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MicroRNA control of Vegf signalling output during vascular development

Lan T. H. Dang[1,2,3], **Nathan D. Lawson**[4,5], and **Jason E. Fish**[1,2,3,5]

[1]Toronto General Research Institute, University Health Network

[2]Department of Laboratory Medicine and Pathobiology, University of Toronto

[3]The Heart and Stroke/Richard Lewar Centre of Excellence in Cardiovascular Research, Toronto

[4]Program in Gene Function and Expression, University of Massachusetts Medical School, Worcester, MA 01605, U.S.A

Abstract

The regulated response of endothelial cells (ECs) to signals in their environment is not only critical for the *de novo* formation of primordial vascular networks during early development (i.e. vasculogenesis), but is also required for the subsequent growth and remodelling of new blood vessels from pre-existing ones (i.e. angiogenesis). Vascular endothelial growth factors (Vegfs) and their endothelial-cell specific receptors play a crucial role in nearly all aspects of blood vessel growth. How the outputs from these pathways affect and coordinate endothelial behaviour is an area of intense research. Recently, numerous studies have highlighted roles for microRNAs in modulating Vegf signalling output in several different contexts. In this review we will provide an overview of how small RNAs regulate multiple aspects of the Vegf signalling pathway. In particular, we highlight areas where identification of microRNAs and their targets has provided new insight into the role of downstream effectors in modulating Vegf output during development. Since Vegf plays a broad role in multiple aspects of endothelial biology and has become a target for therapeutic manipulation of pathological blood vessel growth, microRNAs that affect Vegf signalling output will undoubtedly be major targets of clinical value.

Introduction

Formation of the vertebrate circulatory system requires coordination of diverse cellular behaviors during embryonic development [1]. During vasculogenesis, endothelial cell (EC) progenitors must balance proliferation, differentiation into distinct lineages (e.g. artery and vein), and migration to the appropriate anatomical location to establish a primitive vascular network *de novo* [2]. Subsequent angiogenesis entails the sprouting and growth of new blood vessels from this pre-existing network, which also requires coordination of proliferation and migration and dynamic regulation of 'tip' and 'stalk' cell identities within growing angiogenic sprouts [3]. During development these growth processes must be carefully coordinated with lumen formation and the integration of hemodynamic forces provided by the initial onset of circulatory flow [4]. Not surprisingly, perturbation of any of these steps has detrimental effects on circulatory function in the embryo. Likewise, many of these same processes are utilized in the context of pathological vascular growth [5]. Thus, a better

[5]Corresponding author: Nathan Lawson, PhD, University of Massachusetts Medical School, LRB617, 365 Plantation Street, Worcester, MA 01605, U.S.A. nathan.lawson@umassmed.edu, phone: 508-856-1177. Jason Fish, PhD, Toronto General Research Institute, University Health Network, MaRS Centre, Toronto Medical Discovery Tower, 101 College Street, 3-308, Toronto, Ontario, M5G 1L7, Canada, jason.fish@utoronto.ca, phone: 416-581-7496.

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understanding of the signalling pathways that govern blood vessel growth and homeostasis will undoubtedly have clinical benefits.

Over the last two decades we have learned a great deal about the signals required for vascular growth in both embryonic and adult settings. These studies revealed an important role for many of the central developmental signalling pathways, such as Notch, BMP, and Wnt, in multiple aspects of vascular development and function [1, 5–7]. In addition, several EC-specific pathways have been identified. Most notable in this regard are receptor tyrosine kinases for Vascular endothelial growth factor (Vegf) and angiopoietin ligands [8]. In particular, ligands of the Vegf family play an integral role in nearly every aspect of vascular development and maintenance [9]. Indeed, loss of even a single copy of *Vegfa* completely blocks vessel formation in mouse embryos leading to embryonic lethality [10, 11], while loss of this ligand in the endothelium of adult vasculature causes severe endothelial dysfunction [12], and inhibition of Vegfa signalling potently inhibits pathological angiogenesis [13]. Vegf ligands can elicit a wide range of responses in ECs, including migration, proliferation, survival, differentiation and increased permeability [9]. Given the importance of targeting this pathway in a variety of clinical settings [8], a detailed understanding of how Vegf ligands elicit a particular response *in vivo* is of great importance. Over the past several years, there has been an increasing amount of evidence implicating microRNAs in the control of Vegf signalling output at multiple levels. Indeed, microRNAs are now known to control the expression of Vegf ligands, receptors, and intracellular signalling components of the Vegf pathway, as well as proteins that cross-talk with Vegf receptors. Here, we will review the most recent findings regarding microRNA regulation of Vegf signalling output.

