

# The role of blood flow and microRNAs in blood vessel development

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**ABSTRACT** The circulatory system is the first organ system that develops during embryogenesis, and is essential for embryo viability and survival. Crucial for developing a functional vasculature are the specification of arterial-venous identity in vessels and the formation of a hierarchical branched vascular network. Sprouting angiogenesis, intussusception, and flow driven remodeling events collectively contribute to establishing the vascular architecture. At the molecular level, arterial-venous identity and branching are regulated by genetically hardwired mechanisms involving Notch, vascular endothelial growth factor and neural guidance molecule signaling pathways, modulated by hemodynamic factors. MicroRNAs are small, non-coding RNAs that act as silencers to fine-tune the gene expression profile. MicroRNAs are known to influence cell fate decisions, and microRNA expression can be controlled by blood flow, thus placing microRNAs potentially at the center of the genetic cascades regulating vascular differentiation. In the present review, we summarize current progress regarding microRNA functions in blood vessel development with an emphasis on studies performed in zebrafish and mouse models.

**KEY WORDS:** *microRNA, blood vessel, development, endothelial cell, smooth muscle cell*

## Introduction

The formation of a properly branched vascular system is critical for embryo development and survival. Two successive processes called vasculogenesis and angiogenesis, achieve blood vessel formation during embryonic development. Vasculogenesis is the *in situ* differentiation of angioblasts and endothelial cells from mesenchyme, and their coalescence into tubes of the primary vascular plexus. This process is responsible for the formation of the central axial vessels (including the dorsal aorta), and a meshwork of capillaries. Expansion of the primitive vascular system formed by vasculogenesis occurs through sprouting and intussusception angiogenesis. These distinct angiogenesis processes lead to the remodeling of the primary vascular plexus into a hierarchical vascular tree, consisting of branched arteries, capillaries, and branched veins. Genetic imprinting of arterial-venous identity in embryonic vessels involves Notch signaling (Lawson *et al.*, 2002) counteracted by COUP-TFII, and is reviewed in more detail elsewhere (Gridley, 2007; Swift and Weinstein, 2009). It is established that arteries and veins express distinct molecular markers including neural guidance genes like ephrinB2 and Unc5B in arteries, and EphB4 and neuropilin-2 in veins.

Sprouting angiogenesis involves the budding, and extension of new segments from preexisting vessels, that grow into previously avascular areas. The main driving forces for this process are gradients of vascular endothelial growth factor (VEGF) and hypoxia (Ruhrberg *et al.*, 2002). It is now established that the growing angiogenic sprout consists of genetically distinct cell populations, the so-called endothelial tip and stalk cells (De Smet *et al.*, 2009; Gerhardt *et al.*, 2003). Tip cells display numerous filopodia that explore the local environment for guidance cues. The stalk cells can proliferate and form the lumen of the expanding sprout. The molecular key-players involved in tip-stalk cell differentiation include Notch-delta like 4 signaling (Hellstrom *et al.*, 2007; Phng *et al.*, 2009; Siekmann and Lawson, 2007; Suchting *et al.*, 2007), VEGF receptor signaling (Chappell *et al.*, 2009; Covassin *et al.*, 2006; Tammela *et al.*, 2008), as well as neural guidance gene receptors

*Abbreviations used in this paper:* 3' UTR, 3' un-translated region; CAD, coronary artery disease; COUP-TFII, chicken ovalbumin upstream promoter transcription factor 2; CTGF, connective tissue growth factor; GAX, growth arrest-specific homeobox; HIF-1 $\alpha$ , hypoxia inducible factor-1 $\alpha$ ; HUVEC, human umbilical vein endothelial cell; miRNA, microRNA; MMVEC, myocardial microvascular endothelial cell; SMC, smooth muscle cells; VEGF, vascular endothelial growth factor.

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(Larrivee *et al.*, 2009) that contribute to guidance and positioning of growing sprouts. The molecular processes governing sprout differentiation and vessel guidance will be discussed in more detail by Anne Eichmann, in this issue.

After the start of blood flow, the primitive vascular plexus shows a high degree of plasticity. Vessels with high flow widen, blood vessels with low flow regress, and some vessel segments are disconnected and reused to fashion growth of other vascular compartments (Buschmann *et al.*, 2010; le Noble *et al.*, 2004). While blood vessels with high blood flow in general appear to have less or no endothelial tip cells, increasing blood flow associates with the induction of splitting angiogenesis, also referred to as intussusception (Djonov *et al.*, 2003; Djonov *et al.*, 2000). This process contributes to increasing capillary number, adjustment of vessel branching angles in response to blood flow, and molding out small arterioles and venules from the capillary plexus. For a more comprehensive overview on intussusception, we refer to the chapter by Valentin Djonov, in this issue. The importance of blood flow in regulating arterial-venous differentiation and vessel patterning was recently shown in series of studies using zebrafish, chicken embryo and mouse embryo (Buschmann *et al.*, 2010; le Noble *et al.*, 2004; Lucitti *et al.*, 2007; Nicoli *et al.*, 2010). In mouse and chicken embryo, lack of blood flow perfusion in the yolk sac vascular plexus, for example induced by ablating cardiac activity, results in impaired remodeling of arteries and veins from capillaries. Moreover, in the chicken embryo, exposing arteries to venous blood flow resulted in down-regulation of arterial marker genes including ephrinB2 and neuropilin-1, and emergence of venous markers, including neuropilin-2 and COUP-TFII, in previous arterial domains. Vice versa, veins exposed to arterial flow conditions lost venous markers and instead started to express arterial markers, suggesting that hemodynamic factors can modulate the expression of arterial-venous identity genes. Since mice mutant for such identity genes in general show vascular remodeling defects during early embryogenesis, blood flow may contribute to the maintenance of the expression of these genes allowing AV remodeling to proceed.

