Review Article

MicroRNAs in Postischemic Vascular Repair

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The term angiogenesis describes the growth of endothelial sprouts from preexisting postcapillary venules. More recently, this term has been used to generally indicate the growth and remodeling process of the primitive vascular network into a complex network during development. In adulthood, angiogenesis is activated as a reparative process during wound healing and following ischemia, and it plays a key role in tumor growth and metastasis as well as in inflammatory diseases and diabetic retinopathy. MicroRNAs (miRNAs) are endogenous, small, noncoding RNAs that negatively control gene expression of target mRNAs. In this paper, we aim at describing the role of miRNAs in postischemic angiogenesis. First, we will describe the regulation and the expression of miRNAs in endothelial cells. Then, we will analyze the role of miRNAs in postischemic vascular repair. Finally, we will discuss the role of circulating miRNAs as potential biomarkers in ischemic diseases.

1. Introduction

Postnatal neovascularization occurs in pathological diseases, including diabetic retinopathy, arthritis, and tumor growth and metastasis, as well as during wound healing and postischemic repair [1]. Following occlusion of a major artery, two different types of vascular regrowth responses are activated to contrast the ischemic condition and salvage of injured ischemic tissue: sprouting of capillaries (angiogenesis) and growth of collateral arteries from preexisting arterioles (arteriogenesis) [2]. Angiogenesis is the process of growth of endothelial sprouts from preexisting postcapillary venules. It is initiated by the vasodilation of venules leading to increased permeability, followed by the proliferation and migration of endothelial cells (ECs). Then, venules are divided by periendothelial cells (intussusception) or are separated by transendothelial cell bridges (bridging) to form capillaries [3, 4].

Arteriogenesis was originally defined as the process of collateral arteries formation which follows the occlusion of a major artery [5]. Arteriogenesis is partly driven by shear stress and involves the proliferation and migration of vascular endothelial cells (ECs) and vascular smooth muscle cells (VSMCs). It leads to the production of mature arteries with a fully developed tunica media, which may provide alternative blood perfusion to ischemic areas [6]. microRNAs (miRNAs) are inhibitory regulators of gene expression which act by binding to complementary messenger RNA (mRNA) transcripts. Following initial studies in developmental biology and cancer, miRNAs have recently come into focus of cardiovascular diagnostics and therapeutics [7, 8]. Since miRNAs repress many target mRNAs, deregulation of one single miRNA can result in a cascade of transcriptional and posttranscriptional changes relevant to disease states [9]. miRNAs have been detected in serum and plasma, and circulating miRNA profiles have now been associated with cancer [10], diabetes [11], and heart disease [12] as an emerging class of biomarkers. Here, we discuss the role of miRNAs in postischemic angiogenesis.

2. MicroRNAs Biogenesis

Transcriptions of miRNA genes and protein-coding genes share common regulatory mechanisms [13]. miRNA genes can be embedded in the introns of protein coding genes or can derive from their own transcript units in intergenic regions of the genome. When miRNA genes are located
within introns of protein-coding genes, primary miRNA biogenesis is controlled by the same transcriptional mechanisms as the parent gene. In contrast, an independent miRNA gene will have its own transcriptional controls. Interestingly, multiple miRNAs can be produced within a single long primary nuclear miRNA transcript (pri-miRNA transcript), each of which can act independently [14, 15]. The long primary nuclear miRNA transcript ("pri-miRNA") undergoes maturation by the RNase-III Drosha/Dgcr8 enzyme complex, generating a precursor miRNA ("pre-miRNA") that is exported from the nucleus by exportin-5 [16]. Cytoplasmic pre-miRNAs are cleaved by a Dicer-containing complex [17] to generate a double-strand miRNA-duplex containing the mature miRNA on one strand and a complementary miRNA* on the other strand. Recently, Dicer-independent miRNA biogenesis pathway has also been reported [18]. The specificity of miRNA targeting is defined by Watson-Crick complementarities between positions 2 to 8 from the miRNA (also known as the seed sequence), with the 3′ untranslated region (UTR) of their target mRNAs [19]. Besides the binding through 3′ UTR interactions, some miRNAs have been shown to associate to the open reading frame or to the 5′ UTR of the target genes [19]. miRNAs act through formation of stable complexes with proteins of the Argonaute (Ago) family (in particular with Ago2), the core of the RNA-induced silencing complex (RISC). The RISC blocks the translation initiation by competition with the cytoplasmic cap-binding protein eIF4E (eukaryotic translation initiation factor 4E) [20] or the antiassociation factor eIF6, thus preventing the assembly of the ribosome [21]. Besides these mechanisms, RISC-miRNA complexes can move the miRNAs they bind to the "P-bodies," specialized cytoplasmic compartments enriched in mRNA-catabolizing enzymes, where translational repression or exonucleolytic mRNA degradation or mRNA destabilization may occur [22, 23].

