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REVIEW PAPER

Regulation of angiogenesis by microRNAs in cardiovascular diseases

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Abstract
Non-coding RNAs are functional RNA molecules comprising the majority of human transcriptome. Only about 1.5% of the human genome is transcribed into messenger RNAs (mRNA) that are translated into proteins. Among the non-coding RNAs, miRNAs are extensively studied and miR targets in endothelial cells, perivascular cells, and angiogenic signaling are relatively well defined. MicroRNAs not only regulate transcripts in situ but also function as paracrine mediators in affecting angiogenesis at distant sites. Exosomal miRs are implicated in modulating endothelial cell function and angiogenesis. Thus miRs have been shown to affect tissue microenvironment in a multitude of ways. A comprehensive analysis of the role of miRs in modulation of angiogenesis and their impact on cardiovascular diseases is presented in this review.

Keywords MicroRNA · Non-coding RNA · Angiogenesis · lncRNA · Cardiovascular · MI · Therapeutics

Introduction to non-coding RNAs and their biological role

Non-coding RNAs are functional RNA molecules comprising the majority of human transcriptome. Only about 1.5% of the human genome is transcribed into messenger RNAs (mRNA) that are translated into proteins. The vast majority of the human genome (more than 98%) is made of DNA sequences that are either transcribed into Non-coding RNA or constitute introns or long and short interspersed DNA elements or regulatory DNA elements. Non-coding RNAs can be of different types; microRNA (miRNA), long non-coding RNA (lncRNA), small nuclear RNA (snRNA), small nucleolar RNA (snoRNA), circular RNA, and transfer RNA (tRNA). The complexities and importance of non-coding RNAs in regulating various cellular processes have not been completely understood. Figure 1 summarizes the major functional role of non-coding RNAs. MicroRNAs (miR) arise from introns or exons. While the vast majority of miRs are generated from the coding strand (sense strand), some miRs are produced from the antisense strand of DNA (e.g., miR-199a). Ontogeny of miRs has been recently reviewed by Daugaard and Hansen [1]. Precursors for miRs are transcribed as a several hundred-long transcript by RNA polymerase II, 5′-capped, and polyadenylated (Primary miR, Pri-miR). Some miRs are also generated from splicing process. Pri-miR is processed by Drosha/DGCR8 complex to a shorter form of precursor, (Pre-miR) which is then transported to the cytoplasm by the RanGTPase/Exportin 5 system. Further processing by Dicer complex results in a duplex of about 22 nucleotides in length. Mature duplex miRs bind to Argonaute 1–4 leading to the selection of one of the strands forming the miRISC complex which, can bind to complementary sequences in the 3′-UTR of mRNA and inhibit either translation initiation or mRNA degradation. Recent studies have identified another layer of complexity in the generation of miRs. Variants of miRs are found to be produced by 5′ or 3′ excision or addition of nucleotides. RNA editing (deamination) is also implicated in the generation of miR analogs called isomiRs [2]. Intracellular concentrations of functional miRs are controlled at multiple levels. In addition to transcriptional regulation, miR levels are controlled by processing and degradation. Recent studies have identified another mechanism by which functional miR levels are maintained within the cell. Circular RNAs are a novel lass of RNA molecules generated during 'back splicing' process. Circular RNAs and lncRNAs which have

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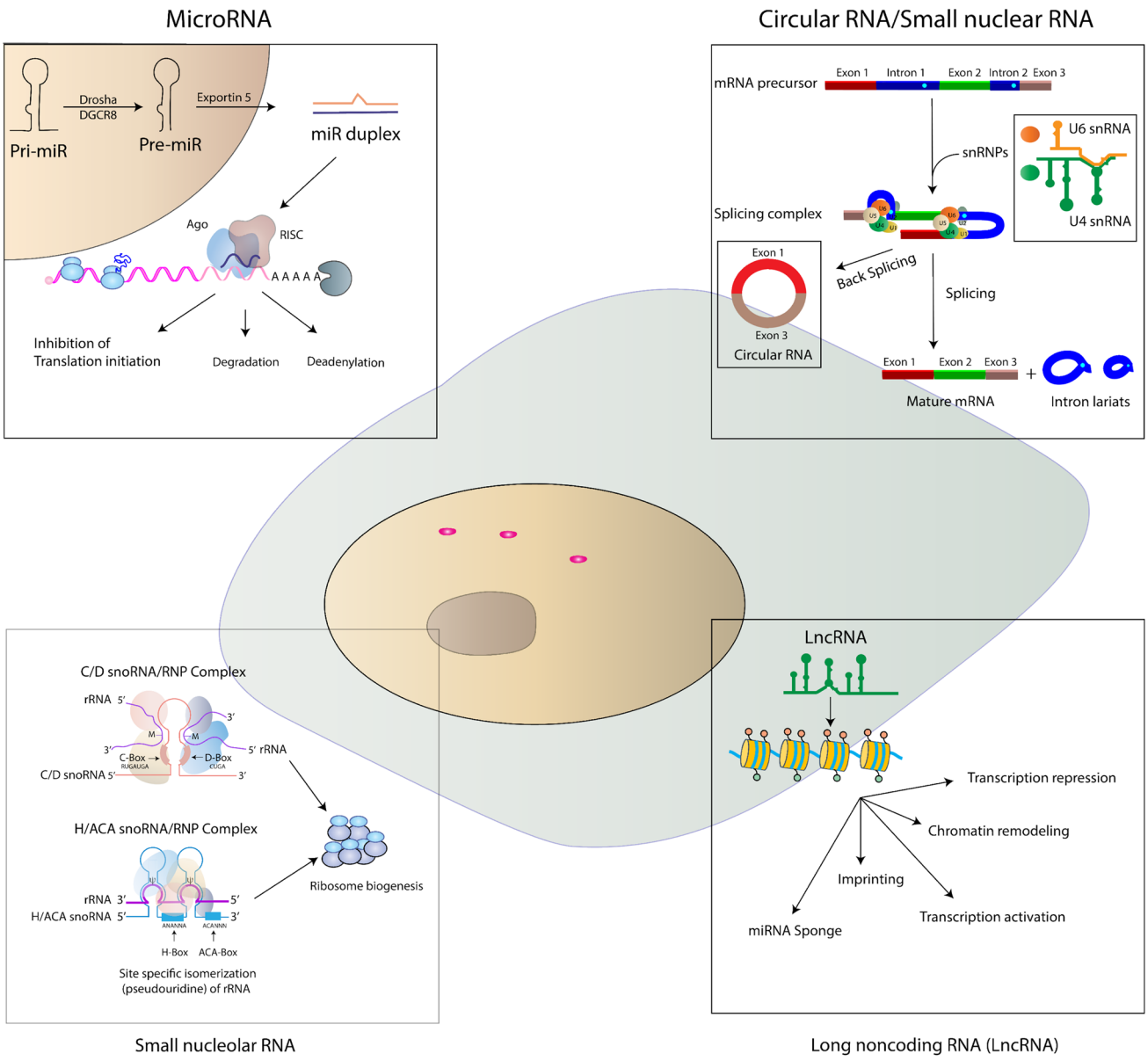