In *Mlc2a*<sup>-/-</sup> mutant mice, which display a selective cardiac contractility defect impeding proper control of cardiac output, yolk sac remodelling does not occur (Lucitti *et al.*, 2007). This could be attributed to the mechanical properties of the early blood, and not to the presence of circulation angiogenic factors or changes in oxygen carrying capacity (Lucitti *et al.*, 2007). Using erythroblast trapping techniques and injection of high molecular weight synthetic sugars, it was demonstrated that the mechanical force, normally imparted by the flow of circulating erythrocytes, is necessary and sufficient to induce vessel remodelling in the yolk sac of early mouse embryo. In neuropilin-1 mutant mice, yolk sac perfusion is initiated, by subsequent arterial-venous network formation severely impaired, suggesting that active neuropilin-1 function is required for the transduction of mechanical forces exerted by blood flow into a remodelling response (Jones *et al.*, 2008).

We recently addressed which hemodynamic signal exerted by blood flow could be responsible for the maintenance of identity genes in developing arteries of the chicken embryo yolk sac vascular plexus (Buschmann *et al.*, 2010). We considered factors related to flow (shear stress), pressure (pressure related cyclic stretch of vessels), and oxygen. The best discrimination between arteries and veins was obtained by calculating the maximal pulsatile increase in shear rate relative to the time-averaged shear rate in the same

vessel: the relative pulse slope index (RPSI). RPSI is significantly higher in arteries than veins. Experimentally lowering RPSI in pre-existing arterioles resulted in loss of arterial markers and emergence of venous markers (Buschmann *et al.*, 2010). *In vitro*, exposure to a pulsatile shear regime augmented arterial marker expression including *hey/hes* transcription factors and ephrinB2, compared to exposure to constant shear. During early embryogenesis, arterial pressures in the developing yolk sac vasculature are extremely low (about 2 mmHg in arteries, less than 1 mmHg in veins), and pressure induced cyclic stretch was limited to the aorta only. Although the role of pressure in vessel remodeling in the adult is well established, the low pressures in the embryonic system, as well as the small differences in pressure between arteries and veins, makes it unlikely that, at this stage of development, endothelial cells can sense an absolute threshold pressure value above which they start to express arterial marker genes. Hypoxia is known to stimulate the expression of arterial markers in endothelial (progenitor) cells *in vitro*. Surprisingly, exposing the developing embryo to global hypoxia, reduced arterial network formation in the yolk sac most likely due to a negative inotropic effect of hypoxia on cardiac activity (Tintu *et al.*, 2009), resulting in reduced cardiac output and flow delivery to the vitelline circulation.

Besides modulating vessel identity, blood flow also contributes to shaping the global pattern of developing arterial-venous networks (le Noble *et al.*, 2004). In the chicken embryo these flow induced morphological changes included 1) flow driven fusion of small calibre vessels into large tubes, a process accounting for the formation of the large vitelline arteries and veins, 2) selective disconnection of endothelial cells from the arterial system and reuse of these endothelial cells to fashion growth of the venous system, 3) shear stress driven guidance of lumenized vessel sprouts. Moreover, in response to changes in arterial blood flow distribution, we noted strong induction of intussusception aiding to patterning of arteries in the direction of the evoked blood flow distribution. Such unique plasticity of the arterial pattern in response to blood flow may be limited to early embryogenesis, and during later stages other cues derived from vascular smooth muscle cells and perivascular nerves clearly contribute to regulating vessel identity and patterning as well (Larrivee *et al.*, 2009).

Although zebrafish embryos can make the major axial vessels like the aorta, and cardinal vein, as well as the segmental sprouts in the absence of perfusion, it was recently shown that blood flow is essential for inducing sprouting events in aortic arch involving activation of the shear stress sensitive gene *kfl2*, activating VEGF signalling (Nicoli *et al.*, 2010). In addition, blood flow was shown to be important for the formation of an arterial collateral circulation, resulting in rerouting of blood flow around an aortic stenosis in gridlock mutant zebrafish (Gray *et al.*, 2007). Besides blood flow, this response depended on circulating myeloid cells, but appeared independent of hypoxia or tissue ischemia.

While in the adult, the vasculature is in general in a quiescent stage, postnatal vascular development occurs in response to a variety of physiological and pathological conditions including tissue ischemia, tissue repair, tumor growth and reproductive functions (Carmeliet, 2003). A special type of vascular adaptation occurs after arterial occlusion (Schaper, 2009). In this case the occlusion provokes a redistribution of blood flow resulting in the formation of a collateral arterial network that bypasses the occlusion and delivers blood flow to the compromised regions. This "biologi-

cal bypass" critically determines the clinical outcome in several ischemic cardiovascular diseases including stroke, heart infarct and peripheral ischemia (Schaper, 2009). It is established that arteriogenesis, the outward remodeling of preexisting arterioles (mainly in the proximal part of the network), and angiogenesis (mainly in the distal part of the network), contribute to restoring flow and oxygenation after an ischemic insult. Increased blood flow appears to be an essential trigger for the arteriogenesis response (Eitenmuller *et al.*, 2006), while tissue ischemia associates with the magnitude of the angiogenic response. Interestingly, inhibition of the biogenesis of miRNAs results in a severely disturbed vascular adaptive response after arterial occlusion (Suarez *et al.*, 2008). Given the regulatory roles of miRNAs in cell fate decisions, and cell cycle control, as well as the observation that flow can regulate miRNA expression, miRNAs are emerging as potentially important targets for therapeutic angiogenesis and arteriogenesis approaches.

### MiRNA structure and function

MiRNAs are single-stranded, non coding, RNA molecules of 18-25 nucleotides in length. The first animal miRNA was described in 1993 by Lee and colleagues in the Victor Ambros lab as a regulator of developmental timing in *Caenorhabditis elegans* (Lee *et al.*, 1993). It was not until 2001 that miRNAs were confirmed to be widespread in various plants and animals, including *Caenorhabditis elegans*, *Danio rerio*, *Homo sapiens*, and the plant *Arabidopsis thaliana* (Lagos-Quintana *et al.*, 2001; Lau *et al.*, 2001; Lee and Ambros, 2001). While the mechanisms used by miRNAs to regulate gene expression remain under debate, it is at present generally accepted that the main function of miRNAs is in gene silencing. For that purpose, a miRNA is complementary to a part of one or more messenger RNAs (mRNAs). In animals, miRNAs are usually complementary to a site in the 3' UTR.