### 3. Expression and Regulation of MicroRNAs in Endothelial Cells

miRNA microarray analysis is, at the moment, the most used and complete technique to analyse global miRNAs expression profile. Using this approach, 28 miRNAs (miRNA-15b, -16, -20, -21, -23a, -23b, -24, -27a, -29a, -30a, -30c, -31, -100, -103, -106, 125a and -b, -126, -181a, -191, -199a, -221, -222, -320, let-7, let-7b, let-7c, and let-7d) have been found to be commonly expressed in ECs cultured under normal conditions. Using this approach, 28 miRNAs (miRNA-15b, -16, -20, -21, -23a, -23b, -24, -27a, -29a, -30a, -30c, -31, -100, -103, -106, 125a and -b, -126, -181a, -191, -199a, -221, -222, -320, let-7, let-7b, let-7c, and let-7d) have been found to be commonly expressed in ECs cultured under normal conditions [24–30]. The miRNA expression profile in ECs could change if cells are studied under stressed conditions, as we recently proved for miRNA-503 (miR-503), which is highly induced in ECs in response to diabetes and ischemia [31]. A highly scalable approach using next generation sequencing (small RNA-seq) would facilitate the analysis of miRNA expression patterns in ECs. The group of Martelli was the first to use this approach to analyze the miRNAs expressed by ECs exposed to hypoxia [32]. Based on experimental evidence, it is possible to divide the endothelial miRNAs into pro- and antiangiogenic miRNAs (Table 1).

### Table 1: microRNAs and target genes involved in neovascularisation.

| Proangiogenic miRNAs | Target genes | References |
|----------------------|--------------|------------|
| miR-126              | Spred1/PK3R2  | [33]       |
| miR-221/222          | c-kit        | [34]       |
| miR-378              | Fus-1/Sufu   | [35]       |
| miR-424              | CUL2         | [36]       |
| miR-132              | p120RasGAP   | [37]       |
| miR-210              | EFNA3        | [27]       |
| miR-130a             | Spred1/PK3R2 | [38]       |
| miR-296              | c-kit        | [28]       |
| miR-17-5p            | Fus-1/Sufu   | [39]       |
| miR-23-27            | CUL2         | [40]       |

| Antiangiogenic miRNAs | Target genes | References |
|-----------------------|--------------|------------|
| miR-200c              | ZEB1         | [41]       |
| miR-217               | SIRT1        | [42]       |
| miR-34                | SIRT1        | [43]       |
| miR-503               | CCNE1/cdc25A | [31]       |
| miR-20a               | VEGF-A       | [44]       |
| miR-92a               | ITGB5        | [45]       |

**miRNAs involved in posts ischemic angiogenesis**

| Ischemic stimuli | miRNAs | References |
|------------------|--------|------------|
| Myocardial infarct | miR-126 | [33] |
| Myocardial infarct | miR-92a | [45] |
| Peripheral ischemia | miR-210 | [46] |
| Peripheral ischemia | miR-503 | [31] |
| Myocardial infarct | miR-92a | [45] |
| Myocardial infarct | miR-100 | [47] |

### 3.1. Proangiogenic MicroRNAs

miR-126 is recognized as the most important miRNA for maintaining vascular integrity during ongoing angiogenesis, as it targets SPRED1 and PIK3R2, two negative regulators of VEGFs signalling [30, 33]. Angiogenic sprouting of vessels requires that the zinc finger transcription factor klf2a induces expression of an endothelial-specific miR-126 [48]. Growth factors increase the expression of the pro-angiogenic miR-130a and miR-296 in ECs [28, 38]. miR-130a stimulates angiogenesis by inhibiting GAX and HOX5A; while, miR-296 acts through the inhibition of hepatocyte growth factor (HGF)-regulated tyrosine kinase [28]. Moreover, EC stimulation with VEGF-A and bFGF lead to a rapid transcription of miR-132. Overexpression of miR-132 increases EC proliferation and in vitro networking by targeting p120RasGAP, a GTPase-activating protein [49]. miR-210 overexpression considerably increased HUVEC migration and the formation of
of 15 years of clinical trials with monotherapeutic prophylactic microvascular approaches, the results have been encouraging. However, despite the remarkable results in animal models, clinical translation has been limited. In fact, the latter two miRNAs have been shown to target proteins containing thrombospondin type I repeats (TSR) in murine models of limb ischemia. MiR-17-5p appears to promote angiogenesis by targeting tissue inhibitor of metalloproteinase 1 (TIMP1) [39]. Recently, miR-23-27-24 cluster has also been reported to have a significant role in angiogenesis [40]. In particular, both miR-23 and miR-27 enhance angiogenesis by targeting the anti-angiogenic proteins Sprouty-2 and Semaphorin-3A [40]. Finally, miR-378 promotes angiogenesis by targeting tumor suppressor candidate 2 (Fus-1) and suppressor of fused (Sufu), thus inducing indirect up-regulation of VEGF and Angiopoietin-1/2 [35].