Fig. 1 Schematic diagram showing major classes of non-coding RNAs. Some of the non-coding RNAs such as PiRNA, tRNA, siRNA, and ScaRNA are not included. Ontogeny and function of non-coding RNAs are briefly illustrated. While SnoRNAs are involved in the ribosomal RNA modification (canonical function), they may also contribute to angiogenesis indirectly by regulating ribosome biogenesis in vascular cells

complementary sequences can act as 'miRNA sponges' to keep them away from 3′ UTR targets present in mRNAs. Thus, miRNA levels and function are governed by coordinated processes acting at multiple levels from biosynthesis to maturation and sequestration.

Other groups of non-coding RNAs include small nuclear RNA (snRNA) such as U6RNA which are involved in splicing of mRNA and small nucleolar RNA (snoRNA). SnoRNAs are of two kinds, C/D and H/ACA box containing snoRNAs. C/D snoRNAs direct methylation of ribosomal RNAs (rRNA), while H/ACA snoRNAs are involved in the modification of specific Uridine residues to pseudouridine in rRNA. The biological roles of snRNAs and snoRNAs in angiogenesis are not known. A recent study has shown non-canonical function of a snoRNA, (SNORA23) in the regulation of pancreatic cancer metastasis to liver [3]. As metastasis is directly dependent on angiogenesis, it is likely that SNORA23 could impact endothelial cell functions either directly or indirectly by affecting ribosome biogenesis.

Only recently, the role of lncRNAs in the regulation of angiogenic process is recognized. lncRNA, JHDM1D-AS1, for example, has been found to regulate tumor

angiogenesis under the conditions of nutrient starvation [4]. Similarly, Maternally Expressed Gene 3 (MEG3) lncRNA guides angiogenesis by targeting AKT pathway [5, 6]. Recent studies have shown that MEG3 lncRNA is regulated by HIF-1α and plays a role in Vascular Endothelial Growth Factor A (VEGF-A)-induced angiogenesis through the stabilization of the Vascular Endothelial Growth Factor Receptor-2 (VEGFR-2) mRNA in the hypoxic endothelium [7]. While some of the lncRNAs modulate angiogenic signaling pathways, other lncRNAs such as MANTIS, control epigenetic regulation in endothelial cells. Using a JARID1B knockdown screen, Leisegang et al. [8], have identified upregulation of MANTIS in endothelial cells. MANTIS is generated from the antisense strand of an intron of Annexin IV gene. Further studies unraveled that MANTIS is recruited to promoters of proangiogenic genes through SWI/SNF-BRG1-BAF155 complex and facilitates nucleosome remodeling and transcriptional upregulation. Man et al. [9] have recently identified that lncRNAs can govern endothelial cell specification, mainly in response to shear stress and angiogenic potential. They reported a set of endothelial cell-enriched lncRNAs, spliced-transcript endothelial-enriched lncRNA (STEEL), and showed their proangiogenic effects in vitro and in vivo through transcriptional upregulation of endothelial nitric oxide synthase (eNOS) and Kruppel-like factor 2 (KLF2), which is a key sensor of shear forces [9]. Functional role of lncRNAs in the development of cardiovascular diseases and their role as potential diagnostic markers and/or therapeutic targets are recently reviewed by Haemmig et al. [10] and Yu et al. [11]. lncRNAs (mainly H19, MEG3, and Metastasis-Associated Lung Adenocarcinoma-Associated Transcript 1-MALAT1) have also been shown to have a significant role to play in acute myocardial ischemia/reperfusion injury and ischemic stroke [12, 13]. lncRNA H19 in fact has been found to improve the survival and angiogenic capacity of mesenchymal stem cells (MSCs) through upregulation of VEGF-A expression by competing with miR-199a-5p [14].

