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VEGF signaling inside vascular endothelial cells and beyond

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Abstract

Vascular endothelial growth factor-A (VEGF-A) has long been recognized as the key regulator of vascular development and function in health and disease. VEGF is a secreted polypeptide that binds to transmembrane tyrosine kinase VEGF receptors on the plasma membrane, inducing their dimerization, activation and assembly of a membrane-proximal signaling complex. Recent studies have revealed that many key events of VEGFR signaling occur inside the endothelial cell and are regulated by endosomal receptor trafficking. Plasma membrane VEGFR interacting molecules, including vascular guidance receptors Neuropilins and Ephrins also regulate VEGFR endocytosis and trafficking. VEGF signaling is increasingly recognized for its roles outside of the vascular system, notably during neural development, and blood vessels regulate epithelial branching morphogenesis. We review here recent advances in our understanding of VEGF signaling and its biological roles.

Introduction

Blood vessels form complex branched tubular networks composed of arteries, capillaries and veins that supply oxygen to all body tissues and remove waste. Endothelial cells (ECs) form the inner lining of mature blood vessels and dictate the vascular branching pattern and vessel wall assembly. ECs are formed very early in embryonic development, before the onset of heart beat, from mesodermal cells that coalesce to form primitive vascular channels. During subsequent growth, this primitive vascular plexus remodels in a process termed angiogenesis that includes EC proliferation. Angiogenic ECs can form new vessels either by splitting the pre-existing ones, a process termed intussusception, or by sprouting, which is currently best understood in terms of molecular signaling mechanisms [1]. Remarkably, these different EC behaviors give rise to highly stereotyped branching networks [2]. A major question is how individual ECs coordinate their behaviors to achieve formation of ordered vessel networks during development.

Despite its complexity, angiogenesis is predominantly regulated by a single growth factor, VEGF-A [3]. VEGF is one of the most important genes regulating multiple biological processes. Indeed, its levels are so exactly maintained that even a 50% reduction results in embryonic lethality. The more becomes known about regulation of its function the more the exquisite network that regulates its biological activities comes to light. At the ligand level, VEGF activities are controlled not just by its own level of expression, but also by expression of various proteins that can bind and sequester it, with the most prominent being VEGFR1. At the receptor level, an equally complicated network controlling VEGFR2 signaling is emerging.

VEGF-A is one of five related growth factors, including VEGF-B, VEGF-C, VEGF-D and placenta growth factor (PlGF), that bind to three high affinity tyrosine kinase receptors, VEGFR1–3, with differing affinity [3,4]. VEGFR1 is considered to be a VEGF-A trap, by virtue of its high ligand affinity and poor kinase activity. Furthermore, alternative splicing generates a soluble form containing the VEGFR1 extracellular domain (sflt1), which will trap VEGF and inhibit VEGF signaling upon secretion from EC [4]. Mouse knockout studies have shown that VEGF-A binding to VEGFR2 is critically required for vascular development, while VEGF-C binding to VEGFR-3 is required for the formation of lymphatic vessels and modulates angiogenic sprouting [5]. Biological roles of individual VEGFs and VEGFR downstream signaling pathways have been extensively reviewed recently [4] and cannot be covered here. Rather, we will focus on novel aspects of regulation of VEGF signaling, including regulation of angiogenic sprouting and VEGFR endocytosis.

VEGF function in the vasculature

Sprouting angiogenesis

Sprouting capillaries designate one EC as the motile capillary ‘leader’, termed tip cell, with the other capillary cells (stalk cells) trailing behind [6–9]. While tip cells guide capillary outgrowth, stalk cells form the capillary lumen, allowing blood flow and oxygen and nutrient transport. Tip cells exhibit a characteristic gene expression profile that includes high levels of platelet-derived growth factor B (PDGFB), the Netrin receptor UNC5B, the Notch ligand DLL4, EC-specific molecule1 (ESM1), the peptide ligand apelin (APLN), angiopoietin2 and other genes [10,11]. Interestingly, however, tip and stalk cell fate are not permanently determined, with EC in the growing vascular sprout continuously overtaking each other, and an individual EC occupying the tip position for limited periods of time [12•, 13•]. Tip and stalk cell positioning is coordinated by the interplay between VEGF and Notch signaling [6–9]. VEGF promotes tip cell selection, while activation of Notch inhibits tip cells formation and promotes the stalk cell phenotype. Notch regulates expression levels of all three VEGFRs: VEGFR2 and 3 levels are decreased by Notch activation, while VEGFR1 levels are increased by Notch. VEGFR1, notably the soluble sflt1 form, negatively regulates tip cell formation [14,15•]. The VEGF-C receptor VEGFR3, which is critical for lymphangiogenesis, also contributes to coordinated tip cell sprouting [16,17•]. Interestingly, inducible deletion of the *Vegfr3* gene in postnatal retinal vessels leads to hypersprouting, while *Vegfr3* haploinsufficiency reduces angiogenesis, suggesting that VEGFR3 activation occurs both in a ligand-dependent and ligand-independent manner

