miRNAs in vascular integrity (Review)

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Abstract. Endothelial cells (ECs) are confirmed as important regulators of vascular integrity, particularly in relation to angiogenesis, wound repair post-injury, and during embryogenesis. Futher, miRNAs have been implicated in EC function and proliferation. Moreover, knockdown of these miRNAs resulted in altered expressions of several important regulators of endothelial biology and angiogenesis including vascular endothelial growth factor receptor 2, endothelial nitric oxide synthase and tubule formation capacity. Several miRNAs have been identified to play a role in the regulation of function, proliferation and growth of vascular ECs. These miRNAs may be important therapeutic targets in the treatment of a range of ischemic diseases, as well as in the regulation of angiogenesis during cancer and tumour progression. The present review discuss some of the important miRNAs having confirmed regulatory role in EC in connection espically with cardiovascular disease.

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Introduction

Cardiovascular diseases (CVDs) associated morbidity and mortality is continuously evolving worldwide (1-3). The term CVD encompasses all diseases of the heart and circulatory system, including stroke, myocardial infarction (MI) and a variety of ischemic diseases. Although the number of people living with CVD has fallen in recent years, there is still need for the development of novel therapeutic strategies to relieve the burden on health services around the world.

Endothelial cells (ECs) play a major role in maintenance of vascular integrity, particular with relation to angiogenesis and wound repair post-injury, as well as in vasculogenesis during *in vivo* embryo development (4). Further, miRNAs have been implicated in EC function and proliferation, as well as in the regulation of angiogenesis and vasculogenesis. Global reduction of miRNAs, via conditional knockdown of the miRNA processing Dicer using siRNAs *in vitro*, altered the expression of several important regulators of endothelial biology and angiogenesis including vascular endothelial growth factor receptor 2 (VEGFR2; KDR) and endothelial nitric oxide synthase (eNOS), as well reducing proliferation and tubule formation capacity (5). *In vivo*, Dicer silencing was also shown to reduce postnatal angiogenesis in response to angiogenic cytokines, such as VEGF (5). Combined transient silencing of both Dicer and Drosha processing enzymes reduced the sprout forming and angiogenic properties of ECs, although only silencing of Dicer had significant effects on angiogenic potential *in vivo* (6). Several miRNAs have been identified to play a role in the regulation of function, proliferation and growth of vascular ECs (7). These include miR-126, miR-10a, the Let-7 cluster, the pro-angiogenic miR-17-92 cluster and the anti-angiogenic miR-221 and miR-222. miRNAs identified to play key roles in the regulation of angiogenesis may be important therapeutic targets in the treatment of a range of ischemic diseases, as well as in the regulation of angiogenesis during cancer and tumor progression.

2. miR-126 in vascular integrity

One of the most studied and extensively characterized EC miRNAs is miR-126. Early miRNA profiling studies found that miR-126 is enriched in tissues with a high vascular component, and expression patterns in zebrafish also showed the expression of miR-126 confined to the vascular system (8).