Among the non-coding RNAs, miRNAs are extensively studied and miR targets in endothelial cells, perivascular cells, and angiogenic signaling are relatively well defined. MicroRNAs not only regulate transcripts in situ but also function as paracrine mediators in affecting angiogenesis at distant sites. A number of studies have shown that miRs are also packaged into vesicles (exosomes) and secreted. Exosomal miRs are implicated in modulating endothelial cell function and angiogenesis [15–17]. Exosomal cargos delivered to secondary target tissues such as liver and lungs are shown to create proangiogenic niche and modulate immune functions at the local milieu as well [18]. Thus miRs have broader impact on tissue microenvironment [19]. In this review, we would like to focus on the role of microRNAs in adaptive neovascularization in response to ischemia and their role in cardiovascular diseases.

MicroRNAs and cardiovascular diseases: role in ischemia and adaptive neovascularization

Cardiovascular diseases are the leading cause of mortality for both men and women, accounting for one in every four deaths in the United Sates [20]. Depending on the area of vessel involvement, it can present in a myriad of effects, ranging from angina and Myocardial Infarction (MI), Transient ischemic attacks, and stroke in the brain to Critical Limb Ischemia (CLI) in the extremities. Recent advances have provided immense understanding of the pathophysiology and have helped revolutionize management of these conditions. However, there is still need for development of new treatment strategies for patients who neither respond to traditional therapy nor are candidates for surgical revascularization procedures. Therapeutic angiogenesis is one promising approach for such patients where novel treatment strategies could be directed. The goal of such therapies would be formation of new blood vessels or maturation of pre-existing vasculature to bypass the blocked arteries and maintain organ perfusion, thereby providing symptomatic relief, improving the quality of life, and preventing adverse organ remodeling. Adaptive neovascularization in ischemia occurs through two distinct processes: angiogenesis and arteriogenesis. Formation of new blood vessels from pre-existing vasculature is angiogenesis, whereas arteriogenesis involves remodeling of pre-existing collateral vessels into functional arteries. Hypoxia is the major physiological signal for angiogenesis but has a limited role in arteriogenesis. Arteriogenesis occurs in response to changes in shear force in the collateral vessels due to arterial occlusion [21]. The relative importance of these two processes depends on the target tissue of involvement. For instance, angiogenesis is pivotal for tumor growth and metastases. On the other hand, arteriogenesis is indispensable for restoring blood flow into ischemic heart and limbs.

In the last decade, the importance of microRNAs in the regulation of both angiogenesis and arteriogenesis has been realized. The fact that Dicer (microRNA processing enzyme) knockout mice die during embryogenesis due to vascular malformations underscores this phenomenon [22]. Moreover, adaptive neovascularization in ischemia occurs in response to multiple growth factors like VEGF, fibroblast growth factors (FGF), platelet-derived growth factor (PDGF) with each of these factors having their distinctive effects on the developing vasculature. Modulating a single growth factor after ischemia has not produced any significant clinical benefit [23–25]. MicroRNAs can modulate the expression and stability of multiple proteins

and hence changing a single microRNA has the potential of simultaneously activating more than one growth factor-mediated signaling pathways. The role of microRNAs in cardiac hypertrophy, atherosclerosis, and metabolism has been reviewed before [26, 27]. This section will focus on the microRNAs regulating neovascularization and maturation in response to an ischemic condition. MicroRNAs can either promote or inhibit neovascularization after ischemia depending on their targets (Fig. 2). The following section summarizes the role and regulation of microRNAs in cardiac and hind limb ischemia (HLI).

Anti-angiogenic microRNAs and adaptive neovascularization

Reverse target prediction analyses of 197 genes important in post-ischemic neovascularization (both angiogenesis and arteriogenesis) revealed enrichment of binding sites for 27 microRNAs from a highly conserved microRNA gene cluster 14q32 [28]. Based on microRNA expression in murine hind limb ischemia (HLI) model using single femoral artery ligation, miR-494 and miR-487b (early responders), miR-329 (late responder), and miR-495 (non-responder) were the representative microRNAs extensively studied from this