Regulation of angiogenesis by microRNAs

Originally identified in *C. elegans* in 1993 [14], microRNAs are a group of highly conserved, non-coding single-strand RNA molecules (~21–23 nucleotides (nts)) that function to fine-tune protein expression through various mechanisms, including the degradation of target mRNA, the inhibition of translation elongation, or the sequestration of target mRNAs away from the translation machinery [15–17]. This is accomplished by the incorporation of the microRNA into the RNA-Induced Silencing Complex (RISC) [18], which is then directed primarily to the 3′ untranslated region (UTR) of the target mRNA via the seed sequence of the microRNA. While microRNAs may affect the expression of hundreds of target genes [19], a small number of target mRNAs may adequately explain the biological effect of a particular microRNA in a given cellular context [20]. Numerous studies have also demonstrated that microRNAs can target several genes within the same pathway to control biological responses [21].

MicroRNAs have been shown to play key roles in vascular development. For example, deletion of *Dicer*, an enzyme required for microRNA biogenesis, results in early embryonic lethality [22], while mice homozygous for a hypomorphic allele of *Dicer* survive to mid-gestation but have major defects in angiogenesis [23]. Conditional deletion of *Dicer* in the endothelium results in reduced vascular growth in postnatal models of tumor angiogenesis and ischemia [24]. While these mice did not display angiogenic defects during development, Dicer was not ablated from all ECs in this study [24], and it remains possible that full deletion of Dicer may well result in developmental vascular defects. In addition to these developmental studies, knockdown of Dicer in cultured ECs has been shown to result in significant defects in their responsiveness to angiogenic factors [25].

Control of VEGF ligand and receptor expression by microRNAs

The Vegf family is composed of several ligands (Vegfa, b, c, d and e and Placental growth factor (Plgf)), each of which has a particular preference for one of three endothelial expressed receptors (Vegfr-1/Flt1, Vegfr-2/Flk1/Kdr and Vegfr-3/Flt4) [9]. Regarding vascular development, Vegfa is the most functionally significant and well-characterized member of the Vegf family. Upon binding to its receptor tyrosine kinase, Vegfr-2, Vegfa can elicit distinct responses, including proliferation, cell survival, permeability, differentiation, and migration [9]. These effects are mediated through the activation of several downstream signalling effectors [9]. Homozygous deletion of *Vegfr-2* [26], much like deletion of its ligand [10, 11], results in an absence of blood vessels and lethality in mice. Vegfr-3, which becomes almost exclusive to lymphatic ECs in late development [27], is also expressed on vascular endothelial tip cells during embryonic and post-natal angiogenesis and responds to Vegfc and Vegfd [28, 29]. *Vegfr-3* deletion also leads to embryonic lethality, which is due to a failure of vascular remodelling [30], and inhibition of Vegfr-3 activity can inhibit tumor angiogenesis [28]. In contrast to Vegfr-2 and Vegfr-3, Vegfr-1 largely functions as a “decoy” receptor to negatively regulate Vegf signalling during development. [31, 32]. The *Vegfr-1* gene encodes an alternative soluble isoform that lacks the intracellular domain and has a much higher affinity for Vegfa than Vegfr-2 [33]. Accordingly, mice bearing a null allele of *Vegfr-1* exhibit extensive vascular over-growth, while those bearing a deletion of only the tyrosine kinase domain display normal vascular development [34]. Importantly, the levels of Vegfr-2/3 versus Vegfr-1 appear to be both spatially and temporally regulated during angiogenesis. Thus, the proper balance and regulation of Vegf receptor levels plays an important role in orchestrating angiogenic sprouting [31, 32].

