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Role of specific microRNAs for endothelial function and angiogenesis

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Abstract

Accumulating evidence indicates that various aspects of angiogenesis, such as proliferation, migration and morphogenesis of endothelial cells, can be regulated by specific miRNAs in an endothelial-specific manner. As novel molecular targets, miRNAs have a potential value for treatment of angiogenesis-associated diseases such as cancers, inflammation, and vascular diseases. In this article, we review the latest advances in the identification and validation of angiogenesis-regulatory miRNAs and their targets, and discuss their roles and mechanisms in regulating endothelial cell function and angiogenesis.

Keywords

microRNA; Endothelial cell; Angiogenesis; Gene regulation

Introduction

MicroRNA (miRNA) is a class of highly conserved, single-stranded, non-coding small RNAs. After maturation, they entry into the RNA interference pathway and regulate gene expression on the post-transcriptional level by inhibiting the translation of protein from mRNA or by promoting the degradation of mRNA [1,2] [Figure 1]. Many studies have shown that miRNAs control cell proliferation, differentiation, and apoptosis in different types of cells [3-5]. Some endothelial-specific miRNAs have been implicated in the regulation of various aspects of angiogenesis, including proliferation, migration and morphogenesis of endothelial cells [6]. In vitro, some miRNAs have been shown to be highly expressed in endothelial cells [7]. Endothelium is the main regulator of angiogenesis and is highly responsive to angiogenic factors. Human umbilical vein endothelial cells (HUVECs) are a valuable in vitro model of angiogenesis because of their ability to form capillary-like structures in response to appropriate

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stimuli [8]. Here, we review the roles of specific microRNAs in regulation of endothelial function and angiogenesis.

Role of Dicer and Drosha in endothelial cell function and angiogenesis

The biological functions of miRNAs depend on their ability to silence gene expression, primarily via degradation of the target mRNA and/or translational suppression [9,10]. miRNAs are generated in a 2-step processing pathway mediated by 2 major enzymes, Dicer and Drosha, which belong to the class of RNase III endonucleases [Figure 1]. The first evidence implicating miRNAs in the regulation of angiogenesis came from analysis of mice homozygous for a hypomorphic allele of Dicer, which indicated that Dicer was essential for mouse embryonic angiogenesis [11]. The mice with a deletion of the first and second exons of the dicer gene (*dicer^{ex1/2}*) died between days 12.5 and 14.5 of gestation and displayed defect in blood vessel formation. Specific silencing of Dicer and Drosha by siRNA technique in endothelial cells significantly reduced endothelial cell migration, capillary sprouting, and tube formation. However, silencing of Dicer but not of Drosha reduced angiogenesis in vivo [11]. Experiments with cultured endothelial cells demonstrated a role for Dicer in several angiogenic processes, including proliferation, migration, capillary sprouting, and formation of endothelial cell networks resembling capillaries [12-15]. In a model of mouse mutant with a hypomorphic Dicer1 allele (*Dicer (d/d)*), Dicer1 deficiency resulted in female infertility. This defect in female *Dicer (d/d)* mice was caused by corpus luteum insufficiency and resulted, at least in part, from the impaired growth of new capillary vessels in the ovary [16]. Dicer knockdown by specific siRNA in human microvascular endothelial cells (HMECs) diminished cell migration and matrigel tube formation by targeting p47phox of the NADPH oxidase complex [14]. In addition, the loss of Dicer in endothelial cells significantly increased the expression of thrombospondin-1 (TSP-1) [13,15], which has been identified as an endogenous angiogenesis inhibitor. The knockdown of Dicer in human endothelial cells has been shown to modulate gene expression of several key regulators of angiogenesis, such as TEK/Tie-2, KDR/VEGFR2, Tie-1, eNOS and IL-8 [12]. In contrast to Dicer, Drosha appears not to be involved in angiogenic processes in vivo [11,13]. The discrepancy between effects of knockdown of Dicer and Drosha on angiogenesis might be due to the involvement of Dicer in other cellular processes, such as regulation of heterochromatin formation [17] and Drosha-independent miRNA processing pathway that could compensate for loss of Drosha [18].

As Dicer and Drosha are essential for miRNA processing, blocking Dicer or Drosha expression were expected to cause the down-regulation of most miRNAs. In fact, only a few miRNAs have been shown to be affected by the inhibition of these enzymes. This incomplete inhibition of miRNA processing might attribute to an insufficient down-regulation of Dicer and Drosha or to a higher stability of some of the miRNAs that could exceed the transient effect of Drosha or Dicer depletion by siRNA [19].