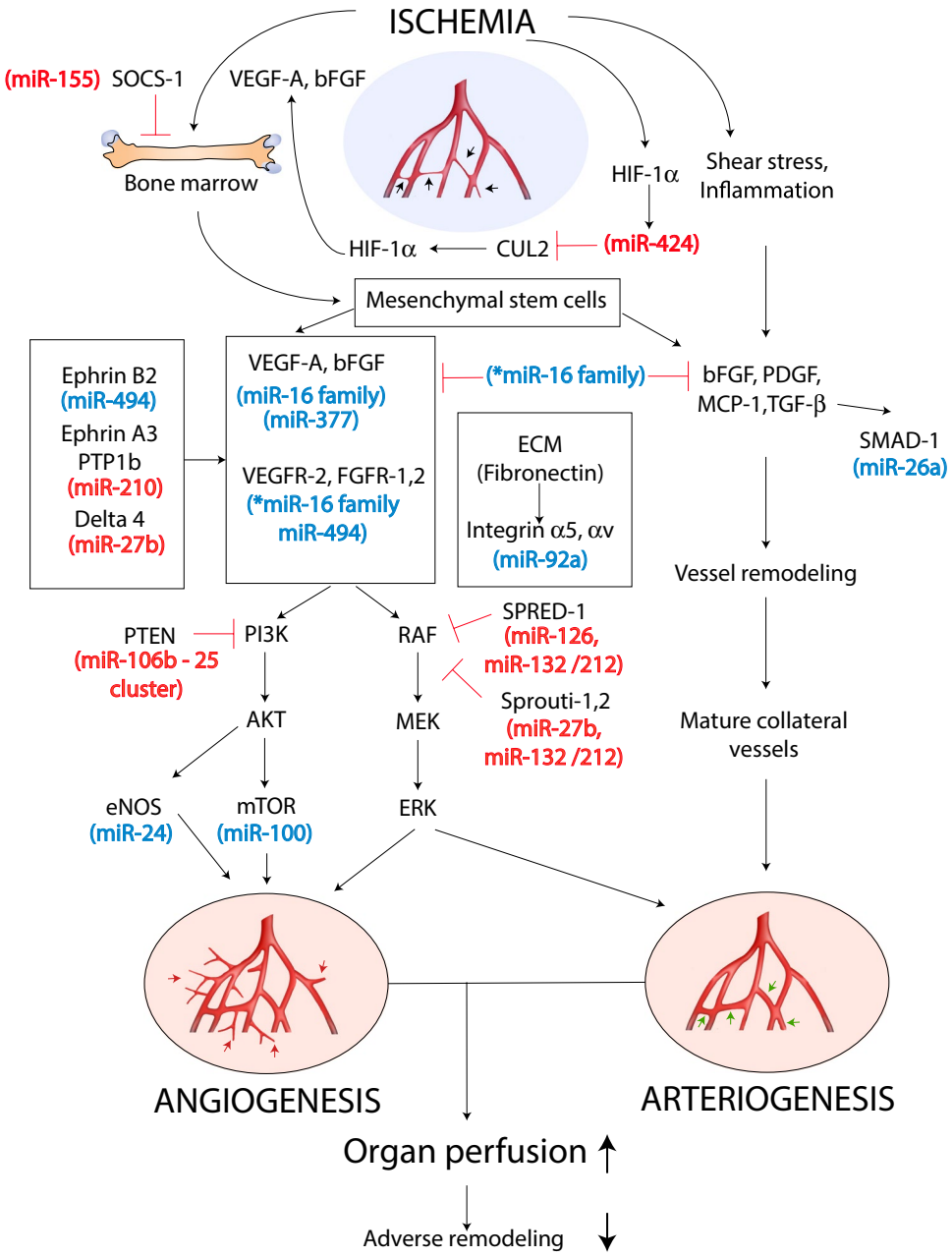

Fig. 2 Summary of microRNAs regulating angiogenesis and arteriogenesis. Black arrows in the ischemic area show pre-existing, immature collateral vessels. Green arrows show shear force-induced, mature, functional, collateral vessels (arteriogenesis). Red arrows show sprouting of new blood vessels from pre-existing vessels (angiogenesis) which are also seeded with endothelial cell progenitor cells mobilized from the bone marrow. MicroRNAs shown in red are proangiogenic and microRNAs shown in blue are anti-angiogenic. MicroRNAs are listed underneath each proven target molecules involved in angiogenesis and arteriogenesis. miR 16 family includes miR-15a, miR-16, and miR-503

cluster. Gene-Specific Oligonucleotides (GSO) were used to systemically inhibit these microRNAs in murine HLI models. There was improved recovery of perfusion in the ischemic limb with inhibition of all four microRNAs, but the most striking impact was seen when miR-329 was inhibited with anti-miR treatment. Complete perfusion recovery by day 7 was seen in mice treated with anti-miR-329, when compared to the control animals. Furthermore, both angiogenesis and arteriogenesis were improved by anti-miR treatment as reflected by the increased capillary density in the ischemic muscles (GSO-329, GSO-487b, and GSO-495) and a trend towards larger collateral vessels, especially when miR-487b and miR-329 were inhibited. The effect of GSO-487b on collateral vessel diameter was not surprising, as it is known to target the anti-apoptotic Insulin Receptor Substrate 1 (IRS1) in the arterial wall, thus affecting vascular remodeling in response to ischemia [29]. Furthermore, miR-494 targets Fibroblast Growth Factor Receptor 2 (FGFR2), Vascular Endothelial Growth factor A (VEGF-A), and Ephrin B2 (EFNB2) while miR-329 targets the proangiogenic transcription factor Myocyte Enhancer Factor 2A (MEF2A), explaining the anti-angiogenic properties of this cluster. Furthermore, 14q32 miR cluster was also found in endothelial microparticles secreted into systemic circulation upon endothelial cell damage in Bicuspid aortic valve disease [30]. As we learn more about the clinical relevance of 14q32 miR cluster in cardiovascular diseases, it is becoming important to understand the molecular mechanisms regulating the expression levels of 14q32 miR cluster. Recent studies suggest that 14q32 miR cluster is epigenetically regulated. Overexpression of this miR cluster correlated with genomic hypomethylation as evidenced by DNA methylation arrays and bisulfite sequencing validation [31]. In contrast, hypermethylation of promoter reduced the expression of 14q32 miR cluster in Type 2 diabetic islets [32] suggesting that methylation status of the regulatory genetic elements could directly impact on the expression levels of 14q32 miR cluster. Furthermore, to conclude, inhibition of 14q32 cluster microRNAs is a promising strategy to improve post-ischemic neovascularization as it affects both angiogenesis and arteriogenesis.