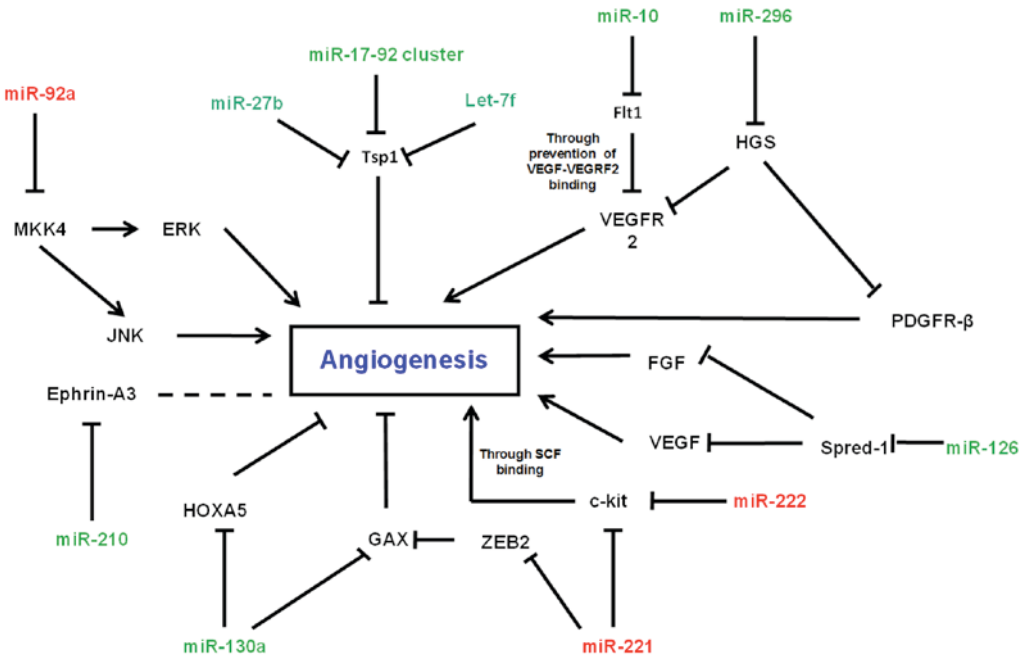

Figure 1. miRNAs play an important role in the regulation of angiogenesis.

Further, a study revealed miR-126 is expressed in primary human umbilical vein ECs (HUVECs), as well as in a number of EC lines (9). Moreover, miR-126 has been reported to be highly enriched miRNA in ECs generated from differentiating mESC (10). Generation of miR-126-null mice resulted in ~40% embryonic lethality, exhibiting a large number of vascular defects, including severe systemic oedema, haemorrhage and vessel rupture, and knockdown in zebrafish using pri-miR-126-specific morpholinos resulted in comprised blood vessel integrity, haemorrhages and compromised endothelial tube formation. Taken together, this data suggested a role for miR-126 in the maintenance of endothelial and vascular integrity during development. Knockdown of miR-126 also resulted in a decrease in angiogenesis during *ex vivo* and *in vivo* assays. To exert its angiogenic affects, miR-126 was also shown to augment MAP kinase pathway activation in response to angiogenic factors such as FGF and VEGF *in vitro*, with knockdown of the miRNA resulting in a reduction in FGF and VEGF-mediated migration, possibly through defective reorganisation of the actin cytoskeleton (10). It was subsequently demonstrated that miR-126 exerts its angiogenic effects through the targeting of the sprouty-related protein Spred-1 (Fig. 1). A role for miR-126 in the regulation of vascular inflammation has also been elucidated, via its targeting and repression of vascular cell adhesion molecule-1 (VCAM-1), an intercellular adhesion molecule expressed by ECs. VCAM-1 expression is upregulated during vascular inflammation, where it functions to mediate the ratio of EC:leukocyte adhesion. Reduced expression of miR-126, in tumour necrosis factor-α (TNF-α) stimulated ECs, increases the expression of VCAM-1, in turn enhancing leukocyte adherence and, ultimately, vascular inflammation (9).

miR-126 is located within intron 7 of epidermal growth factor-like domain 7 (Egfl7), a gene which is highly expressed in ECs, and the expression of both strands of the miR-126 stem loop (miR-126-3p and -5p) mirrors the expression of Egfl7 (11). This suggested that miR-126 is processed from the pre-mRNA-Egfl7 transcript. A 5' region upstream of the Egfl7/miR-126 locus was then found to regulate expression of miR-126, and *in silico* analysis subsequently revealed two Ets binding sites (EBS) within this region. The Ets factors are a family of transcription factors, and several members, including Ets-1 and -2, have been shown to play important roles in vasculo- and angiogenesis (12). Ets-1 and -2 were found to bind to the Egfl7/miR-126 5' region to activate transcription in ECs, therefore suggesting that these transcription factors play an important role in the regulation of miR-126 expression and, therefore, the regulation of it angiogenic affects (11).