Angiogenesis-regulatory microRNAs and their targets

Angiogenesis-regulatory miRNAs can be divided into the pro-angiogenic miRNAs that promote angiogenesis and the anti-angiogenic miRNAs that inhibit angiogenesis (Table 1).

Pro-angiogenic microRNAs

miR-126—miR-126 has been shown to be specifically and highly expressed in human endothelial cells [20]. It is located in chromosome 9q34.3 within the host gene encoding for epidermal growth factor like-7 (*Egfl-7*) that is specifically expressed in endothelial cells with high levels in the immature vessels. Loss of *Egfl-7* function in zebra fish embryos specifically blocked vascular tubulogenesis [21]. miR-126 regulates many aspects of endothelial cell biology in vitro, including cell migration, organization of the cytoskeleton, and capillary

network stability. In vivo, the knockdown of miR-126 in zebra fish resulted in the loss of vascular integrity and hemorrhage during embryonic development. The mice deficient in miR-126 (*miR-126^{Δ/Δ}*) exhibited delayed angiogenic sprouting, widespread hemorrhaging, and partial embryonic lethality [22-24]. In addition, miR-126 mutant mice that successfully completed embryogenesis displayed diminished angiogenesis and increased mortality after coronary ligation in a model for myocardial infarction [23]. Adult *miR-126^{Δ/Δ}* mice exhibited 50% impairment of corneal vascularization relative to *Egfl7^{Δ/Δ}* or wild-type mice. *miR-126^{Δ/Δ}*, but not *Egfl7^{Δ/Δ}*, mice exhibited incompletely penetrant embryonic lethality, edema and vascular leakage [24]. Moreover, recent work demonstrates the potential role of miR-126 in arteriogenesis and angiogenesis [20]. miR-126 is functionally active in EC in vitro and it could be specifically repressed using antagomirs specifically targeting miR-126. In vivo, mice treated with a high dose of antagomir-126 had a markedly reduced angiogenic response [20].

miR-126 regulates endothelial cell angiogenic activity in response to angiogenic growth factors such as VEGF and bFGF, through targeting multiple proteins that modulate angiogenesis and vascular integrity [22-24]. Sprouty-related protein family members function as membrane-associated suppressors of growth factor-induced ERK activation [25]. *Spred-1* contains a predicted target sequence for miR-126, and it plays a major role as a mediator of the pro-angiogenic actions of miR-126. miR-126 functions in part by directly repressing negative regulators of *Spred-1* [22,23]. Furthermore, miR-126 regulating VEGF and other growth factor signaling may be reinforced by the target of PI3 kinase regulatory subunit 2 (*PI3KR2/p85*), which negatively regulates the activity of PI3 kinase [26]. Importantly, siRNA-mediated knockdown of *PIK3R2* in cells with reduced miR-126 levels was able to rescue the defect in VEGF-dependent Akt/PKB phosphorylation, indicating the *PIK3R2* as a target of miR-126 [22,24]. In addition, miR-126 may be involved in vascular inflammation by suppressing vascular cell adhesion molecule- 1 (VCAM-1) expression and thereby decreasing leukocyte interactions with endothelial cells [27].

miR-17-92 Cluster—In the human genome, this cluster encodes seven miRNAs, namely, miR-17-5p, miR-17-3p, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92-1[28]. miR-17-92 promotes cell proliferation, suppresses cancer cell apoptosis, and induces tumor angiogenesis [5,29,30]. A potent tumor angiogenesis promoting activity has been attributed to the miR-17-92 cluster which is significantly upregulated in Myc-induced tumors.[31]. Enhanced neovascularization correlated with downregulation of anti-angiogenic thrombospondin-1 (Tsp1) and related proteins, such as connective tissue growth factor (CTGF). Both Tsp1 and CTGF are predicted targets for repression by the miR-17-92 cluster. In particular, miR-18 preferentially suppresses CTGF expression, whereas miR-19 targets the potent angiogenesis-inhibitor Tsp-1. Indeed, miR-17-92 knockdown partially restored Tsp1 and CTGF expression. Transduction of Ras-only cells with a miR-17-92–encoding retrovirus reduced Tsp1 and CTGF levels [31,32]. miR17-5p controlled endothelial cell proliferation and motility by targeting the anti-angiogenic factor tissue inhibitor of metalloproteinase 1 (TIMP-1). miR17-5p downregulated TIMP-1 expression and activity [16]. In the model of mouse mutant with a hypomorphic *Dicer1* allele (*Dicer (d/d)*), the impaired angiogenesis in the corpus luteum in *Dicer (d/d)* mice was associated with a lack of miR17-5p and let7b. Furthermore, injection of miR17-5p and let7b into the ovaries of *Dicer (d/d)* mice partially normalized TIMP1 expression and recovered the vascularity in the ovaries CL in vivo [16]. In addition, angiogenesis is mostly an adaptive response to tissue hypoxia, which induces the accumulation of hypoxia inducible factors (HIF) to activate the expression of many angiogenic genes [33]. HIF-1α has been identified as a novel direct target for miR-17-92 cluster, and induction of miR-17-92 may play a role at least in part in c-myc-mediated repression of HIF-1 α [30].