3.2. Antiangiogenic MicroRNAs. The antiangiogenic miR-221/222 was discovered by Poliseno et al. during the first screening of miRNAs in HUVECs [34]. Overexpression of miR-221/222 inhibits the angiogenesis responses to stem cell factor (SCF) by targeting the SCF receptor c-kit [34]. Belonging to the miR-17-92 cluster, miR-20a, and miR-92a display antiangiogenic activity, respectively, targeting VEGF-A and integrin β5 (ITGB5) transcripts [44, 45]. Oxidative stress upregulates miR-200c in ECs, which translates in the downmodulation of the miR-200c target ZEB1 [41]. miR-217 and miR-34 regulate the expression of silent information regulator 1 (SirT1). Inhibition of either miR-217 or miR-34 in ECs promotes angiogenesis via an increase in SirT1 activity [42, 43]. Finally, miR-503 expression in ECs is upregulated under anti-angiogenic conditions mimicking diabetes mellitus and ischemia [31]. miR-503 targets cdc25A and cyclin E1 (CCNE1) and its overexpression inhibits EC proliferation, migration, and network formation on Matrigel. Conversely, blocking miR-503 activity restores the angiogenic process in diabetic and ischemic limb muscles. [31].

4. MicroRNAs in Postischemic Angiogenesis

4.1. Limb Ischemia. Peripheral artery disease (PAD) induces limb muscle hypoperfusion and favors the formation and aggravation of skin ulcers. When the wound healing capacity is lost, PAD patients progress to a critical condition, identified as critical limb ischaemia (CLI), which usually requires minor or major amputations of the affected limb [56]. Despite improvements in surgical treatments, a significant portion of patients with CLI are not eligible for revascularization, and no medical therapy has been shown to be capable of reducing the need for amputation [57]. Diabetes aggravates PAD and CLI. Furthermore, postischemic recovery is delayed in diabetic patients due to diabetes-induced impairment of reparative angiogenesis. A laboratory-based study showed that conditional inactivation of Dicer in ECs reduced the spontaneous angiogenic responses to limb ischemia, demonstrating that endothelial miRNAs are required for reparative neovascularisation [26]. Recently, miRNA expression in murine models of limb ischemia has been profiled, showing upregulation of miR-92a and -100 [47, 58]. MiR-92 represses the ITGB5 target gene, thus impairing angiogenesis both in vitro and in vivo [45]. Conversely, miR-100 functions as an endogenous repressor of the serine/threonine protein kinase mammalian target of rapamycin (mTOR), increasing EC proliferation, sprouting activity and network formation on Matrigel [47]. Moreover, we found miR-503 levels to be remarkably higher in ischemic muscles of diabetic mice and of diabetic patients with CLI in comparison to nondiabetic and/or nonischemic controls [31]. In summary, it is evident that a number of miRNAs are modulated by ischemia and participate in postischemic angiogenesis.

4.2. Acute Myocardial Infarction. Myocardial ischemic injury results from severe impairment of the coronary blood supply usually produced by thrombosis or other acute alterations of coronary atherosclerotic plaques. Rapid formation of collateral vessels that bypass the obstructed coronary artery is necessary for the survival of the myocardial region that surrounds the necrotic infarct tissue and for the subsequent myocardial repair process. The most convincing data in the cardiac response to ischemia are for miR-126, miR-210, and miR-92a. Neovascularization at 3 weeks postmyocardial infarct (MI) is inhibited in miR-126 knockout mice in comparison with wild type [33]. Moreover, miR-126 is downregulated in the MI area, but upregulated in the MI border zone [33]. In contrast, miR-210 is upregulated in the ischemic myocardium [46]. MiR-210 expression is induced by HIF1α, and it has been associated with increased survival of cardiomyocytes and increased angiogenesis in a murine model of myocardial...
4.3. miRNAs-Based Therapeutic Strategies in Vascular Repair.