Another microRNA deemed important in the regulation of post-ischemic neovascularization is miR-100. miR-100 has earlier been found to be upregulated in patients suffering from heart failure secondary to idiopathic dilated cardiomyopathy, and impaired cardiac microcirculation is considered to have a role in the pathogenesis of idiopathic dilated cardiomyopathy [33, 34]. Consistent with these findings, miR-100 was downregulated during vascular remodeling over time (up to 7 days) after ischemia in the murine HLI model and systemic delivery of miR-100 antagomiR before induction of HLI improved restoration of blood flow in the ischemic limb compared to treatment with control antagomiRs [35]. Inhibition of miR-100 improved perfusion by increasing capillary and arterial density in the ischemic limb muscles. miR-100 is highly expressed in endothelial and vascular smooth muscle cells. Based on gene expression analyses and bioinformatics screening, it was found to target the proangiogenic protein kinase, mammalian target of rapamycin (mTOR) [35, 36]. As expected, mTOR inhibitor, rapamycin, abrogated the proangiogenic effects of antagomiR-100 in the murine HLI model.

Another important group of miRs generated from a single, polycistronic transcript is miR17-92 cluster. The miR17-92 cluster is upregulated in hypoxic tissues and serves a proangiogenic role. miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92a constitute this conserved microRNA cluster. The proangiogenic function of this cluster is associated with miR-18a and miR-19a, which inhibit release of anti-angiogenic molecules such as thrombospondin-1 (TSP-1) and connective tissue growth factor (CTGF) from the hypoxic cells [37]. Endothelial microRNA expression profiling showed that endothelial cells also express miR-17-92 cluster, particularly miR-92a. Surprisingly, overexpression of miR-92a in endothelial cells inhibited vessel sprouting. Biogenesis of this cluster in embryonic stem cells has recently been found to involve another post-transcriptional processing step, the progenitor-miRNA. Pro-miRNA generation depends on the conformational state of the primary miRNA transcript upstream of cleavage by the endonuclease, Drosha. This would help explain the mechanisms underlying this differential expression of individual microRNAs belonging to this cluster [38]. miR-92a targets the proangiogenic integrins α5 and α_v thereby, affecting endothelial cell matrix interactions [39]. Interestingly, miR-92a levels increased in a mouse model of acute MI after occlusion of left coronary artery, and antagomir-92a treatment in the same settings improved perfusion in the peri-infarct zone which led to a decrease in the infarct size and improved cardiac function. Furthermore, systemic administration of antagomiR-92a in the murine HLI model improved restoration of blood flow and reduced toe necrosis. Similar results have been observed with encapsulated antagomiR-92a microspheres and Locked nucleic acid (LNA)-92a delivered into the coronary arteries in a pig model of acute MI [40, 41]. These studies suggest that miR-92a can be targeted for therapeutic intervention in MI.

MicroRNA-214 is upregulated after MI in the peri-infarct zone both in murine as well as human tissues [42]. It is considered to have a cardio-protective role as it prevents ischemia–reperfusion injury by inhibiting sodium–calcium exchanger 1 thereby, reducing calcium overload in the heart [43]. However, miR-214 was also upregulated in chronic, failing human hearts and in mouse models of heart failure and hypertrophy. Earlier studies have shown coronary angiogenesis to be beneficial for adaptive hypertrophy in response

to pressure loads in the heart. Reduced angiogenesis was found to interfere with coordinated tissue growth leading to dilated heart and heart failure [44]. Adenovirus-mediated silencing of miR-214 in murine models of heart failure and hypertrophy led to increased capillary density, improved cardiac function, and reduced cardiac fibrosis. These studies further support that angiogenesis in the failing heart lowers adverse remodeling [45]. miR-214 was found to target a key Unfolded Protein Response (UPR) transcription factor, spliced XBP1. Upstream sensors of UPR such as IRE1alpha, PERK, and ATF6alpha regulate VEGF-A transcription and secretion thereby enforcing a positive correlation between UPR and angiogenesis [46, 47].

In the past decade, transplant of mesenchymal stem cells (MSCs), derived typically from the bone marrow, into the ischemic myocardium to augment the angiogenic response through paracrine mechanisms has shown great promise. MSCs are an ideal choice for cell-based regenerative therapy because they are proangiogenic and a natural component of the host response to ischemic insult [48]. However, MSCs are highly sensitive to ischemic conditions and suffer from low rates of retention following engraftment. Genetically engineered MSCs, that are resistant to hypoxic insult, may help to overcome these issues. MicroRNA profiling of MSCs exposed to hypoxia has revealed that miR-377 decreased when cells are grown under low oxygen levels. miR-377 targets VEGF-A and thus has an anti-angiogenic effect on the endothelial cells. MSCs genetically engineered to overexpress or knockdown miR-377 using lentiviral vectors were transplanted into ischemic hearts in rats. Anti-miR-377 MSCs resulted in higher capillary and arterial density, lower fibrosis, and better cardiac function compared to miR-null MSCs, while MSCs overexpressing miR-377 had opposite effects [49]. These studies confirm the negative effects of miR-377 and reducing the levels of miR-377 using antagomiRs is a clinically useful strategy to improve the functional life of MSCs in regenerative medicine.