ECs are highly sensitive and responsive to Vegf gradients. These gradients are established through the alternative splicing of Vegfa transcripts, which results in an array of protein variants with diverse functional properties, such as differential diffusion rates, as well as variable association with the extra-cellular matrix (ECM), leading to distinct abilities to activate cell signalling pathways and to induce vascular morphogenesis [35]. In humans, Vegfa isoforms contain 121, 165, 189 or 206 amino acids; all except $Vegfa_{121}$ contain a basic stretch near the carboxyl terminus with variable affinity for heparan sulfate proteoglycans (HSPGs) and Neuropilin-1 (co-receptors for Vegfa) [35]. As a result $Vegfa_{121}$, which is unable to bind HSPGs, is freely diffusible and can influence EC proliferation, though contributing little to EC migration [36]. On the contrary, $Vegfa_{165}$ and $Vegfa_{189}$, which have strong affinities for HSPGs, are tightly associated with the ECM, forming a gradient to allow for the directional migration of ECs by promoting filopodia extensions [36]. One possible explanation for the vast array of cellular responses elicited by different Vegfa isoforms is the preferential activation of one downstream signalling effector over another. For example, unlike its soluble counterpart, matrix-bound Vegfa can induce prolonged tyrosine kinase activity of Vegfr-2 leading to increased phosphorylation of p38/Mitogen Activated Protein Kinase (MAPK), thereby up-regulating angiogenic sprouting. This is mediated by interaction between Vegfr-2 and β1-integrins [37].

Given the considerable dynamic control of Vegf ligand and receptor expression during vascular development, it is not surprising that microRNAs can control this pathway by targeting transcripts encoding these proteins (Fig. 1). Since enhanced expression of Vegfa occurs in diseases such as cancer and diabetic retinopathy and drives pathological vascular growth [38], alterations in microRNA expression may contribute to these diseases. Indeed, microRNA (miR)-93 [39] and miR-200b [40] were recently found to be down-regulated by hyperglycemic conditions. These microRNAs both target the *Vegfa* 3′ UTR, and knock-down of these microRNAs enhances Vegfa expression *in vivo* [39, 40]. MiR-15a also directly represses Vegf and Fibroblast growth factor 2 (Fgf2) in ECs to control angiogenesis [41], and

miR-20b represses Vegf expression in tumor cells to affect their cell survival in hypoxic conditions [42]. MicroRNAs are also induced down-stream of Vegf signalling itself [24]. For example, Vegf induces the expression of miR-16 and miR-424, which share a common seed sequence [43]. These microRNAs act in a negative feedback loop to control angiogenesis through combined targeting of Vegfa, Vegfr-2 and the FGF receptor, FGFR1 [43]. Other microRNAs, such as miR-296, indirectly regulate Vegfr-2 expression [44]. Vegfr-2 turnover is regulated by hepatocyte growth factor-regulated tyrosine kinase substrate (HGS), which mediates sorting of ligand/receptor complexes to lysosomes for degradation [45]. MiR-296 expression is enhanced in tumor vasculature and is induced by Vegf signalling, and through its repression of HGS expression in ECs, miR-296 facilitates enhanced expression of Vegfr-2 on the cell surface and potentiates Vegf signalling [44].

Control of intracellular signalling downstream of Vegf by microRNAs

In addition to regulation at the level of ligand and receptor, several studies reveal that intracellular signalling effectors utilized downstream of Vegf are targeted by microRNAs (Fig. 1). In particular, this work has underscored the importance of both the phosphoinositide-3-kinase (PI3K) and MAPK/ERK signalling pathways in modulating Vegf signalling outputs during vascular development.