Let-7 family—Let-7 and its family members are highly conserved across species in sequence and function, and misregulation of let-7 leads to a less differentiated cellular state and the development of cell-based diseases such as cancers [34]. Let-7 is highly expressed in HUVECs [8,13]. Inhibition of let-7f significantly reduced sprout formation in vitro [13]. Let-7b can control EC proliferation and motility to affect tube formation by suppressing the expression and activity of the anti-angiogenic factor TIMP-1 [16]. Although in silico analysis of target genes using miRanda software predicted that let-7 family might regulate the expression of TSP-1, in fact, inhibition of let-7f induced only a minor, not significant, increase in TSP-1 expression [13].

miR-130a—miR-130a expression was undetectable in quiescent HUVECs and strongly upregulated after exposure to fetal bovine serum [35]. miR-130a is a regulator of the angiogenic phenotype of vascular ECs through its ability to modulate the expression of homeobox gene GAX (growth arrest-specific homeobox) and homeobox A5 (HOXA5) [35]. GAX is expressed in both vascular ECs and smooth muscle cells (VSMCs), and has been shown to be a major regulator of EC phenotype in response to both pro-angiogenic and anti-angiogenic signals [36]. miR-130a contains important regulatory sequences in the GAX 3'-UTR that control the down-regulation of GAX and HOXA5 expression in response to mitogens, proangiogenic factors, and pro-inflammatory factors. A 280-bp fragment from the GAX 3'-UTR contain 2 miR-130a targeting sites [35]. After exposure to mitogens, rapid down-regulation of GAX expression required the miR-130a sequences in the GAX 3'-UTR. In addition, miR-130a could down-regulate the expression of HOXA5, and antagonize HOXA5 function in ECs [35].

miR-210—miR-210 up-regulation is a crucial element of endothelial cell response to hypoxia, affecting cell survival, migration, and differentiation [37]. In normoxic endothelial cells, miR-210 overexpression could stimulate VEGF-driven cell migration and the formation of capillary-like structures on Matrigel, whereas its blockade decreased the cell migration in response to VEGF and inhibited the formation of the capillary-like structures [37]. miR-210 affects angiogenesis mainly by targeting HIF and ephrin-A3. HIF-1 α induced the expression of miR-210 in endothelial cells [38,39]. Hypoxia is a key determinant of tissue pathology during tumor development and organ ischemia. Two recent studies showed that miR-210 was up-regulated in rat models of cardiac hypertrophy/cardiac failure [40] and brain transient focal ischemia [41].

Ephrin-A3, eph-related receptor tyrosine kinase ligand 3, is a direct target of miR-210 in hypoxia since miR-210 was necessary and sufficient to down-modulate its expression [37]. Ephrin-A3 modulation by miR-210 has significant functional consequences. The expression of an Ephrin-A3 allele that is not targeted by miR-210 prevented miR-210-mediated stimulation of both tubulogenesis and chemotaxis. Down-regulation of Ephrin-A3 expression by miR-210 modulates endothelial cell angiogenic response to hypoxia [37]. In the developing cardiovascular system, Eph and ephrin molecules control the angiogenic remodeling of blood vessels and lymphatic vessels and play essential roles in endothelial cells as well as in supporting pericytes and vascular smooth muscle cells [42].