Currently, about 97 clinical trials are being carried out to evaluate the use of MSCs in cardiovascular diseases (http://www.clinicaltrials.gov). Recruitment of MSCs to tissues undergoing remodeling and angiogenesis is positively modulated by growth factors such as bone morphogenic proteins (BMP). BMP signaling through its receptor activates SMAD signaling and regulates angiogenesis [50]. SMAD1 knockout mice die during embryogenesis and suffer from severe vascular defects, consolidating the importance of this pathway in vasculogenesis [51]. miR-26a is highly expressed in the endothelium and has been found to negatively regulate SMAD1 signaling axis (SMAD1-Id1-p21/p27), thereby inhibiting endothelial proliferation and angiogenesis. Moreover, circulating levels of this microRNA are increased in patients suffering from acute coronary syndromes and in murine models of acute myocardial infarction (MI). Systemic administration of locked nucleic acid (LNA) miR-26a (antagomiR) in murine model of acute MI increased the levels of SMAD1 in the heart which functionally translated into greater angiogenic response, decreased infarct size, and improved cardiac function [52].

An exceptional microRNA, which inhibits angiogenesis but promotes arteriogenesis, is miR-155. Dichotomous effects of miR-155 are not surprising as arteriogenesis and angiogenesis are entirely different processes with their own stimulatory cues and regulators. miR-155 expression levels were downregulated in the ischemic endothelium after femoral artery occlusion in the murine HLI model. Additionally, miR-155 inhibited endothelial proliferation; sprouting and matrigel plugs injected in miR-155 knockout mice had more neovascularization compared to wild-type mice. Surprisingly, when miR-155 knockout mice were subjected to femoral artery occlusion, there was slower recovery in perfusion despite increased angiogenesis in the knockout animals. The reason being fewer infiltrating myeloid cells in the ischemic limb leading to altered inflammatory milieu and impaired arteriogenesis. miR-155 primarily targets Angiotensin II type 1 receptor (AGTR1) on endothelial cells and Suppressor of Cytokine Signaling-1 (SOCS-1) in the inflammatory cells. The anti-angiogenic effects are mediated solely by inhibition of AGTR1 while targeting SOCS-1 gives the myeloid cells a proarteriogenic phenotype, thereby explaining this divergent effect of miR-155 [53].

Some of the recent studies have shown miR-24 as an important regulator of post-ischemic neovascularization. Its role in this regard has, however, been controversial. Systemic delivery of antagomiR-24 or adenoviral decoy of miR-24 delivery into the myocardium before induction of MI in the mice was associated with decreased infarct size, improved neovascularization and cardiac function [54, 55]. In contrast, intramyocardial delivery of Pre-miR-24 improved post-ischemic cardiac function in a similar setting [56]. miR-24 targets proangiogenic genes such as p21 protein-cdc42/Rac-activated kinase (PAK4), endothelial nitric oxide synthase (eNOS), and global transcription factor binding protein 2 (GATA2), thereby explaining its anti-angiogenic properties [54, 55]. Its effects on cardiomyocyte survival are explained by another target, the pro-apoptotic BCL2-like 11 apoptosis facilitator (BIM) [56]. After MI, miR-24 levels increased in the peri–infarct endothelial cells but the levels went down in cardiomyocytes and fibroblasts in the same area. Thus miR-24 seems to differentially affect various cell populations at the infarct site. Based on these studies, it is safe to conclude that inhibition of miR-24 after MI accelerates recovery due to a dominant proangiogenic effect on the endothelial cells and a pro-apoptotic effect on the fibroblasts.

Regenerative cellular therapy using progenitor cells is being tested as a novel approach to improve organ perfusion in response to an ischemic insult for “no-option” patients

suffering from insufficient coronary or peripheral circulation [57, 58]. CD34 + mononuclear cells enriched from peripheral blood or bone marrow are most commonly used for this indication. However, most patients in this disease cohort also are diabetic, and regenerative potential of these progenitor cells from diabetic patients is limited [59]. Progenitor cells derived from patients suffering from critical limb ischemia (CLI) are enriched in certain microRNAs like miR-15a and miR-16. Intriguingly, genetically engineered progenitor cells to express anti-miR-15a and anti-miR-16 increased their regenerative potential and improved hind limb perfusion in mice after femoral artery ligation. miR-15a and miR-16 levels in the serum have also been found to correlate with the incidence of restenosis in diabetic patients suffering from CLI 12 months after revascularization [60]. Similarly, another microRNA, miR-503, has been implicated as a therapeutic target in diabetic patients with limb ischemia. miR-503 levels in the plasma and lower limb muscles were significantly higher in diabetic patients suffering from CLI compared to non-ischemic healthy controls. Moreover, delivery of an adenoviral decoy miR-503 (anti-miR-503) to ischemic muscles of diabetic mice increased neovascularization and restored limb perfusion to the same levels as non-diabetic controls [61]. Interestingly, miR-15a, miR-16, and miR-503 are a part of the extended microRNA16 family with overlapping targets because of common seed sequence [62]. Members of this family affect multiple proangiogenic growth factor-mediated signaling pathways because they commonly target VEGF-A, FGF-2, Vascular Endothelial Growth Factor Receptor-2 (VEGFR-2), Fibroblast Growth Factor Receptor-1 (FGFR-1), and AKT3 [60, 62, 63]. In a Tie-2 promoter-driven transgenic mouse model with endothelial selective overexpression of miR-15a, there was reduced led angiogenesis and local perfusion after HLI because of mechanisms discussed above [63]. Expression of this family of microRNAs has been shown to increase after growth factor stimulation, thus creating an effective feedback loop [64].

Having defined the role of microRNAs that primarily have an inhibitory role to play in adaptive neovascularization in response to ischemia, the following section will review the other end of this spectrum, angiogenesis promoting miRs (angiomiRs).