PI3K activity is an essential downstream effector of Vegf signalling during vascular development [46]. Class I PI3Ks are heterodimers composed of a catalytic subunit, which can be encoded by 4 different genes (p110α, β, δ, and γ: referred to hereafter as *pik3ca*, *b*, *d*, and *g*) and a regulatory subunit, for which 5 different genes have been identified (*pik3r1-5*) [47]. Notably, Pik3r1, 2, and 3 possess SH2 domains that mediate direct interactions with upstream receptor tyrosine kinases. *Pik3ca*, *b*, and *g*, as well as most regulatory subunits are expressed in ECs [48, 49] and appear to have distinct roles in modulating Vegf output. For example, *pik3ca* is essential for Vegfa-mediated endothelial migration during developmental angiogenesis [46], while *pik3cg* plays an important role in Vegfa-stimulated vascular permeability in adult vasculature [49]. PI3Ks phosphorylate membrane bound phosphoinositol-4,5-bisphosphate to generate phosphoinositol-1,4,5-trisphosphate, which serves as a docking and activation site for downstream signalling molecules, such as Akt1, a serine/threonine kinase. Given the importance of PI3K signalling in cell survival and growth, constitutive activation of this pathway is typically associated with cellular transformation in non-endothelial cell types. In many of these cases, activation is due to the loss of PI3K regulatory subunits, which are thought to exist in a 1:1 ratio with the catalytic subunits and usually inhibit kinase activity in the absence of upstream activation [50]. Several recent findings suggest that these regulatory subunits are important targets of microRNAs to regulate Vegf signalling output in ECs. In particular, miR-126 and miR-221 have been found to target Pik3r2 [51, 52] and Pik3r1 [53], respectively.

MiR-126 control of Vegfr-2 signalling

MiR-126 was the first EC-specific microRNA identified and is located within intron 7 of the *Egfl7* gene [51, 54]. Deletion of *miR-126* in mice (without altering the expression of the host gene), results in severe defects in blood vessel development, including delayed angiogenic sprouting, haemorrhage and partial early embryonic lethality [52, 54]. These defects are similar to phenotypes associated with loss of Vegf signalling, in both the mouse [55] as well as the zebrafish [51], suggesting that miR-126 acts to modulate Vegf signalling output. Indeed, knockdown of miR-126 in ECs results in decreased phosphorylation of Akt, as well as ERK1/2 in response to Vegf treatment [51, 52, 54]. Furthermore, miR-126 targets transcripts encoding Pik3r2 [51, 52] and Spred1 [51, 52, 54, 56], an inhibitor of MAPK signalling, providing a direct link between this microRNA and the Vegf signalling pathway.

Interestingly, results from zebrafish suggest that miR-126-mediated repression of Spred1 and Pik3r2 may be context dependent. Unlike mammalian genomes, which encode one copy of *miR-126* that does not appear to be regulated by blood flow [57], the zebrafish genome encodes two copies of *miR-126*, one of which is induced by blood flow in the aortic arches where it is required for Vegfa-dependent angiogenic remodelling [58]. In this case, Spred1 over-expression mimics the loss of miR-126, while reduction of Spred1 levels in *miR-126* deficient embryos rescues aortic arch remodelling. Thus, Spred1, but not Pik3r2, is required for flow-mediated angiogenic remodelling of the aortic arches in the zebrafish. Further investigation using compound mouse knockouts deficient for *miR-126* and its targets will be insightful to determine the context-dependent requirements for Spred1 and Pik3r2 in mediating ERK and PI3K signalling downstream of Vegfa during embryonic and postnatal angiogenesis.

