by targeting IGF-1, Hsp20 and Ets2 [166]. These data are relevant in the pathophysiology of diabetic cardiomyopathy, because they can partly explain the defective angiogenesis seen in diabetic hearts. Of note, exosomal levels of miR-126 are also significantly reduced in this model [166].

Another interesting study indicates the paracrine role of cardiomyocyte-derived miR-199a-rich vesicles on endothelial function [167]. It showed that miR-199a stimulates protein arginine methyltransferase I (PRMT)-mediated synthesis of a well-known NO synthase inhibitor, asymmetric dimethylarginine, by targeting Ube2i and Ube2g1. The final result is reduced NO production by ECs [167].

Overall, these findings emphasize an appealing role for extracellular vesicle-mediated miRNA transfer as part of EC-cardiomyocyte crosstalk. The latter might be an interesting point for therapeutic interventions in cardiomyopathies. Recent insights in the mediator role of miRNAs in this bidirectional communication seems a promising field, however, current understanding is still limited.

9. Future prospects: targeting cardiac disease through endothelial miRNA modulation

Research over the last two decades revealed an important role for miRNAs in multiple diseases, including cancer, neurological disorders, and immune diseases. As is the case in these pathologies, miRNAs are also involved in disease progression, prognosis, diagnosis, and treatment response in cardiac disorders [168].

Modulation of endothelial miRNAs can affect the course of different diseases, as is shown in cancer pathology. For instance, upregulation of the endothelial miR-17-92 cluster promotes tumor-angiogenesis and acts as an oncogenic cluster, while beneficial effects are observed *in vivo* when expression is reduced (reviewed in [169]). Considering endothelial dysfunction as a central mediator of many cardiovascular disorders, such as atherosclerosis, cardiac infarction, and heart failure, cardiovascular diseases could benefit from endothelial miRNA modulation as well. Additionally, the pivotal role of ECs in modulating cardiac function also

implies that cardiomyocytes can indirectly be targeted by modulating endothelial miRNAs [3, 128, 170, 171].
For therapeutic and diagnostic purposes, endothelial miRNAs could be promising targets for the following reasons: (i) they are highly stable in body fluids, including the circulation, (ii) they play a role in normal myocardial physiology as well as in pathophysiology of the cardiovascular system, by influencing intracellular cell biology and intercellular communication, (iii) they can readily be taken up in target cells [172]. Furthermore, endothelial miRNAs influence multiple targets simultaneously, which implicates that it could be possible to modulate multiple pathway levels and to achieve a larger effect size compared to standard single-target drugs. On the flip side, a lower target specificity could result in more side effects [173, 174].

Along with advancing technologies, several RNA interference therapy tools are currently available to modulate miRNA expression, which enables functional miRNA studies and provides opportunities for therapeutic approaches. The principal miRNA modulating tools are miRNA inhibitors (antimiRs or antagomiRs) and miRNA mimics [175, 176]. MiRNA inhibitors are synthetic single-stranded oligonucleotide probes that bind to target miRNAs or miRNA binding sites in order to block their action. MiRNA mimics act in a similar way as endogenous miRNAs to increase effect size [177].
In most instances, systemic delivery is necessary for RNA interference. Delivery of miRNA therapeutics to ECs is of particular interest; because of their unique position lining the blood vessels, ECs are ideally suited to internalize systemically administered miRNAs. Moreover, ECs readily take up microparticles, exosomes, and nanovectors used for stabilizing miRNA therapeutics [178]. Nevertheless, many issues remain to be resolved at the bench, before effective clinical therapy can be achieved. Examples of these issues include cell-type-specific delivery, off-target effects, dosage, and stability. Stability is often circumvented by addition of chemical modifications, but in the case of miRNA mimics these modifications might lead to sterical hindrance during loading on the RISC-complex; this is not an issue when using inhibitors, which bind to miRNAs itself. Toxicity, on the other hand, is often the result of long-term off-target effects. For that reason, various vehicles and delivery packages are in development, such as nanocarriers, gold particles, miRNA sponges, modified naked oligonucleotides, and even exosomal delivery methods to improve either (targeted) cellular uptake, stability, and toxicity [179]. In some cases, these vehicles can be labeled with molecules targeting specific cells, like ECs, for increased cell-specific uptake [180]. An example includes nanoparticles labeled with a VCAM-1 binding protein - VHPK peptide- that are used to target inflamed ECs [176, 181].