miR-378—miR-378 functions as an oncogene by enhancing angiogenesis, tumor cell survival, and tumor growth. Recent work demonstrated that miR-378 transfection reduced caspase-3 activity, enhanced cell survival, tumor growth, and angiogenesis through repression of the expression of two tumor suppressors, Sufu (suppressor of fused) and Fus-1 (tumor suppressor candidate 2, TUSC2) [43]. In most of the cell lines tested, high level of Sufu expression was correlated with low level of miR-378 expression and vice versa. Cell survival assays showed that the reintroduction of Sufu into miR-378-expressing cells reversed the effect of miR-378 on cell survival. In the presence of the 3'-UTR, the effect of Sufu was repressed by miR-378, which promoted cell survival, confirming that the Sufu 3'-UTR is a target of miR-378.

Transfection of Fus-1 siRNA reduced Fus-1-triggered cell death, whereas reintroduction of Fus-1 reversed the effect of miR-378-mediated effect on cell survival. These findings suggest that Sufu- and Fus-1-mediated pathways are essential for miR-378-enhanced cell survival [43].

miR-296—Recent work indicates that miR-296 is a critical component of the angiogenic process [44]. Angiogenic growth factors enhance the level of miR-296 in primary cultures of human brain microvascular ECs. The miR-296 level is also elevated in primary tumor ECs isolated from human brain tumors compared to normal brain ECs. Down- and upregulation of miR-296 results in the inhibition and induction, respectively, of morphologic characteristics associated with angiogenesis of human ECs. Sequence-specific inhibition of miR-296 by intravenous injection of cholesterol-conjugated antagomirs results in decreased neovascularization of tumors in mice. Moreover, inhibition of miR-296 with antagomirs reduces angiogenesis in tumor xenografts in vivo [44]. Altogether, these findings support a role for increased miR-296 levels in promoting angiogenesis in tumors. The hepatocyte growth factor-regulated tyrosine kinase substrate (HGS) has been identified as a target for miR-296. Since HGS mediates the degradative sorting of PDGFR as well as VEGFR and EGFR, it seems likely that increased levels of these growth factor receptors in angiogenic blood vessels are due at least in part to the miR-296 downregulation of HGS expression [44]. In addition, EGF was also capable of inducing miR-296, suggesting a complex growth factor-growth factor receptor crosstalk mechanism that combinatorially increases miR-296 levels [44].

Anti-angiogenic microRNAs

miR-221/miR222—miR-221 and miR-222 belong to the same family and control common targets. These genes are located in close proximity on Xp11.3 chromosome, and might be regulated in a coordinated manner [45]. The two miRNAs have been shown to inhibit endothelial cell migration, proliferation, and angiogenesis in vitro by targeting stem cell factor (SCF) receptor, c-Kit [8,46]. SCF dose-dependently promotes survival, migration, and capillary tube formation by HUVECs [7]. In vivo, miR-221/miR-222-transfected cells were no more able to form tubes or to heal wounds, in response to SCF [8]. High glucose treatment of HUVECs induced expression of miR-221 and impaired endothelial cell migration, and reduced expression of c-kit [46]. Furthermore, antisense miR-221 oligonucleotide reduced expression of miR-221, restored c-kit protein expression in HUVECs, and abolished the inhibitory effect of high glucose on HUVECs transmigration. These findings suggest that miR-221-c-kit pathway may play an important role in diabetes-associated vascular dysfunction [46]. In addition, miR-221/miR-222 indirectly regulate endothelial nitric oxide synthase (eNOS) expression [12]. miR221/222 overexpression in Dicer-knockdown ECs restored the elevated eNOS protein levels that induced by Dicer silencing. Since prediction sites for these miRNAs were not found in eNOS 3'UTR, the regulation of eNOS protein levels by miR-221/222 is likely to be indirect [12].