MiR-221 control of Vegfr-3 signalling

Similar to miR-126, recent data suggest that miR-221 also acts to modulate Vegf receptor signalling through regulation of a PI3K regulatory subunit. Although previously characterized in other systems [59], miR-221 was identified through deep sequencing efforts as an endothelial-enriched microRNA in zebrafish embryos [53]. Knockdown of miR-221 does not affect initial blood vessel development but causes angiogenesis and lymphatic defects [53] that are remarkably similar to loss of Vegfr-3, a receptor for the Vegfc ligand [29]. Furthermore, epistasis studies suggest that miR-221 acts within the Vegfc/Vegfr-3 signalling pathway and in parallel to Vegfa/Vegfr-2. Mosaic analysis revealed that miR-221 deficient cells do not contribute well to the tip cell position in developing angiogenic sprouts. By contrast, overexpression of miR-221 induces tip cell behaviors, such as enhanced proliferation and migration. The effect of miR-221 on cell proliferation appears to be largely due to repression of cyclin-dependent kinase inhibitor 1B (Cdkn1b), and miR-221 also targets Pik3r1, which was previously shown to interact with Vegfr-3 [60]. Both reduction and over-expression of Pik3r1 result in inhibition of angiogenesis [53], suggesting that precisely tuned levels of this regulatory subunit are required for optimal PI3K output.

These studies suggest that a common mechanism by which microRNAs regulate intracellular growth factor signalling is by precisely tuning the level of PI3K regulatory subunits. Studies showing that Pik3r1 and Pik3r2 are targeted by several different microRNAs during growth factor signalling in non-endothelial cell types further suggest that this may be a general theme [61–63]. Interestingly, in these contexts, microRNA-mediated repression of the regulatory subunit is associated with reduced proliferation and apoptosis. By contrast, both miR-126 and miR-221 promote endothelial growth and angiogenesis suggesting that they fine-tune, rather than simply inhibit, PI3K activity to elicit context-dependent Vegf signalling outputs. Moreover, these microRNAs appear to control output through two distinct receptors: miR-126 regulates Vegfr-2, while microRNA-221 regulates Vegfr-3 signalling. These studies would further imply that these receptors each utilize different PI3K signalling complexes. In general, these microRNAs could provide a mechanism by which a cell may respond differentially to, or appropriately integrate, signalling in response to Vegfa and/or Vegfc.

MiR-132 control of Ras activity

Ras is a key regulator that acts downstream of the Vegf receptor to mediate activation of the MAPK/ERK pathway, and recent findings have suggested that Ras activity in ECs is controlled by miR-132 during tumor angiogenesis. Activity of Ras is controlled by opposing GTPase activating proteins (GAPs) and GTP exchange factors (GEFs) that dictate whether Ras is an a GTP-associated active form, or a GDP-associated inactive form. MiR-132 was found to be highly expressed in tumor ECs, but not in normal ECs, and its induction is

driven by angiogenic factors such as Vegf and Fgf2 [64]. Silencing of miR-132 expression decreases the miR-132 target, p120RasGAP [65], which negatively regulates Ras, and contributes to vascular development and remodelling [66]. Induction of miR-132 expression in ECs may act as an angiogenic switch during tumor neovascularization, and antagonism of miR-132 markedly decreases tumor angiogenesis in mouse models [64]. Furthermore, the role of miR-132 appears to be EC specific, as its inhibition has little effect on the expression of p120RasGAP in other cell types [64].

MicroRNAs regulate signalling pathways that cross-talk with Vegfr-2

Cross-talk with several signalling pathways also contributes to the precise modulation of Vegf signal output to govern appropriate EC responses [1]. Several recent studies have revealed that these pathways are also targeted by microRNAs in ECs (Fig. 1). These include components of the Slit/Robo pathway, which are widely known for their roles as repulsive cues during neuronal guidance [67, 68], the Notch pathway, which plays a central role in switching off angiogenic behaviors induced by Vegf signalling [29], as well as integrins, which can modulate Vegfr-2 signalling [69].