Role of proangiogenic microRNAs in adaptive neovascularization

Chronic heart failure due to ischemic cardiomyopathy, like diabetes, impairs the regenerative potential of angiogenic progenitor cells. Human CD34 + progenitor cells from healthy controls when transplanted into athymic, nude mice induce a robust angiogenic response after MI thereby, improving cardiac function while a similar cell population derived from heart failure patients failed to induce neovascularization or cardiac repair after ischemia. miRNA profiling of circulating progenitor cells from heart failure patients revealed a significant downregulation of miR-126 compared to healthy controls. Knockdown of miR-126 in zebrafish resulted in abnormal vascular development and hemorrhage due to loss of vascular integrity [65]. Further, endothelial-specific knockout of miR-126 in mice resulted in partial embryonic lethality due to leaky vessels and hemorrhaging. The knockout mice that did survive suffered from defects in neovascularization after cardiac ischemia [66]. Additionally, anti-miR-126 transfection into progenitor cells from healthy controls impaired their regenerative potential while overexpressing miR-126 in the progenitor cells derived from heart failure patients restored their angiogenic potential and accelerated cardiac recovery following ischemia in athymic mice [67]. miR-126 promotes angiogenesis by targeting SPRED1, which inhibits ERK activation in response to growth factor signaling [65, 67].

miR-210, a hypoxamir, has been extensively studied in various cancers. In addition to cancers, cardiomyocytes also show upregulation of the hypoxamir miR-210 when exposed to low oxygen conditions. Cardiomyocytes overexpressing miR-210 had a survival advantage and showed lesser apoptosis when exposed to hypoxia [68]. The fact that the protective effects of Ischemic preconditioning of mesenchymal stem cells is dependent on miR-210 further corroborates these findings [69]. miR-210 has anti-apoptotic and proangiogenic effects by targeting PTP-1b and EPHA3, respectively [68, 70, 71]. As a result, when miR-210 minicircle vectors were injected into the myocardium after induction of MI in mice, cardiac function improved after 8 weeks because of increased neovascularization and post-ischemic repair [68]. Another hypoxamir, mir-424/mu-miR-322, has been found to be significantly upregulated in rat models of experimental myocardial infarction and PAD. Endothelial cells exposed to hypoxia upregulate C/EBP-α levels. The heterodimeric complex of C/EBP-α and RUNX-1 then transactivates PU.1 expression. PU.1 is an ETS-domain containing transcription factor which is restricted to myeloid and B-cell differentiation. PU.1 binds to PU-box (purine rich sequences) found in promoters and coordinates transcriptional activation of target genes. MicroRNA-424 promoter has a PU-box and PU.1 was indeed found to drive the expression of mir-424 in endothelial cells. Interestingly, miR-424 targets cullin-2 (CUL2), a scaffolding protein which is an integral component of the E3 ubiquitin ligase complex (VHL/CUL2/Elongin-B/C and RBX-1 complex) that ubiquitinates lysine residues on HiF-1α and targets it for proteasomal degradation. Compared to miR-210, another hypoxamir found to be upregulated ubiquitously in multiple cell types under hypoxia, miR-424 was only found to be upregulated in

endothelial cells under hypoxia. Further, through targeting CUL2, this was an important discovery of a new pathway of HIF-1α regulation and hence modulation of the angiogenic response [72].

Expression of the miR-106b-25 cluster, including miR-106b, miR-93, and miR-25, increases with ischemia in the murine HLI model. Furthermore, post-ischemic neovascularization after HLI is impaired in the miR-106b-25 knockout mice but reintroduction of this cluster locally in the ischemic limbs did not improve perfusion. This proves that the effects of this cluster on angiogenesis are not limited to just the ischemic limb. Interestingly, bone marrow-derived stromal cells (BMDCs) from the knockout mice have decreased survival, migration, and stemness and secrete less cytokines, thereby impairing adaptive neovascularization in response to ischemia. miR-106b-25 cluster targets PTEN and hence increases AKT phosphorylation in response to proangiogenic factors [73]. miR-27b (part of the miR-23-24-27 cluster) stimulates tip cell specification and endothelial sprouting by targeting negative regulators like Delta 4, Semaphorin 6A, and Sprouty 2 [74, 75]. In murine model of MI, miR-27b mimics injected into the myocardium-stimulated angiogenesis, increased cardiac function and decreased adverse remodeling. Similarly, miR-27b mimics introduced locally into the murine ischemic muscles stimulated neovascularization and restored perfusion. In addition, miR-27b mimics altered the inflammatory milieu significantly. Besides increasing recruitment of BMDCs to the healing site, miR-27b also inhibited macrophage infiltration into the ischemic tissues [76].

Lastly the microRNA family miR-132-212 is upregulated after ischemia in the murine HLI model. Moreover, HLI experiments in miR-132-212 knockout mice show delayed recovery of blood flow in the ischemic limb because of impaired collateral remodeling in these mice. miR-132-212 family increases growth factor-induced RAS-MAPK signaling by targeting negative regulators like Spred1, Rasa1, and Sprouty1 [77]. Endothelial cell ERK activation regulates arteriogenesis [78].