3. miR-17-92 cluster in vascular integrity

The miR-17-92 cluster, consisting of miR-17, -18a, -19a/b, -20a and -92a, has also been extensively studied in the context of ECs and angiogenesis. Originally, it was discovered that this cluster plays a role in tumor angiogenesis. The above cluster of miRNAs have been observed to be upregulated also in colonocytes. In this system the miRNAs target the anti-angiogeneic thrombospondin-1 (Tsp1) and the related protein connective tissue growth factor (CTGF), to cause an increase in angiogenesis, and cells transduced with the miR-17-92 cluster formed larger, better-perfused tumors (13). On the other hand a study by Doebele *et al* showed that the overexpression of individual members of the cluster, specifically miR-17, -18a, -19a and -20a, resulted in reduction in angiogenic sprouting. However, same inhibitors of the similar miRNAs caused an increase in angiogenesis *in vitro*, suggesting an anti-angiogenic role for this cluster (14). This ties in with previous work showing that miR-92a targets several proangiogenic proteins, including integrin subunit α5, to exert an anti-angiogenic effect, with overexpression of this miRNA blocking angiogenesis *in vivo* and *in vitro* (15). More recently, it was shown that histone deacetylase 9 (HDAC9) displays a

proangiogenic affect, regulated through the transcriptional repression of the miR-17-92 cluster (16). Taken together, the data suggest a varied role for the miR-17-92 cluster in the context of EC function and angiogenesis.

4. miR-210 in vascular integrity

Reduced miR-210 expression has been shown to inhibit EC growth and induce apoptosis, suggesting a proangiogenic role for this miRNA. In hypoxic conditions, it was found that the level of miR-210 was increased in HUVECs when compared to cells in normoxic conditions (17). In normoxic ECs *in vitro*, overexpression of miR-210 simulated the formation of capillary-like structures on Matrigel, and VEGF-mediated migration. Knockdown of this miRNA inhibited these same functions, and also inhibited cell growth and caused increased apoptosis. *In silico* analysis revealed Ephrin-A3 (EFNA3), a gene post-transcriptionally downregulated by hypoxia, as a potential target for miR-210, and luciferase assays using the 3'-UTR of the EFNA3 mRNA confirmed this. Downregulation of this gene was proven to be necessary for miR-210-regulated angiogenesis. Lou *et al* further elucidated the mechanism of miR-210 and its role in angiogenesis by investigating the role of this microRNA in regulating angiogenesis in response to brain ischemic injury. miR-210 was significantly upregulated in adult rat ischemic brain cortexes *in vivo*, where the expression of Notch-1 signaling molecules, which facilitate the migration of ECs, were also increased. Using lentiviral-mediated overexpression, it was shown that miR-210 activated the Notch signaling pathway, facilitating the migration of ECs inducing *in vitro* capillary formation (18). This has also been shown in the normal adult mouse brain, where overexpression of miR-210, using stereotactic injection of a lentiviral vector, increased neurogenesis and angiogenesis, associated with a local increase in the levels of VEGF (19). Overall, data suggest that miR-210 could be used as a therapy, or a therapeutic target for the modulation of angiogenesis in the treatment of ischemic diseases. Indeed, miR-210 has been used as a therapy *in vivo* to enhance wound healing, and tissue repair, processes in which angiogenesis plays a key role (20).

5. miR-10 in vascular integrity

Interestingly, it was also suggested that miR-10 played a role in angiogenesis, after it was discovered that heparin, an antithrombotic drug, impairs angiogenesis through the inhibition of miR-10, therefore inducing upregulation of its mRNA target Hoxd10 (21). Indeed, further investigation showed an increase in the expression of miR-10a in $CD144^+$ $VEGFR2^+$ ECs generated from mESCs when compared to $CD144^-$ $VEGFR2^-$ non-ECs (22). Knockdown of all four miR-10 isoforms (a-d) during zebrafish development resulted in an impairment in angiogenesis and the development of the intersegmental vessels, whereas overexpression lead to enhanced angiogenic potential *in vivo*. *In vitro* experiments demonstrated the direct targeting of the 3'-UTR of Flt1, also known as VEGFR1, and its soluble splice variant sFlt1 by miR-10, with miR-10 depletion leading to an increase in Flt-1 expression both *in vivo* and *in vitro*. Flt-1 binds VEGF and is thought to function to inhibit angiogenesis by sequestering VEGF to prevent binding to VEGFR2 (22).