Experimental evidence showing the distinct roles of miRNAs in physiological and pathophysiological processes is just emerging. It has become clear that miRNAs play diverse roles in fundamental biological processes, such as cell proliferation, differentiation, growth, apoptosis, stress response, and tumorigenesis. In particular, miRNAs have been implicated in various aspects of animal development, such as neuronal, muscle and cardiovascular development (Kloosterman and Plasterk, 2006; Stefani and Slack, 2008).

In the present review, current progress regarding the understanding of miRNA role in blood vessel development is summarized. It appears that miRNAs and the signaling pathways involved in vascular development coordinate perfectly the formation of the complex structured arterial-venous vascular network, and increasing experimental evidence indicates that miRNAs play important roles in the regulation of gene expression in blood vessel development and angiogenesis related diseases.

### MiRNA discovery, biogenesis and fundamental function

In 1993, Lee and Feinbaum discovered that *C. elegans* gene *lin-4* did not code a protein but instead produced a pair of short RNA transcripts (Lee *et al.*, 1993). The two small *lin-4* transcripts of approximately 22 and 61 nucleotides were found to contain sequences complementary to a repeated sequence element in the 3' UTR of *lin-14* mRNA, suggesting that *lin-4* regulates *lin-14* translation via an antisense RNA-RNA interaction (Lee *et al.*, 1993;

Wightman *et al.*, 1993). But the term microRNA was only introduced in 2001 when the abundance of these tiny regulatory RNAs was discovered and reported in a set of three articles (Lagos-Quintana *et al.*, 2001; Lau *et al.*, 2001; Lee and Ambros, 2001). At present, miRNAs have been confirmed in various animals, including *C. elegans*, zebrafish, chicken, mouse and human (Cheng *et al.*, 2007; van Rooij *et al.*, 2006).

MiRNAs can be encoded by independent genes which contain their own miRNA gene promoter and regulatory units, but also can be processed from a variety of different RNA species, including introns, exons, 3' UTRs of mRNAs, long non-coding RNAs, snoRNAs and transposons (Bartel, 2004; He *et al.*, 2008; Saraiya and Wang, 2008). As much as around 40% of miRNA genes may lie in the introns of protein coding genes or even in exons (Rodriguez *et al.*, 2004). These genes are usually in a sense orientation and as a consequence could be regulated together with their host genes (Baskerville and Bartel, 2005; Cai *et al.*, 2004; Kim and Kim, 2007; Rodriguez *et al.*, 2004; Weber, 2005).

MiRNA genes are usually transcribed by RNA polymerase II, but a small amount of miRNA can also be transcribed by RNA polymerase III, such as those from transfer RNA (tRNA) (Faller and Guo, 2008; Lee *et al.*, 2004). The transcription products range from hundreds to thousands nucleotides in length which are much longer than the mature miRNAs. They are usually capped with a modified nucleotide at the 5' end and polyadenylated (Cai *et al.*, 2004; Lee *et al.*, 2004). Each primary transcript called a pri-miRNA is processed into a short stem-loop structure, which is named pre-miRNA. Afterwards, the pre-miRNA is exported from the nucleus to cytoplasm in a process involving the nucleocytoplasmic shuttle Exportin-5. Finally the hairpin is cleaved by the RNase III enzyme Dicer into a RNA duplex. Then one of the double strands is incorporated into the RNA-induced silencing complex (RISC), where by base pairing to the 3' UTR of its target mRNA, results in gene silencing.

The mechanisms used by miRNAs to regulate gene expression are still subject of debate. Indeed, increasing evidence indicates that miRNAs repress protein expression at several different levels: (a) Deadenylation followed by mRNA degradation; (b) compete for eIF6/60S ribosomal subunits; (c) compete for the cap binding; (d) block mRNA circularization by deadenylation; (e) premature termination of translation (ribosome drop-off); (f) inhibition of translation elongation; and (g) co-translational protein degradation (Carthew and Sontheimer, 2009; Chekulaeva and Filipowicz, 2009; Eulalio *et al.*, 2008). MicroRNAs might also silence their targets by sequestering mRNAs in discrete cytoplasmic foci known as mRNA processing bodies or P bodies, which exclude the translation machinery (Eulalio *et al.*, 2008). In addition, animal miRNAs can induce significant degradation of mRNA targets despite imperfect mRNA-miRNA base pairing (Behm-Ansmant *et al.*, 2006; Giraldez *et al.*, 2006).

It is estimated that about one-third of the genes are regulated by miRNAs. The complexity of miRNA-dependent gene expression is further extended by the fact that more than one miRNA can cooperatively bind to the same 3' UTR and that each miRNA can regulate hundreds of targets (Doench *et al.*, 2003). Like transcription factors, miRNAs regulate diverse cellular pathways and are widely believed to regulate most biological processes in plants and animals, ranging from housekeeping functions to responses to environmental stress. Covering this vast body of work is beyond

the scope of this article; the cited reviews provide valuable insight (Bartel and Chen, 2004; Stefani and Slack, 2008; Yekta *et al.*, 2008). Both basic and clinical studies suggest that miRNAs are important regulators of cell differentiation, growth, proliferation and apoptosis (Ambros, 2004; Hwang and Mendell, 2006; Jovanovic and Hengartner, 2006). Therefore miRNAs could be pivotal regulators in normal development and physiology, as well as in disease development.

### MiRNA expression signature in blood vessels

Tissue-specific expression manner is one important characteristic of miRNA expression. As a matter of fact, one miRNA may be highly expressed in one tissue but have no or low expression in other tissues (Lagos-Quintana *et al.*, 2002). At present, the systematic profile of miRNAs expressed in endothelial cells and smooth muscle cells, of different anatomical locations, or angiogenic conditions, remains to be performed.

Recently, the expression of 360 human miRNAs was profiled using real-time PCR in HUVECs. But comparative analysis was performed for only 23 miRNAs (Bonauer *et al.*, 2009). Polisino *et al.* performed a large-scale analysis of miRNA expression in HUVECs, and identified 27 highly expressed miRNAs (Poliseno *et al.*, 2006). Tamia A. Harris *et al.* measured the expression of miRNA in endothelial cells using microarray. Total RNA from HUVECs was size fractionated, labeled with a fluorescent dye, and hybridized on a microarray chip. Of the 500 miRNA probes on the microarray chip, the 26 miRNA with the highest level of expression in HUVECs were identified. The most frequently expressed vascular miRNA is miR-126 (Harris *et al.*, 2008).