At present, several miRNA-targeting therapies are being tested in clinical trials for a broad spectrum of diseases [182, 183]. The first miRNA-targeting therapeutic to enter clinical phase III is called Miravirsen, a suppressor of miR-122 that is applied for treatment of hepatitis C [184]. In the field of endothelial miRNAs, a phase I clinical trial using MRG110, a suppressor drug of miR-92,is ongoing for the treatment of heart failure and wound healing [185]. So far, only a few miRNA-targeting drugs are in the pipeline for cardiovascular diseases. Examples are MGN-2677, an inhibitor of miR-143/145, and MGN-5804, an inhibitor of miR-378, for treatment of cardiometabolic diseases [186]. However, no candidate entered clinical phase III study at the time of writing [187].

Thus, treatment options are arising, but a lot of knowledge gaps and problems at the bench remain to be resolved. Missing evidence leads to inadequate mechanistic insights and incomplete understanding of the functional relevance of endothelial miRNAs for their individual target genes in a spatial and temporal manner. Furthermore, there is a heterogeneity among EC populations in different tissues [188] and potentially among ECs in a single organ. Considering heterogeneity of miRNA expression in different ECs, in depth research has yet to start, but it is likely that single-cell RNA sequencing will add significantly to our knowledge on this subject [189].

The ability of miRNAs to bind their targets with different degrees of sequence complementarity and to modulate multiple targets simultaneously, also complicates the identification of direct target genes and the determination of their functional effects at the pathway level. Moreover, numerous other factors influence the sensitivity of mRNAs for miRNA-mediated gene regulation, such as the abundancy and availability of miRNAs and their corresponding targets. This creates an extra layer of complexity that is largely unexplored. Studies focusing on the exploration of tissue-specific miRNA expression signatures and miRNA interactions might elucidate some of these unanswered topics.

Concerning circulating miRNAs, many aspects are still uncertain and require further research, such as the mode of extracellular miRNA uptake and the ability of antagomirs to target circulating miRNAs enclosed in vehicles or bound to complexing molecules [190].

At present, optimization and standardization of workflows is occurring and new technologies are arising to assist miRNA research and to address these questions. For instance, big-data bioinformatics will greatly aid the unravelling of the miRNA interactome and its highly complex network regulation at both RNA and protein level. Meanwhile, single-cell sequencing technologies enhance the discovery of spatial and temporal-specific expression patterns of miRNAs and their target genes [190]. Functional studies as well as target identification will also benefit from better miRNA interference tools and miRNA identification methods.

In summary, limitations still overshadow therapeutic benefit, and numerous open questions remain to be answered. Nevertheless, these issues will probably be resolved with technical improvement and with established standardized workflows, that will increase our mechanistic insights. In the years to come, miRNA-based therapeutics targeting the endothelium will most likely become a new line of treatment for specific cardiovascular diseases.