MiR-218 control of Slit/Robo signalling

The Slit family of ligands (Slit1-3) bind to their cognate Roundabout (Robo) receptors (Robo1-4) to control cell migratory behaviors. While this pathway is best known for its regulation of neuronal guidance [67, 68], this ligand/receptor system also impinges on the Vegf signalling pathway in ECs. For example, Slit2 can activate Robo1 and Robo4 receptors, which are expressed on ECs [70], to elicit divergent EC responses. Slit2 activates a Robo4-dependent signalling pathway in ECs that represses Vegf signalling by inhibiting the small GTPase, Arf6 [71, 72]. In contrast, Robo1 potentiates Vegf signalling by enhancing phosphorylation of Vegfr-2[73]. Recently, a microRNA family (miR-218) was found to be intronically encoded in the *Slit2* and *Slit3* genes [73, 74]. Intriguingly, Slit-encoded miR-218 targets the Slit receptor, Robo1, as well as components of the HSPG biosynthetic pathway and negatively regulates angiogenic responses in ECs [73, 74]. HSPGs have previously been shown to enhance Slit binding to Robo receptors [75], and they also influence Vegf ligand/receptor interactions [35]. Since miR-218 does not target Robo4, this microRNA may alter the activation of Robo1- vs Robo4-dependent pathways, which have opposing effects on Vegf signalling. Knockdown of miR-218 in the mouse retina results in defective angiogenesis [74], and knockdown of miR-218 in zebrafish results in defects in the Vegf-dependent migration of the endocardium to the midline during the early stages of heart development [73], illustrating the requirement of the Slit/miR-218/Robo1 pathway for normal cardiovascular development [73]. The contribution of this pathway to pathological vascular growth remains to be determined.

Notch, microRNAs and Vegf

In sprouting blood vessels, Notch signalling plays a vital role in reducing the angiogenic response normally induced by Vegf [29, 76, 77]. In this case, Vegfa stimulates migration of leading tip cells from a pre-existing blood vessel. During this process, Vegfa induces expression of the Notch ligand, Dll4 in the tip cell, which activates Notch signalling in adjacent cells, reducing their migration and proliferation. This provides an elegant mechanism to license a restricted number of cells to sprout from a pre-existing vessel, and allows the growing sprout to maintain its connection to the patent blood vessel. Notch activation is thought to inhibit Vegfa-stimulated migration and proliferation by downregulating Vegfr-3 signalling [29], while also inducing the expression of soluble Vegfr-1 [78], which presumably acts as a sponge to bind surrounding Vegfa to prevent activation of Vegfr-2 [31].

Several studies suggest that cross-talk between Notch and Vegf signalling is further modulated by microRNAs. As mentioned above, miR-221 promotes tip cell behavior, in part through regulation of *Pik3r1* and modulation of signalling downstream of Vegfr-3 [53]. Interestingly, Notch activation represses the expression of miR-221 [53]. Also, excessive tip cell behaviors normally associated with loss of Notch signalling are blocked by loss of miR-221. Thus, Notch appears to affect the output of Vegf-3 signalling, in part through the regulation of miR-221 levels. Notch signalling components themselves have also been identified as microRNA targets during angiogenesis. In zebrafish embryos, miR-27b was found to repress the transcript encoding Dll4. Accordingly, miR-27b-deficient zebrafish embryos have reduced filopodia formation and impaired sprouting [79], similar to embryos with activated Notch [29]. This is associated with increased expression of Dll4 and upregulation of Vegfr-1 [79]. Interestingly, recent studies in cultured human cells and mice have shown that the miR-23/27/24 cluster is highly expressed in endothelial cells and that miR-23 and miR-27 are also capable of coordinately repressing Sprouty2 [80], a negative regulator of Raf1 [81], and Semaphorin 6A and D [80, 82], which inhibit Vegfr-2 signalling [83]. Thus, similar to miR-126 and miR-221, miR-27 can control the expression of multiple targets to modulate Vegf signalling output.