[17••]. Mechanistically, VEGFR-3 reinforces Notch signaling by activating expression of Notch target genes via the transcription factor FoxC2, thereby restricting formation of new tip cells [17••]. In mouse retinas, VEGF-C is produced by macrophages [17••], which are situated at vessel branch points and promote anastomosis of newly formed vessel branches [18]. In zebrafish, the Wnt signaling regulator R-spondin1 has been shown to induce VEGF-C expression in ECs, and to promote intersegmental vessel sprouting via VEGFR3 signaling [19]. Wnt/ β catenin signaling can also influence angiogenic sprouting by upregulating Dll4-Notch signaling, but this effect is context-dependent and occurs during early mouse embryonic development, but not at postnatal stages [20]. Finally, Wnt-signaling regulated production of sflt1 by retinal myeloid cells restrains formation of vessel sprouts in the deeper layers of the retinal vasculature [21], revealing complex and stage-dependent effects of Wnt signaling on angiogenic sprouting.

Vascular guidance receptors contribute to angiogenic sprouting by fine-tuning the VEGF-Notch balance. In zebrafish, PlexinD1 signaling inhibits VEGF's proangiogenic effects via production of sflt1 [22], thereby ensuring proper formation of intersegmental vessel sprouts. In mice, PlexinD1 is induced by VEGF signaling in retinal tip cells, where it limits Dll4 expression in tip cells, thereby modulating Notch activation [23]. In ischemic retinas, PlexinD1 signaling normalizes retinal neovascularization by counteracting VEGF-induced vascular out-growth [24]. These data link PlexinD1 signaling to VEGF signaling, but show that the signaling readout depends on the cellular context. The Netrin receptor UNC5B also modulates VEGF-induced retinal and corneal neovascularization. UNC5B interacts with a number of binding partners in addition to Netrin, including the vascular guidance receptor Robo4. Robo4-UNC5B signaling counteracts VEGF-driven angiogenesis and vascular permeability, a mechanism driven at least in part by competition for downstream activating targets including Src family kinases [25•]. Ephrin-B2 and Neuropilin-1 (Nrp1) regulate VEGFR endocytosis and trafficking, as described below.

VEGF signaling regulation by endocytosis

Until fairly recently, tyrosine kinase (TK) receptor signaling was thought to occur exclusively on the plasma membrane where following its ligand binding a receptor signaling complex would assemble. In this scheme of things levels of the receptor on the membrane and the ligand in the immediate cellular environment were thought to be the primary controls of signaling. Ligand binding was thought to simultaneously activate signaling and initiate its termination by stimulating endocytosis and receptor degradation. This turned out to be an over-simplification, as many signaling events associated with TK receptor signaling actually occur after its endocytosis. It has become clear that the process of endocytosis and intracellular trafficking can affect signaling in a number of subtle ways [26]. Here we will review new information on endocytosis-dependent regulation of VEGFR2 signaling.

One of the challenges of VEGFR2 biology is that the receptor exists in a bewildering array of complexes with other transmembrane proteins such as VEGFR3, Nrp1, VE-cadherin, Ephrin-B2, thrombospondin receptor CD47 [27] and certain integrins [4], which can affect its signaling and endocytosis. On the intracellular side, VEGFR2 is in close proximity to a number of phosphotyrosine phosphatases (PTP) including CD148, VE-PTP and PTP1b that can

also affect its function. The interactions between these various proteins affect all aspects of VEGFR2 function and will be explored below.