There are two major approaches to developing miRNA-based therapeutics: miRNA antagonists and miRNA mimics. miRNA antagonists are generated to inhibit endogenous miRNAs that show a pathogenic gain-of-function in diseased tissues. miRNA mimics are used to upregulate expression of beneficial miRNAs. Inhibition of miR-92a, miR-100 and miR-503 or increase of miR-210 can promote angiogenesis, thus improving postischemic blood flow recovery in limb ischemia or myocardial infarction.

Finally, miR-92a expression increases after experimental MI and inhibits neovascularisation [45].

miRNAs in Blood

5. Circulating MicroRNAs

The discovery of miRNAs in biological fluids is opening new frontiers in the miRNA arena. Mitchell et al. first demonstrated that circulating miRNAs could be detected reliably in serum and showed that miRNA profiles correlate with specific diseases. Moreover, miRNAs appear protected from RNAse and remain stable in the blood [66]. Therefore, their stability makes miRNAs concentrations well suited for being tested in patient samples as potential biomarkers of different pathological conditions. Recent studies identified miRNAs in different types of microvesicles (MVs) secreted from cultured cells, thus supporting the idea that MVs may serve as physiological carriers of miRNA [11, 67, 68]. In line with this hypothesis, MVs released from cells were proved able to alter gene expression in neighbouring and distant cells.
in vitro and in vivo by transferring miRNAs [69, 70]. Two distinct processes of MVs release from the cells have been described. MVs may derive from the endosomal membranes that are extruded from the cell surface of activated cells as exosomes [71]. Furthermore, high-density lipoprotein (HDL) transports endogenous miRNAs and delivers them to recipient cells with functional targeting capabilities [72]. Interestingly, a recent study demonstrated that the majority of circulating miRNAs are complexed with protein rather than with vesicles. In particular, Ago2 was present in human plasma and eluted with plasma miRNAs. This finding raises the possibility that cells release a functional RISC complex into the circulation [73].

Recent reports suggest that serum levels of cardiac miRNA correlate with the severity of heart injury and may represent a good biomarker of MI. Corsten et al. described surprisingly increased plasma levels of miR-208b and -499 after MI. However, studies on circulating miRNAs and MI have led to contrasting conclusions on miR-208. Wang et al. suggested miR-208 as a candidate biomarker for MI in humans [74], while Adachi et al. found very low levels of miR-208a and miR-208b in human hearts [75]. The latter result was confirmed by D’Alessandra et al., who reported that miR-208 was detectable only in 30% of MI patients and always at very low levels [76]. In contrast, miR-499 was detectable in all control subjects and increased in all patients with MI [76]. Finally, we found that the plasma levels of miR-503 were dramatically increased in diabetic patients with CLI in comparison with controls. Due to the low number of patients in the study, further analysis is required to investigate miR-503 as a potential circulating biomarker of ongoing ischemia in diabetic subjects [31].

6. Future Research

Great interest focuses on exploiting miRNAs for therapeutic purposes and as clinical biomarkers. As therapeutic targets for vascular diseases, miRNAs could represent a major breakthrough because a single miRNA can regulate several target genes, thus influencing multiple molecular pathways. In consideration of the above, miRNAs antagonists or mimics could become an important new class of drugs to regulate angiogenesis. Moreover, manipulation of miRNAs structures might increase their delivery efficiency. In particular, the promising results obtained with modified microparticles or nanoparticles for drug delivery in cancer [37] should be extended to investigate their capacity for promoting vascular regeneration in ischemic tissues. A potential drawback in the therapeutic use of miRNAs is their possible “off-target effects.” Since not all targets of a particular miRNA are disease-related, any therapeutic perturbation of miRNA expression will likely have side effects.

As biomarkers, circulating miRNAs offer a great potential for the diagnosis and prognosis of different diseases. However, there are still limitations in the technology and various issues regarding miRNA measurement and quantification in the circulation. First, circulating miRNA profiling has to be verified in larger and independent studies in order to translate the findings into clinical biomarker. Then, in order to obtain reliable and reproducible results, there is a need to determine suitable normalization methods for blood-based miRNA investigations. Finally, the mechanisms by which miRNAs are released into the circulation and whether circulating miRNA molecules have any functional capabilities need further characterization.

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