To study the biological functions of miRNA in vascular disease, Ruirui Ji *et al.* determined the miRNA expression in rat carotid arteries through miRNA microarray analysis (Ji *et al.*, 2007). Overall, 140 miRNAs of the 180 (more than 70%) arrayed were found with 49 being highly expressed. Currently, around 200 miRNAs were detected in human endothelial cells by different methods.

### Effects of interrupting miRNA biogenesis on vascular development

Constitutive expression of Dicer and Drosha protein was noted in cytoplasm and nucleus of endothelial cells respectively by using immuno-cytochemistry and Western blot (Kuehbachner *et al.*, 2007; Suarez *et al.*, 2007). The importance of miRNAs in endothelial cell function and blood vessel development was discovered by disrupting the function of Dicer and Drosha, two RNase III enzymes required for miRNA biogenesis.

The depletion of Dicer using siRNA in endothelial cells altered several key regulators of endothelial cell biology and angiogenesis including growth factor receptors, chemokines, cytokines and several other angiogenesis related genes, such as Tie-1, Tie-2, Id3, eNOS, IL-8, ENG (Endoglin), AKT1 and VEGFR2. Genetic silencing of both Dicer and Drosha significantly reduced capillary sprouting and proliferation of endothelial cells. Knockdown of Dicer resulted in significant impairment of the cord forming activity under base conditions and after stimulation with serum or VEGF in HUVECs and EA.hy.926. In the *in vivo* matrigel plug assay, vessel like structures were significantly reduced in Matrigel plugs with Dicer knockdown HUVECs. In contrast, Drosha siRNA transfection

did not significantly affect sprouting angiogenesis of Matrigel plugs *in vivo* (Kuehbachner *et al.*, 2007; Shilo *et al.*, 2008; Suarez *et al.*, 2007). In this case, the less dramatic effects of knockdown of Drosha, might be explained by the mirtrons- short hairpin introns that provide an alternative source for animal microRNA biogenesis and use the splicing machinery to bypass Drosha cleavage in initial maturation (Chan and Slack, 2007).

The complete loss of Dicer function results in profound developmental defects in both zebrafish and mouse. In zebrafish, strong expression of maternal *dicer1* mRNA and ubiquitous expression up to 2 dpf has been detected through whole-mount *in situ* hybridizations. Target-selected inactivation of the *dicer1* gene in zebrafish resulted in the miRNA accumulation being stopped after a few days and developmental arrest around day 10 (Wienholds *et al.*, 2003). Maternal-zygotic *dicer* mutants that lack both maternal and zygotic Dicer undergo axis formation and differentiate multiple cell types but display abnormal morphogenesis during gastrulation, brain formation, somitogenesis and heart development. Endothelial and hematopoietic precursor cells were present as judged from the expression of the markers *fli-1* and *scl*, but endocardial *fli-1* expression was reduced and blood circulation disrupted in MZ-dicer mutants (Giraldez *et al.*, 2005).

It was found in two independent studies that Dicer is essential for normal mouse development. Bernstein *et al.*, reported that loss of Dicer1 leads to lethality in early development, with Dicer1-null embryos depleted of stem cells. At E7.5, a subset of Dicer mutant mice embryos appeared small and morphologically abnormal, although distinctions between the embryonic and extra-embryonic regions were recognizable. It was suggested that Dicer mutant mice was growth arrested before the body plan is configured during gastrulation (Bernstein *et al.*, 2003). In another study, Dicer-ex1/2 homozygous embryos displayed a retarded phenotype and died between days 12.5 and 14.5 of gestation. Interestingly, it was found that blood vessel formation and maintenance in *dicer-ex1/2* embryos and yolk sacs were severely compromised, suggesting a possible role for Dicer in angiogenesis. This finding was confirmed by the altered expression of *Vegf*, *Flt1*, *Kdr*, and *Tie1* in the mutant embryos. Taken together, it was indicated that Dicer exerts its function on mouse embryonic angiogenesis probably through its role in the processing of miRNA that regulate the expression levels of some critical angiogenesis regulators in the cell (Yang *et al.*, 2005).

To overcome the early lethality, a conditional Dicer mutant mouse was created by Harfe *et al.* using the Dicer conditional allele and transgenic mouse lines that express Cre in the limb mesoderm, they proved an initial characterization of the role that Dicer plays in vertebrate limb development (Harfe *et al.*, 2005). Cardiac-specific deletion of Dicer leads to rapidly progressive dilated cardiomyopathy, heart failure, and postnatal lethality. It was also indicated in this report that expression level of several important proteins in heart was altered by loss of Dicer (Chen *et al.*, 2008). To address the importance of endothelial-specific miRNAs in postnatal angiogenesis, Suárez Y *et al.* generated two mouse models that were homozygous for the conditional floxed Dicer allele and expressed Cre-recombinase under the regulation of Tie2 promoter or Tamoxifen (TMX)-inducible expressed Cre-recombinase under the regulation of vascular endothelial cadherin promoter to achieve specific inactivation of Dicer in endothelial cells. Through these models, it was shown that reduction of endothelial miRNAs by cell-specific inactivation of Dicer reduced postnatal angiogenic

responses to a variety of stimuli, including exogenous VEGF, tumors, limb ischemia, and wound healing (Suarez *et al.*, 2008). Deletion of Dicer in vascular smooth muscle (VSM) results in late embryonic lethality at embryonic day 16 to 17, and associates with extensive internal hemorrhage. The loss of VSM Dicer results in dilated, thin-walled blood vessels caused by a reduction in cellular proliferation. In addition, blood vessels from VSM-deleted Dicer mice exhibited impaired contractility because of a loss of contractile proteins (Albinsson *et al.*, 2010).

### Individual miRNAs in endothelial cell biology

Various miRNAs are thought to be expressed in a tissue-specific manner and, thus, may play tissue-specific roles. Tissue specificity is a peculiar feature of miRNAs and may have relevance also during angiogenesis, where highly coordinated multi-step processes are required. Currently a number of miRNAs were identified to be expressed in endothelial cells, and involved in blood vessel development (Table 1).