MiR-92a control of integrin expression

Endothelial cells interact with several ECM proteins, including fibronectin, which can affect angiogenesis. For example, fibronectin can facilitate the migration of tip cells by enhancing Vegfr-2 signalling [84]. Fibronectins signal through several integrin proteins that are expressed on ECs. The importance of integrins for vascular growth is illustrated by the embryonic lethality and major vascular defects that are observed in Integrin-α5 (*ITGA5*) knock-out mice [85]. Recently, miR-92a was shown to control angiogenesis through the targeting of *ITGA5* in the endothelium [86]. Over-expression of miR-92a inhibits sprout formation and vascular network formation *in vitro*, and also represses vascular invasion of matrigel plugs loaded with angiogenic factors *in vivo*. Of particular interest, inhibition of miR-92a in mouse models of hind limb ischemia or myocardial infarction results in enhanced angiogenesis and tissue regeneration, strongly suggesting that miR-92a normally suppresses angiogenic signalling through its targeting of *ITGA5* [86].

Emerging areas of microRNA biology with possible implications for Vegf signalling and angiogenesis

In general, microRNAs are thought to act cell-autonomously to repress their target transcripts. The aforementioned examples of microRNA regulation have focused on this well-known role for microRNAs. However, recent findings suggest that microRNAs may act as paracrine or even endocrine factors. Furthermore, microRNA target transcripts in a given cell may also serve as sponges to titrate microRNA levels and function rather than being functional targets themselves. While little is known concerning the importance of these two new concepts in the context of Vegf signalling and angiogenesis, there are hints from recent studies that they will be relevant in this context. Therefore, we provide a brief overview of these new aspects of microRNA biology.

Several studies have now shown that microRNAs are abundant and relatively stable in circulation, suggesting that they may play a paracrine role in controlling gene expression [87, 88]. MicroRNAs have been associated with a variety of carriers, including lipid-encapsulated microvesicles (MV), such as exosomes (50–100 nm), microparticles (0.1–1 μm) and apoptotic bodies (0.5–2 μm), as well as HDL and LDL complexes, and these carriers can deliver microRNAs to recipient cells [89–91]. Additionally, circulating Ago2-containing protein complexes that contain microRNAs may be the main carriers of

circulating microRNAs [92], but it is unclear whether these non-lipid-encapsulated complexes can be delivered to cells. Tumor cells can secrete microRNA-containing MVs that are taken up by ECs to alter EC phenotype, including their angiogenic properties [93–95]. It is currently not known whether secreted microRNAs contribute to vascular development, but this is an exciting prospect. A recent study has implied that levels of EC-enriched microRNAs, such as miR-126 and miR-92a may be reduced in vascular diseases such as coronary artery disease [96], but whether this is a cause or consequence of disease is not currently known. Further analyses in larger cohorts of age-matched patients will be necessary to clarify these initial findings.

While the functional effects of microRNAs are typically thought to be manifested through the regulation of protein levels from a target transcript, recent observations suggest that many target transcripts may serve a different purpose. In this case, mRNAs containing microRNA binding sites can affect the expression of other mRNAs in a protein coding-independent manner via competition for the binding of microRNAs to their 3′ UTRs [97, 98]. These RNAs have been named competing endogenous RNAs (ceRNAs). Their function was first illustrated by the finding that a pseudogene for *PTEN*, *PTENP1*, can affect the expression of the PTEN gene by acting as a microRNA decoy [99]. Considering the central role that PTEN plays in controlling PI3K activity, an important output for Vegf signalling [100], this finding will likely be relevant to vascular growth. It has subsequently been shown that multiple coding transcripts can control PTEN expression by titrating away microRNAs from the *PTEN* 3′ UTR [101, 102]. A long non-coding RNA has also been shown to regulate muscle differentiation by acting as a ceRNA for miR-133 [103]. It will be interesting to determine whether ceRNAs affect angiogenic signalling by competition for important pro- or anti-angiogenic microRNAs. CeRNAs represent another layer of regulation that may impact on the dynamic control of angiogenic signalling pathways.