Conflicts of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Abbreviations

ATM= ataxia telangiectasia mutated

bFGF= basic fibroblast growth factor

BMP= bone morphogenetic protein

cGMP= cyclic guanosine monophosphate

CTRP9= C1q-tumor necrosis factor–related protein-9

CX43= connexin 43

Dlk1= delta-like 1 homolog

EC= endothelial cell

EGF= endothelial growth factor

eNOS= endothelial nitric oxide synthase

FGFR2= fibroblast growth factor receptor 2

HIF1α = hypoxia-inducible factor 1 alpha

hNRP= heterogeneous nuclear ribonucleoprotein

id1= inhibitor of DNA binding 1

IGFR1= Insulin-like Growth Factor Receptor 1

Irak1= Interleukin-1 receptor-associated kinase 1

ITGA5= integrin subunit alpha 5

16K PRL= 16KDa N-terminal prolactin

KLF2= krüppel-like factor 2

KLF4= krüppel-like factor 4

miRISC= microRNA induced silencing complex

miRNA= microRNA

MKK4= MAP kinase kinase 4

mRNA= messenger RNA

mTOR= mammalian target of rapamycine

NgBR= nogo B receptor

NO= nitric oxide

NSMase2 = neural sphingomyelinase 2

PAK4= P21 activated kinase 4

PIK3= Phosphoinositide 3-kinases

PIK3R2= phosphoinositol-3 kinase regulatory subunit 2

PKG= protein kinase G

PRMT1= protein arginine methyltransferase 1

Pten= phosphatase and tensin homolog

Ptpb1= Polypyrimidine Tract Binding Protein 1

RGS16= regulator of G-protein signaling 16

RISC= RNA induced silencing complex

ROS= reactive oxygen species

SDF= steroidogenic derived factor

SDF-1= stromal cell-derived factor 1

sGC= soluble guanylyl cyclase

SPRED1= Sprouty-related EVH1 domain-containing 1

Spry1= Sprouty RTK Signaling Antagonist 1

Spry2= Sprouty RTK Signaling Antagonist 2

TGF-β= transforming growth factor beta

TGFβR1= transforming growth factor beta receptor 1

TNFα= tumor necrosis factor alpha

VCAM= vascular cell adhesion molecule

VEGF(R)= vascular endothelial growth factor (receptor)

Figure source file numbering

- **Figure 1.tif** *=2-column fitting*

Title:

Figure 1: Paracrine secretion of endothelial cells

Legend:

Figure 1: *Endothelial cells modulate various processes in neighboring cells in the myocardium by secreting different paracrine factors, including small molecules, peptides, proteins, and miRNAs.*

- **Figure 2.tif** *= 2-column fitting*

Title:

Figure 2: Endothelial-cardiomyocyte crosstalk

Legend:

Figure 2: *Overview of the endothelial-cardiomyocyte crosstalk with examples of endothelial-derived factors and corresponding targeting miRNAs and cardiomyocyte-derived factors. Paracrine signals from endothelial cells influence cardiomyocyte processes and reciprocal signals from cardiomyocytes influence endothelial cells. This bidirectional communication is important for normal cardiovascular homeostasis, but is also important in pathophysiology. ET1= endothelin-1, NO= nitric oxide, PGI2= prostaglandin I2, IL6= interleukin 6, NRG1= neuregulin 1, FGF= fibroblast growth factor, IGF1= insulin growth factor 1, VEGF= vascular endothelial growth factor, CTRP9= C1q-tumor necrosis factor–related protein-9.*

- **Figure 3.tif** = 1.5-column fitting

Title:

Figure 3: Biogenesis of miRNA

Legend:

Figure 3: *MiRNA-synthesis starts with the transcription of pri-miRs from a miRNA gene. Enzymatic cleavage by Drosha results in pre-miRs, which are transferred to the cytoplasm and cleaved by Dicer enzyme resulting in mature miRs. One of the two strands is loaded on RNA-induced silencing complex (RISC) for post-transcriptional modulation of target mRNAs, while the other strand is degraded.*

- **Figure 4.tif** = 2-column fitting

Title:

Figure 4: Endothelial miRNAs in angiogenesis

Legend:

Figure 4: *Endothelial miRNAs involved in the regulation of angiogenesis. The major pro-angiogenic RAS/RAF and PI3K/Akt pathways are represented in blue, while modulators of these pathways are represented in purple. MiRNAs can interfere at multiple levels of the angiogenic process. Pro-angiogenic miRNAs are colored in green and anti-angiogenic miRNAs in red.*

- **Figure 5.tif** = 2-column fitting

Title:

Figure 5: miRNAs involved in endothelial-cardiomyocyte crosstalk

Legend:

Figure 5: *The trafficking of miRNAs (with their corresponding target genes) in the endothelial cell-cardiomyocyte crosstalk by vesicle-mediated transport. Vesicle-mediated paracrine signaling is a newly discovered method of paracrine signaling.*

Highlights

- Multiple endothelial miRNAs function as regulators of physiological and pathological angiogenesis
- Endothelial miRNAs modulate paracrine signaling in the myocardium
- MiRNAs modulate endothelial-cardiomyocyte crosstalk