Through *in situ* hybridization with LNA (locked-nucleic acid) probes, miRNA-126 was detected in blood vessel and heart of zebrafish (Wienholds *et al.*, 2005). After that, several *in vitro* and *in vivo* experiments showed that miR-126 was also expressed in endothelial cells of mammals. It was found that in HUVECs the most frequently expressed miRNA was miR-126. Studying miR-126 expression in primary human endothelial cells and other cell lines, showed that endothelial cells from veins, arteries, skin, and brain all express miR-126. However, other cell lines such as vascular smooth muscle cells (VSMC) or leukocyte cell lines do not (Fish *et al.*, 2008; Harris *et al.*, 2008; Wang *et al.*, 2008b). Up to date, miR-126 was shown to play several roles in endothelial cell biology and blood vessel development. Transfection of endothelial cells with an oligonucleotide that decreases miR-126 permits an

increase in TNF- $\alpha$ -stimulated VCAM-1 expression. Conversely, overexpression of the precursor of miR-126 increases miR-126 levels and decreases VCAM-1 expression. In addition, decreasing endogenous miR-126 levels increases leukocyte adherence to endothelial cells (Harris *et al.*, 2008; van Solingen *et al.*, 2009). Knockdown of miR-126 in zebrafish resulted in loss of vascular integrity and hemorrhage during embryonic development. MiR-126 functioned in part by directly repressing negative regulators of the VEGF pathway, including the sprouty-related protein SPRED1 and phosphoinositol-3 kinase regulatory subunit 2 (PIR3R2/p85- $\beta$ ) (Fish *et al.*, 2008). In mice, it was found that miR-126 originates from the Egfl7 pre-mRNA. Targeted deletion of miR-126 in mice causes leaky vessels, hemorrhaging and partial embryonic lethality, due to a loss of vascular integrity and defects in endothelial cell proliferation, migration and angiogenesis. MiR-126 enhances the proangiogenic actions of VEGF and FGF and promotes blood vessel formation by repressing the expression of Spred-1 (Wang *et al.*, 2008b). In addition, miR-126 deletion inhibited VEGF-dependent Akt signaling by depression of the p85 $\beta$  subunit of PI3 kinase (Kuhnert *et al.*, 2008). Moreover, according to the latest report, by using two-photon imaging of living zebrafish embryos, it was observed that flow is essential for angiogenesis during AA development requiring miR-126. It was shown that angiogenic sprouting of Aortic arch vessels requires a flow-induced genetic pathway in which the mechano-sensitive transcription factor klf2a induces expression of an endothelial-specific microRNA, miR-126, to activate VEGF signaling (Nicoli *et al.*, 2010).

The miR-17-92 cluster is highly conserved in vertebrate evolution, and encodes miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1 and miR-92a-1 within an 800 bp intronic region on chromosome 13 (in *Homo sapiens*) (Olive *et al.*, 2010). Interestingly, not only the sequence of each miRNA component is highly conserved across species; the organization of these miRNAs within the miR-17-92 family also exhibits a high level of conservation. The functional significance of this conservation is still unclear. It possibly reflects the evolutionary path through which gene duplications followed by subsequent loss of individual miRNA components shaped the formation of this polycistronic miRNA family (Olive *et al.*, 2010). MiRNAs encoded by the miR-17-92 cluster and its paralogs are known to act as oncogenes. Expression of these miRNAs promotes cell proliferation, suppresses apoptosis of cancer cells, and induces tumor angiogenesis (Mendell, 2008). These findings on miR-17-92 indicate that miRNAs are integrated components of the molecular pathways that regulate tumor development and tumor maintenance (Olive *et al.*, 2010). A potent tumor angiogenesis promoting activity has been attributed to the miR-17-92 cluster, which is significantly unregulated in Myc-induced tumors. VEGF levels were unaffected by Myc, but enhanced neovascularization correlated with down-regulation of anti-angiogenic thrombospondin-1 (Tsp1) and related proteins, such as CTGF. Both Tsp1 and CTGF are predicted targets for repression by the miR-17-92 miRNA cluster. In particular, miR-18 preferentially suppresses CTGF expression, whereas miR-19 targets the potent angiogenesis-inhibitor Tsp1 (Dews *et al.*, 2006). In addition, over expression of c-myc leads to down regulation of HIF-1 $\alpha$  and induction of miR-17-92, the latter of which was reported to be a transcriptional activation activity, suggesting that the induction of miR-17-92 may play a role at least in part in c-myc-mediated repression of HIF-1 $\alpha$  (Taguchi *et al.*, 2008). Moreover, Angelika *et al.*, reported miR-17-92 cluster is

TABLE 1

#### MicroRNAs IN ENDOTHELIAL CELL BIOLOGY

MicroRNA	Functions	Relevant targets	References
miR-126	Maintain vascular integrity, development; activate Vegf signaling	VCAM-1, SPRED1, PIR3R2/p85- $\beta$ ,	Fish <i>et al.</i> , 2008, Harris <i>et al.</i> , 2008, Nicoli <i>et al.</i> , 2010, van Solingen <i>et al.</i> , 2009, Wang <i>et al.</i> , 2008b
miR-17-92 cluster	Induce tumor angiogenesis, promote cell proliferation, suppress apoptosis, inhibit sprouting	Tsp1(miR-19), CTGF(miR-19), Jak1 (MiR-17/20)	Dews <i>et al.</i> , 2006, Mendell, 2008, Olive <i>et al.</i> , 2010 Doebele <i>et al.</i> , 2010
miR-92a	negative regulator of growth of new blood vessels	the integrin subunit alpha5	Bonauer <i>et al.</i> , 2009
miR-221/222	inhibit the survival, migration, and capillary tube formation in EC	c-Kit, SCF, eNOS(Indirect )	Minami <i>et al.</i> , 2009, Murohara <i>et al.</i> , 1998, Poliseno <i>et al.</i> , 2006, Suarez <i>et al.</i> , 2007
miR-210	stimulate the formation of capillary-like structures and migration of ECs	ephrin-A3, neuronal pentraxin 1, HIF-1 alpha	Fasanaro <i>et al.</i> , 2008, Ivan <i>et al.</i> , 2008, Pulkkinen <i>et al.</i> , 2008
miR-296	promoting angiogenesis in tumors	HGS	Hua <i>et al.</i> , 2006, Wurdinger <i>et al.</i> , 2008
miR-130a	Promote angiogenesis in vascular ECs	GAX and HOXA5	Chen and Gorski, 2008
miR-378	enhance cell survival, tumor growth, and angiogenesis	Sufu and Fus-1, VEGF	Hua <i>et al.</i> , 2006, Lee <i>et al.</i> , 2007
miR-320	impair angiogenesis	IGF-1	Wang <i>et al.</i> , 2009