6. miR-221 and miR-222

Just as important as miRNAs with potential proangiogenic functions, the miRNAs with anti-angiogenic functions may also be useful targets in the control of angiogenesis in a disease setting, such as in the regulation of tumor angiogenesis during cancer, as well as in cardiovascular and ischemic diseases. Similarly to those involved in the positive regulation of angiogenesis, anti-angiogenic miRNAs function via the targeting of an array of angiogenic-associated mRNAs (Fig. 1).

Located intergenically, miR-221 and miR-222 are transcribed as a cluster, and have been shown to share an identical seed sequence. Both of these miRNAs have been described as anti-angiogenic, and were shown to regulate c-kit, the receptor for stem cell factor (SCF), at the protein level (23). SCF is an angiogenic factor, which has been shown to promote survival, migration and capillary formation of HUVECs *in vitro* (24). Overexpression of miR-221 and miR-222 caused a reduction in survival, migration and capillary formation *in vitro* mediated through reduction in the levels of c-kit. Another study showed that miR-221 and miR-222 regulate the expression of eNOS, with overexpression of these miRNAs inhibiting the increase in eNOS expression observed after Dicer knockdown (5).

It has also been shown that miR-221 indirectly upregulates a potential master regulator of EC angiogenic phenotype namely GAX, so as to inhibit angiogenesis (25). It was hypothesised that this upregulation was mediated through the downregulation of an intermediate protein, and this was subsequently discovered to be ZEB2, a zinc-finger nuclear factor which primarily acts as a transcriptional repressor (26).

7. Other notable pro- and anti-angiogenic miRNAs

Similarly to miRNAs already mentioned, other angiogenesis-regulating miRNAs also function via the direct or indirect targeting of growth factors and their receptors. One such miRNA is the proangiogenic miR-296, which was found to be upregulated when human brain microvascular ECs were treated with angiogenic factors, and also shown to be expressed at higher levels in primary highly angiogenic ECs derived from human brain tumors, when compared to normal brain ECs (27).

In this context miR-296 was found to target hepatocyte growth factor-regulated tyrosine kinase substrate (HGS), a protein involved in the mediation of the degradation of a number of growth factor receptors, including platelet-derived growth factor receptor β and VEGFR2, therefore increasing the levels of signaling by angiogenic factors (28).

Moreover, miR-15b and -16, along with miR-20a and -20b, are also thought to exert their antiangiogenic effects via the targeting of VEGF (29). Although the study was performed in a human nasopharyngeal carcinoma cell line, all four of these miRNAs were downregulated in response to hypoxia, in contrast to the concomitant upregulation of VEGF. These data suggested a role for the four miRNAs in the regulation of angiogenesis, and this has been supported by evidence in a number of other studies (30,31). In the same study miR-378 has

been noted to regulate the expression of VEGF (29). Further, studies showed a proangiogenic role for miR-378, through its propensity to cause an increase in cell survival and a decrease in apoptosis, in a similar way to miR-210 (32). MiR-100, a miRNA expressed in both ECs and vascular smooth muscle cells (VSMCs), was also found to be downregulated during hypoxia, much like miR-15 and miR-16. *In vivo*, miR-100 was significantly downregulated after the induction of hind limb ischemia in mice, and it was found to control angiogenic sprouting and the proliferation of vascular cells *in vitro* (33). Mammalian target of rapamycin (mTOR), a gene previously demonstrated to be involved in angiogenesis and proliferation of ECs in response to hypoxia (34), was found to be a direct target of miR-100 (4) and this was confirmed in the context of ECs, indicating a role for this miRNA in the negative regulation of EC angiogenesis. More recently, this has also been shown in the context of graft-versus-host disease (GvHD), whereby miR-100 antagonism increased neovascularisation and, therefore, increased disease severity (35).

A global knock out of miRNA processing enzymes in human ECs also lead to the identification of miR-27b and Let-7f as possible proangiogenic miRNAs (6).

8. Conclusion

It was concluded from the above studies that miRNAs have significant role in the control of vascular EC function and angiogenic phenotype. This information could further help in development of novel therapies, not only for the treatment of ischemic diseases, but also for angiogenesis regulation in the context of cancer.

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