Endothelial progenitor cells (EPCs) play an important role in the maintenance of vascular integrity. MicroRNA (miR)-221/222 is closely linked to the proliferation of endothelial cells. A recent study shows that levels of miR-221/222 were significantly higher in the coronary artery disease (CAD) group than in the non-CAD group, and were weakly negatively correlated with EPC number in the CAD group. Lipid lowering therapy with atorvastatin markedly increased EPC numbers and decreased miR-221/222 levels in patients with CAD [47].

miR-328—Computational predictions indicate that *miR-328* potentially targets a number of cell adhesion molecules including hyaluronan receptor CD44 [48]. CD44 has been implicated in a variety of biological processes including angiogenesis, wound healing, leukocyte extravasation at inflammatory sites, and tumor metastasis [49]. miR-328 was found to have

several potential target sites in CD44 3'-UTR [48]. Recently, Wang et al demonstrated that miR-38 controls zonation morphogenesis by targeting CD44 [48]. In A431 human epidermoid carcinoma cells, expression of *miR-328* repressed CD44 expression, reduced cell adhesion and migration, and reduced formation of capillary structure, and the miR-328-mediated CD44 actions was validated by anti-CD44 antibody, CD44 siRNA, and CD44 expression constructs, suggesting that the CD44 pathway is essential for miR-328-mediated cell activities [48].

miR-15b, miR-16, miR-20—miR-15b and miR-16 are located in the same cluster on chromosome 3 [50]. miR-20a is a member of the miR-17-92 cluster, and miR-20b is a member of the miR-106a cluster located on X chromosome [50,51]. In CNE cells from a human nasopharyngeal carcinoma cell line, expression of miR-15b, miR-16, miR-20a and miR-20b was markedly down-regulated under hypoxia [50]. The down-regulation of these miRNAs in CNE cells might be mediated by the accumulation of the tumor suppressor p53 or the stabilization of HIF-1 α during hypoxia. The p53 gene might be one of the critical factors which cause the changes in miRNA levels during hypoxia. VEGF is a fundamental regulator of normal and abnormal angiogenesis. Transfection with miR-15b, miR-16, miR-20a, and miR-20b resulted in a 26–51% decrease in VEGF protein expression [50]. In addition, miR-15b and miR-16 may be essential for cell apoptosis by targeting Bcl-2 and the caspase signaling cascade [51].

Conclusion

Although more than 700 human miRNAs have been identified in the human genome, a few specific miRNAs that regulate endothelial cell function and angiogenesis have been validated. Cell-specific targeting with miRNAs is an important area of investigation to be developed. As a single miRNA can bind to multiple targets whereas single gene may be regulated by multiple miRNAs, further studies need to analyze the complex interactions between endothelial-specific miRNAs and their targets during angiogenesis. Identification of miRNAs and their targets and better understanding of in vivo mechanisms will lead us towards development of miRNA drugs designed against specific molecular targets for clinical therapy, its immense therapeutic potential needs to be harnessed further.

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Figure 1. The biological synthesis of microRNA and its role in angiogenesis

miRNA genes are transcribed into pri-miRNAs and processed by Drosha to yield 70- to 90-nt pre-miRNA transcripts that form hairpin structures. Drosha acts in the nucleus, cleaving the pri-miRNA near the base of the hairpin stem to yield the pre-miRNA sequence. The pre-miRNA is then exported to the cytoplasm where the stem is cleaved by Dicer to produce a miRNA duplex. Only one strand (red) is preferentially assembled into the RNA-induced silencing complex (RISC), which subsequently acts on its target by translational repression or mRNA cleavage. Some specific miRNAs that regulate endothelial cell function and angiogenesis have been validated in vitro or in vivo.

Table 1
Angiogenesis-regulatory miRNAs and their targets

microRNA	Biological function	Targets	Ref.
Pro-angiogenic			
miR-126	Maintain vascular development, regeneration and integrity	Spred-1, PIK3R2/p85- β , VCAM-1	20, 22, 24, 27
miR-17-92 cluster	Promote tumor angiogenesis	TSP-1, CTGF, TIMP-1, HIF-1 α ?	16, 30, 31,
Let-7b,f	Regulate sprout formation	Let-7b--TIMP1; Let-7f--TSP-1?	13, 16
miR-130a	Regulate angiogenic phenotype of EC	Homeobox gene: GAX, HOXA5	35
miR-210	Promote EC migration and capillary-like structure formation	Ephrin-A3, HIF-1 α	37, 39
miR-378	Promote tumor angiogenesis	SuFu, Fus-1	43
miR-296	Promote EC migration and tube formation, and tumor angiogenesis in vivo.	HGS	44
Anti-angiogenic			
miR-221/miR-222	Inhibit EC migration, proliferation	c-kit, eNOS	8, 12, 46
miR-328	Reduce formation of capillary structure	CD44	49
miR-15b/miR-16	Induce cell apoptosis	VEGF, Bcl-2?	51, 52
miR-20a/miR-20b		VEGF	51