highly expressed in human endothelial cells. Recent work reveals essential functions for these miRNAs not only in tumor angiogenesis but also during normal development of blood vessels. MiR-92a, a component of this cluster was recently identified as a negative regulator of growth of new blood vessels. Forced overexpression of miR-92a in endothelial cells blocked angiogenesis *in vitro* and *in vivo* with targeting mRNAs corresponding to several proangiogenic proteins, including the integrin subunit alpha5. In mouse models of limb ischemia and myocardial infarction, systemic administration of an antagomir designed to inhibit miR-92a led to increased blood vessel growth and functional recovery of damaged tissue (Bonauer *et al.*, 2009). It was demonstrated that overexpression of miR-17, -18a, -19a and -20a significantly inhibited 3D spheroid sprouting *in vitro*, whereas inhibition of miR-17, -18a and -20a augmented endothelial cell (EC) sprout formation. Inhibition of miR-17 and miR-20a *in vivo* using antagomirs significantly increased the number of perfused vessels in matrigel plugs, whereas antagomirs that specifically target miR-18a and miR-19a were less effective. MiR-17/20 targets several pro-angiogenic genes, such as Janus kinase 1 (Jak1) (Doebele *et al.*, 2010).

MiR-221 and miR-222 genes are located in close proximity on chromosome 13.3. They were found to be expressed in but not confined to endothelial cells in several studies. It has been demonstrated that miR-221 and miR-222 affect c-Kit expression and the angiogenic properties of its ligand, stem cell factor (SCF). As a consequence, they inhibit the survival, migration, and capillary tube formation in HUVECs (Poliseno *et al.*, 2006). Another study indicated that miR-221/222 regulates endothelial nitric oxide synthase protein (eNOS) levels after Dicer silencing, however miR-221/222 were not found to target the 3' UTR of eNOS. So, the regulation of eNOS by miR-221/222 is likely to be an indirect effect (Suarez *et al.*, 2007). It is established that eNOS, also known as nitric oxide synthase 3 (NOS3), generates NO in blood vessels and is involved in regulating vascular formation and function in several aspects (Murohara *et al.*, 1998). Interestingly, a study from Minami *et al.*, suggests the physiological role of miR-221/222 is closely linked to the proliferation of endothelial cells. It was shown that levels of miR-221/222 were significantly higher in the CAD group than in the non-CAD group and levels of miR-221/222 were weakly negatively correlated with Endothelial progenitor cells (EPCs) number in the CAD group (Minami *et al.*, 2009). Collectively, these reports suggest miR-221/222 play an antiangiogenic role *in vitro*, although the *in vivo* functions are undetermined.

MiR-210 overexpression in normoxic endothelial cells stimulated the formation of capillary-like structures on Matrigel and vascular endothelial growth factor-driven cell migration. Conversely, miR-210 blockade via anti-miRNA transfection inhibited the formation of capillary-like structures stimulated by hypoxia and decreased cell migration in response to vascular endothelial growth factor. MiR-210 overexpression did not affect endothelial cell growth in both normoxia and hypoxia. HIF-1 $\alpha$  induced the expression of miR-210 in endothelial cells (Ivan *et al.*, 2008). However, anti-miR-210 transfection inhibited cell growth and induced apoptosis, in both normoxia and hypoxia. Ephrin-A3 was determined to be one relevant target of miR-210 in hypoxia since miR-210 was necessary and sufficient to down-regulate its expression. Moreover, luciferase reporter assays showed that Ephrin-A3 was a direct target of miR-210. In summary, miR-210 up-regulation is a crucial element of the endothelial cell response to hypoxia, affecting cell survival,

migration, and differentiation (Fasanaro *et al.*, 2008). An active form of the HIF-1 $\alpha$  induced the expression of miR-210, showing the involvement of the HIF-1 signaling pathway in miR-210 gene transcription. Furthermore, miR-210 was shown to bind to the predicted target sites of ephrin-A3 or neuronal pentraxin 1, causing repression in luciferase reporter activity (Pulkkinen *et al.*, 2008).

MiR-130a is located on chromosome 11 and the expression of this miRNA was found to be rapidly up regulated under high serum culture conditions in endothelial cells *in vitro*. It has been shown that miR-130a regulates GAX expression and plays a big role in modulating GAX activity in endothelial cells. The homeobox gene GAX inhibits angiogenesis in vascular endothelial cells. A genome-wide search for other possible miR-130a binding sites revealed a miR-130a targeting site in the 3' UTR of the anti-angiogenic homeobox gene HOXA5, whose expression and anti-angiogenic activity are also inhibited by miR-130a. In conclusion, miR-130a is a regulator of the angiogenic phenotype of vascular endothelial cells largely through its ability to modulate the expression of GAX and HOXA5 (Chen and Gorski, 2008). Thomas Würdinger *et al.*, reported a role for miR-296 in promoting angiogenesis in tumors. Growth factor-induced miR-296 contributes significantly to angiogenesis by directly targeting the hepatocyte growth factor-regulated tyrosine kinase substrate (HGS) mRNA, leading to decreased levels of HGS and thereby reducing HGS-mediated degradation of the growth factor receptors VEGFR2 and PDGFR $\beta$  (Hua *et al.*, 2006; Würdinger *et al.*, 2008).