Conclusion and clinical implications

It is evident that the Vegf signalling pathway is highly regulated at multiple levels. Recent studies demonstrate that microRNAs provide an additional layer of regulation by titrating the levels of proteins that are involved in the transduction of angiogenic signals. The general theme from these studies is that microRNAs are important nodes for controlling particular endothelial behaviors downstream of Vegf. Based on these observations, targeting microRNAs to manipulate subtle aspects of vascular growth in a clinical setting will be highly desirable. This is of particular importance since many early angiogenesis inhibitors that bluntly target Vegf ligand/receptor interactions or Vegfr kinase activity result in side effects, such as hypertension and proteinuria [104]. MicroRNAs can be targeted therapeutically [105], and several findings from mouse models indicate that targeting microRNAs, including those implicated in Vegf signalling, may be useful in clinical settings where precise control of vascular growth is desired. For example, silencing miR-126 has been shown to impair neoangiogenesis following myocardial infarction [54] or hind limb ischemia [106]. Interestingly, the host transcript for *miR-126*, *Egfl7*, is highly upregulated in tumor endothelium [107], but the role of miR-126 in tumor angiogenesis has not yet been addressed. Antagonism of miR-132 [64] or miR-296 [44], which are upregulated in tumor endothelium, has promising inhibitory effects on tumor angiogeneis. In contrast, inhibiting miR-92a enhances vascular growth in the setting of myocardial infarction and hind limb ischemia [86]. These exciting results provide an impetus to further understand the role of microRNAs in modulating the signalling output of Vegf.

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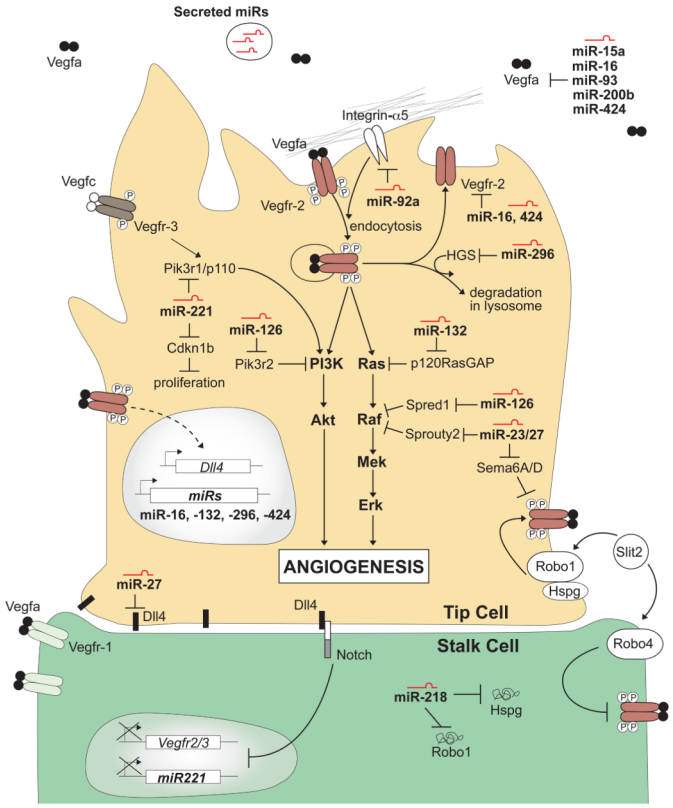

Fig 1. MicroRNAs impinge on Vegf signalling to regulate angiogenesis

Binding to their receptor tyrosine kinases (Vegfr-2 and Vegfr-3), Vegfa and Vegfc can drive angiogenesis through the activation of several downstream signaling effectors (e.g. PI3K and MAPK/ERK). The output of these effectors is modulated by microRNAs. In particular, recent evidence highlight the important roles of microRNAs in controlling Vegf signalling by titrating the levels of Vegf ligand (miR-15a, 16, 93, 200b, and 424), Vegf receptors (miR-16, and 424), as well as positive and negative regulators of the Vegf signal transduction cascade (miR-23, -27, -126, -132, -218 and -221). The identification of these regulatory microRNAs has emphasized the importance of fine-tuning both the PI3K and MAPK signaling pathways downstream of Vegf signaling and has provided new insights into how different outputs can be modulated through post-transcriptional control.