MicroRNAs and therapeutics

MicroRNAs are integral in maintaining tissue homeostasis and have potential to cause disease when dysregulated. Hence, there is a potential for therapeutic modulation of microRNAs. Therapeutic modulation utilizing RNA interference, including short interfering RNAs (siRNA) and short hairpin RNAs (shRNA) have proven to be effective tools in recent human trials, for instance, Inclisiran is a long-acting siRNA targeted towards Proprotein Convertase Subtilisin–Kexin type 9 (PCSK9), and has been shown to reliably lower LDL levels for 90–180 days after one subcutaneous injection and a Phase 2 trial utilizing Inclisiran as lipid-lowering therapy and evaluating its safety is currently underway [79].

While siRNA and shRNA-based therapies target a single gene, modulation of microRNAs can target many genes and potentially affect an entire pathway and hence these therapies can be very potent. However, there are some inherent limitations on microRNA-based therapeutics. MicroRNAs can have numerous targets and can have divergent biological effects depending on the cell type targeted (as noted for miR-24; anti-angiogenic for endothelium and anti-apoptotic for cardiomyocytes), off-target effects with these therapies are common [80]. Furthermore, systemic delivery of these therapies can be associated with innate immune activation causing side effects and can further decrease the bioavailability of these agents. Hence, targeting and delivery of these therapies to the desired organ and the cell type is of utmost importance.

MicroRNA-based therapeutics to modulate post-ischemic angiogenic response are successful in improving perfusion and tissue repair in multiple animal models, as described in previous sections. In order to restore the function of a microRNA that is downregulated in the ischemic tissues, two strategies in the form of microRNA mimics or microRNA expression vectors can be used. microRNA mimics are synthetic double-stranded RNA molecules that would function as the endogenous microRNA molecules once processed into a single-stranded mature miRNA within the cell. For instance, liposomal injections of miR-34a utilized this treatment strategy for patients with advanced hepatocellular carcinoma. Unfortunately, this Phase I study was terminated because of serious immune-related adverse events. A prospective observational study utilizing ex-vivo supplementation of miR-21 mimics in diabetic wounds to improve wound healing is currently underway. Another strategy to restore microRNA function is through microRNA expression vectors, which have the inherent advantage of tissue-specific expression as the expression of the microRNA can be engineered with tissue-specific promoters [81]. On the other hand, strategies to antagonize anti-angiogenic microRNAs include antagomirs, microRNA sponges, and Locked Nucleic Acid (LNA) anti-miRs. Antagomirs are single-stranded 23 nucleotide-long RNA molecules that are complementary to the targeted miR and inhibit miR function by binding. They have chemical modifications in the form of cholesterol conjugation, 2′-*O*-methyl linkages to enhance cellular uptake and decrease degradation by nucleases, respectively. Antagomirs are still being studied in preclinical studies, probably because of the high doses required for effective miR inhibition [82]. LNA anti-miRs are antisense oligonucleotides with some nucleotides substituted by bicyclic RNA analogs with a ribose ring that is locked in a ring conformation through chemical substitution.

This conformation ensures effective blocking through complete Watson–Crick binding to the targeted microRNA, which is then cleaved by RNAse H. LNA anti-miRs, owing to their structure, are resistant to degradation and are taken up by cells efficiently, thus bypassing the need for delivery vehicles, as needed for most other formulations mentioned above. Miravirsen is an LNA anti-miR directed towards miR-122 that successfully transitioned into clinical trials for the treatment of Hepatitis C infection, with Phase 2 trial showing efficacy in inducing sustained virological response for patients with chronic Hepatitis C genotype 1a patients without any dose-limiting adverse events [83]. miR sponges are expression vectors expressing RNA molecules with multiple tandem binding sites complementary to the seed region of the targeted microRNA. They have the potential to inhibit the entire family of microRNAs because of conservation of seed sequence and potentially can have off-target effects for the same reason. microRNA sponges are still being evaluated in preclinical studies. Compared to microRNA therapeutics, lncRNA-based therapies lag behind and the reasons are multifold. Unlike miRs, lncRNAs can fold into secondary and tertiary structures making it difficult to predict the sequence to target. Additionally, lncRNAs are localized primarily in the nucleus, which makes it hard to target the therapies effectively. It may be possible to link antisense sequences to nuclear targeting peptides to direct them to the correct intracellular compartment. Success of such strategies depends on selective uptake, escape from intracellular vesicles, and degradation. Alternatively, nanoparticles and nanocarriers can be used to selectively deliver therapeutic antagonists of lncRNAs.

In almost all of the therapeutic strategies to restore or target microRNAs discussed above, effective delivery of the therapy to the target tissue and cytosol, evading degradation by nucleases, activation of the immune system, or clearance by the liver or kidney, still remains elusive. Vector-based delivery mechanisms (adenoviral, liposomal, polymer-based nanoparticles and natural micro vesicles/exosomes) have hence recently gained attention to target these therapies efficiently to the tissue of interest. A novel technique utilizing ultrasound-based sonoporation has been shown to be effective for miRNA delivery into the myocardium in a preclinical rat model. In this study, antagomiR containing gas-filled albumin-shelled microbubbles were successfully targeted to the ischemic myocardium utilizing ultrasound-triggered microbubble destruction, which increased vascular and cell permeability locally in the myocardium [84]. It is believed that innovative targeting strategies like ultrasound-triggered microbubble destruction, and local delivery of microRNA-based therapies during cardiac and peripheral catheterization procedures will be the future of cardiovascular medicine. These therapies would help revolutionize the care of patients suffering from chronic limb ischemia and ischemic heart failure by improving perfusion and thereby targeting adverse tissue remodeling.

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