Previous studies have shown that miR-378 is expressed in a number of cancer cell lines (Jiang *et al.*, 2005) and is involved in the expression of VEGF (Hua *et al.*, 2006). It was suggested that miR-378 transfection enhanced cell survival, tumor growth, and angiogenesis through repression of the expression of two tumor suppressors, Sufu and Fus-1. The tumors formed by miR-378-transfected cells contained larger blood vessels than those formed by the vector-transfected cells. Although the number of blood vessels per unit field did not seem to increase, it is probable that the total number of blood vessels in the larger tumors had to be higher to allow expansion of the tumor. The formation of the large vessels may be important in facilitating such expansion. These results are in agreement with the previous studies that show that miR-378 binds to VEGF 3' UTR competing with miR-125a for the same seed region and promotes VEGF expression (Lee *et al.*, 2007).

Up-regulation of miRNA-320 was observed in MMVEC from Goto-Kakizaki (GK) rats using real-time reverse transcription-polymerase chain reaction (RT-PCR). Transfection of a miR-320 inhibitor into MMVEC from GK rats confirmed that miR-320 impaired angiogenesis. The proliferation and migration of diabetic MMVEC improved after transfection with the miR-320 inhibitor. In addition, the miR-320 inhibitor significantly increased the expression of IGF-1 protein, but had no effect on the expression of IGF-1R. The results indicate that up-regulation of miR-320 in MMVEC from GK rats may be responsible for the inconsistency between the expression of IGF-1 protein and mRNA and therefore related to impaired angiogenesis in diabetes (Wang *et al.*, 2009).

### Individual miRNAs in smooth muscle cell biology

The vessel wall consists of two major cell types, endothelial and smooth muscle cells that closely interact with each other. Vascular smooth muscle cells form layers within the vessel wall,

and by contracting or relaxing, control the blood pressure and organ perfusion. VSMCs are able to switch between a differentiated and a dedifferentiated state (Owens *et al.*, 2004). Under normal physiologic conditions VSMCs are differentiated and contractile. In the presence of vascular injury and growth factors VSMCs can change their phenotype to a dedifferentiated state, and start to proliferate. Although the modulation of VSMC phenotype is believed to be essential for the repair of vascular injury, deregulation of this process can lead to pathologic changes in the vascular wall. Such remodeling of VSMCs is often observed in atherosclerotic lesions, after chronic exposure to high blood pressure, or other hemodynamic stress conditions. Therefore VSMC differentiation is a key cellular event in the pathogenesis of a variety of vascular diseases including arteriogenesis, atherosclerosis, restenosis and hypertension (Doran *et al.*, 2008; Owens *et al.*, 2004; Rzucidlo *et al.*, 2007).

Recent reports demonstrated a crucial role for miRNAs in VSMC differentiation. miR-143 and miR-145 are abundantly expressed in VSMCs of arteries (Cheng *et al.*, 2009; Ji *et al.*, 2007), and the heart (Boettger *et al.*, 2009; Cordes *et al.*, 2009). During postnatal development the expression in heart is down regulated. To highlight the importance of miR-143 and miR-145 in VSMCs, Boettger and colleagues generated a mouse mutant for the microRNA cluster miR-143/145. Analysis of homozygous miR-143/145 mutant mice revealed a decrease in contractile VSMCs, an increase in proliferative precursors and formation of neointimal lesions (Boettger *et al.*, 2009). Contractile behavior in other organs including, the gastrointestinal motility was not affected. Closer characterization of the physiology of the mutant mice exhibits defects in the agonist-induced SMC contractility in arteries. Changes of the blood pressure were also observed in another study (Xin *et al.*, 2009), and angiotensin I-converting enzyme (ACE), which is implied in promoting vasoconstriction and alteration of VSMC phenotype, by converting angiotensin-I into the vasoactive hormone angiotensin-II, was suggested to be a potential target. Comparison of healthy with injured murine carotid arteries showed a decreased expression of miR-143 and miR-145 in the diseased artery (Cordes *et al.*, 2009). Moreover down-regulation of miR-145 in dedifferentiated cultured SMCs was found. Overexpression of miR-145 led to inhibition of PDGF-induced VSMC proliferation (Cheng *et al.*, 2009). The vascular remodeling in response to injury was also tested in the miR-143/145-null mice from Xing. Ligation of the carotid artery of miR-145<sup>-/-</sup> mice showed virtually no formation of neointima, whereas in miR-143<sup>-/-</sup> a reduced formation compared to wild type mice was observed (Xin *et al.*, 2009).

At the molecular level it is postulated that miR-145 promotes binding of myocardin to serum response factor (SRF) and thereby enhancing the transcription of SMC differentiation genes. On the other hand miR-145/143 blocks the expression of Ets-like gene 1 (ELK-1) and Krüppel-like factor 4 (KLF4), transcription factors necessary for stimulating the SMC proliferation (Wang *et al.*, 2008a; Yoshida *et al.*, 2008). However, additional targets (KLF-4, KLF-5) and regulatory functions may exist (Boettger *et al.*, 2009) (Nagai *et al.*, 2005). Additional targets involved in VSMC phenotype switching, regulation of actin polymerization and cell migration were identified using TargetScan and PicTar prediction algorithms (Xin *et al.*, 2009) but remain to be validated *in vivo* (Xin *et al.*, 2009). The potential targets for miR-143 and miR-145 are listed in Table 2.

Rat carotid arteries express miR-221 and miR-222 in the vascular

wall (Poliseno *et al.*, 2006). Microarray analysis revealed an up-regulation of miR-221 and miR-222 in vessel walls with neointimal lesion formation (Ji *et al.*, 2007). This up-regulation of miR-221 and miR-222 was further confirmed by qRT-PCR of rat carotid arteries after angioplasty *in vivo*. In addition, cultured proliferative VSMCs stimulated by PDGF or serum showed an increased expression of miR-221 and miR-222 (Lin *et al.*, 2009). A study by Davis and colleagues (Davis *et al.*, 2009) demonstrated an induction of miR-221 upon PDGF treatment in human primary pulmonary artery smooth muscle cells (PASMCs). The expression of miR-222 was weakly inhibited after PDGF treatment in PASMCs, suggesting that miR-221 and miR-222 are differentially regulated by PDGF in PASMC (Davis *et al.*, 2009). Knockdown of miR-221 and miR-222 with antisense oligonucleotides resulted in decreased VSMC proliferation *in vitro* and *in vivo*, suggesting that miR-221 and miR-222 control VSMC proliferation (Lin *et al.*, 2009).

MiR-221 and miR-222 share the same seed and appear to have identical targets and similar functions. Computational analysis predicted the potential targets p27 (Kip1) and p57 (Kip2) (Felicetti *et al.*, 2008; Fornari *et al.*, 2008; Galardi *et al.*, 2007; le Sage *et al.*, 2007; Visone *et al.*, 2007). Gain-of-function and loss-of-function approaches confirmed both genes as targets for miR-221 and miR-222. In proliferative VSMCs, p27 (Kip1) and p57 (Kip2) were down regulated and therefore likely involved in the miR-221 and miR-222 mediated effect on VSMC proliferation (Liu *et al.*, 2009). However, the partially inhibition of cell proliferation in p27 (Kip1) and p57 (Kip2) depleted cells indicates a possible role for other unidentified gene targets. Targets for miR-221 include c-kit and p27 (Felli *et al.*, 2005; Fornari *et al.*, 2008; Galardi *et al.*, 2007; le Sage *et al.*, 2007; Visone *et al.*, 2007). C-kit causes inhibition of SMC-specific gene transcription by reducing the expression of myocardin, whereas p27Kip1 promotes cell proliferation upon vascular injury (Chen *et al.*, 1997). It is postulated that the miR-221-p27Kip1 axis might play a role in neointima formation after vascular injury (Liu *et al.*, 2009).

MiR-21 is expressed in vascular smooth muscle cells, as well as endothelium, cardiomyocytes and fibroblasts (Cheng *et al.*, 2007; Ji *et al.*, 2007; Roy *et al.*, 2009; Suarez *et al.*, 2007). MiR-21 appears to play a crucial role in vascular adaptation in response to reactive

TABLE 2

**MicroRNAs IN SMOOTH MUSCLE CELL BIOLOGY**

MicroRNA	Functions	Relevant targets	References
miR-145	Promote contractile phenotype of VSMC	KLF4, Camk1ld , KLF5	Cheng <i>et al.</i> , 2009, Cordes <i>et al.</i> , 2009
	Modulate actin dynamics and cytoskeletal assembly	zinc finger proteins, KLF4, KLF5, Srgap1, Srgap2, Add3, Ssh2 phosphatase, MRTF-B , Argaggp12, Tpm4, Ace	Boettger <i>et al.</i> , 2009, Xin, <i>et al.</i> , 2009
miR-143	Promote contractile phenotype of VSMC	ELK1	Cordes <i>et al.</i> , 2009
	Modulate actin dynamics and cytoskeletal assembly	zinc finger proteins, KLF4, KLF5, Srgap1, Srgap2, Add3, Ssh2 phosphatase, MRTF-B, Argaggp12, Tpm4, Ace	Boettger <i>et al.</i> , 2009, Xin, <i>et al.</i> , 2009
miR-221	Promote proliferative phenotype of VSMC	p27(Kip2), c-Kit, p57 (Kip2)	Davis <i>et al.</i> , 2008, Liu <i>et al.</i> , 2009
miR-222	Promote proliferative phenotype of VSMC	p27 (Kip1) and p57 (Kip2)	Liu <i>et al.</i> , 2009
miR-21	Protective effect on VSMC necrosis	programmed cell death 4 (PDCD4), phosphatase and tensin homology deleted from chromosome 10 (PTEN)	Ji <i>et al.</i> , 2007, Lin <i>et al.</i> , 2009

oxygen species (ROS). It is well established that ROS play a central role in a variety of vascular disorders including atherosclerosis (Vandenbroucke *et al.*, 2008). In line with this, hemodynamic stress conditions, as well as balloon denudation in arteries, is associated with increased expression of miR-21 (Cordes *et al.*, 2009; Ji *et al.*, 2007). Knockdown of miR-21 in these injured arteries inhibited neointimal lesion formation (Ji *et al.*, 2007). *In vitro*, inhibition of miR-21 increased VSMC apoptosis, and decreased proliferation. In line with a regulatory role in adaptation to ROS, inhibition of miR-21 in H<sub>2</sub>O<sub>2</sub> induced VSMCs revealed increased apoptosis and cell death, whereas overexpression of miR-21 resulted in reduced apoptosis and cell survival.

Computational analysis indicated that programmed cell death 4 (PDCD4) is a potential target gene of miR-21. PDCD4 is involved in the regulation of the apoptosis of VSMCs (Cheng *et al.*, 2009; Lin *et al.*, 2009). Overexpression of miR-21 results in decreased PDCD4 expression in H<sub>2</sub>O<sub>2</sub> challenged VSMCs, and binding of miR-21 to PDCD4 was confirmed *in vitro*. Moreover, the miR-21 overexpression-mediated protective effect on VSMC apoptosis and death was blocked after forced overexpression of a PDCD4 construct lacking the miR-21 binding sites, suggesting that PDCD4 may act as a functional target gene of miR-21 (Lin *et al.*, 2009). Another potential target gene of miR-21 is phosphatase and tensin homology deleted from chromosome 10 (PTEN) (Ji *et al.*, 2007). PTEN is known to modulate cell survival/ apoptosis, hypertrophy and contractility (Oudit *et al.*, 2004).

## Concluding remarks

Understanding the miRNA expression profile in developing vessels is essential and invaluable for evaluating miRNAs biological functions. At present, expression profiles of miRNA in developing blood vessels are still incomplete. Large scale miRNA expression profiling in combination with loss- and gain-of-function screens *in vivo*, using zebrafish and target validation are feasible approaches to help understanding the full repertoire of action of miRNAs in angiogenesis and arteriogenesis. Furthermore, miRNAs play an important role in vascular adaptation to arterial occlusion, and adverse smooth muscle cell remodeling in the context of atherosclerosis, and vascular injury. *In vivo* studies in mice have shown that it is possible to therapeutically target miRNA function. Aptamers with miRNA modulators attached can be designed to bind to specific proteins that are readily internalized by endothelial cells or smooth muscle cells. It is expected that such approaches will yield therapeutic levels of miRNA drugs within the vascular target cells.

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