VEGFR2 endocytosis, trafficking and processing

Upon binding VEGF-A, VEGFR2 is endocytosed via a classical clathrin-mediated pathway and is then targeted either for recycling back to the plasma membrane or for sequential proteasome and lysosomal degradation. Cargo movement from clathrin-coated vesicles to Rab5 endosomes is thought to be controlled by adaptor proteins APPL 1 and 2 [28], that in turn interacts with a PDZ protein synectin (GIPC1) [29], that links it to myosin-VI [30] (Figure 1). The exact route chosen depends in part on the cargo: VEGF-A_{165a}, an isoform capable of binding both VEGFR2 and Nrp1 directs VEGFR2 to the Rab5/Rab4/Rab11 recycling pathway while non-Nrp1-binding isoform, VEGF-A_{165b}, directs it towards degradation via the Rab7 pathway [31]. Interestingly, Nrp1 targeting of endocytosed VEGFR2 to the Rab5/Rab11 pathway requires the presence of its PDZ binding domain, suggesting a synectin-dependent process [31].

Remarkably, initiation of VEGFR2 endocytosis requires the presence of Ephrin-B2 (Figure 1). Virtually no uptake of VEGFR2 occurs in Ephrin-B2 absence resulting in a profound effect on its signaling [32••]. Furthermore, Ephrin-B2 plays a similar role in endocytosis of VEGFR3 [33••]. While the mechanism of this effect is not clear, it is interesting to note that Ephrin-B2 is a PDZ binding proteins and the presence of its PDZ domain is required for its effect on EC morphology and migration and lymphangiogenesis [34–36].

Upon entry into cell, VEGFR2, as many other TK receptors, continues to signal. The effectiveness of signaling, however, is dependent on its location in a specific endosomal compartment and subsequent trafficking. In the absence of synectin, newly endocytosed VEGFR2 remains for a prolonged period of time in EEA1-positive endosomes before moving to the Rab5 endosomal compartment, the process that requires synectin/myosin-VI-dependent trafficking. This results in decreased phosphorylation of Y₁₁₇₅ site that is critical to activation of PLC γ /ERK signaling. As a result, ERK phosphorylation is reduced and shortened in duration. Interestingly, phosphorylation of other critical VEGFR2 tyrosine residues remains unchanged and, consequently, activation of other VEGFR2 signaling pathways such as Src and Pi3K/Akt remains unaffected [37••]. The primary phenotype observed in synectin null mice and zebrafish is decreased arterial morphogenesis and reduced arterial lumen size and branching both during development as well as in adult tissues following arterial ligation [38]. The reduced phosphorylation can be traced to the action of PTP1b, an ER-resident PTP that, nevertheless, has been shown to affect activation and trafficking of multiple TK receptors [39]. Knockdown of PTP1b expression in synectin^{-/-} ECs fully restores VEGFR2-dependent ERK activation and suppression of its activity *in vivo* restores arteriogenesis in synectin null mice [37••].

These findings imply that VEGFR2-driven ERK activation occurs predominantly in endosomal compartments. This in fact is in agreement with recent studies showing the existence of an ERK scaffold complex in late endosomes that consists of MEK partners MP1 and p14, which are anchored to the endosome and bind MEK1, ERK1 and ERK2 via p18 adaptor protein [40].

While the link between VEGFR2 endocytosis and exocytosis has not been yet explored, a protein involved in VEGFR2 endocytosis, Golgi-localized target membrane-soluble N-ethylmaleimide attachment protein receptor (t-SNARE) syntaxin 6, appears to play a key role in VEGFR2 signaling. Syntaxin 6 achieves that by regulating VEGFR2 trafficking through trans-Golgi network and a reduction in its levels results in decreased VEGF-induced EC proliferation, migration and tube formation while *in vivo* an inhibitory form of syntaxin 6 reduces VEGF-induced angiogenesis and permeability [41].

Intracellular proteolysis

Another level of VEGFR2 regulation is proteolytic cleavage that releases nearly complete cytoplasmic domain of VEGFR2 [42]. Remarkably, the proteolytically cleaved fragment is able to activate PLC γ and ERK signaling on its own, further demonstrating an intracellular site of ERK activation by VEGFR2.

Non-vascular effect of VEGF

VEGF functions in the nervous system

VEGF-mediated blood vessel growth is essential to sustain development and function of the nervous system [43]. Branching of nerves and blood vessels is interdependent, and nerve-secreted VEGF controls neurovascular co-patterning by acting on vascular Nrp1 to promote arteriogenesis adjacent to sensory nerves in embryonic limb skin [2]. Blood vessels form a vascular niche to sustain adult neurogenesis, providing neurotrophic signals as well as oxygen and nutriment supply [44]. In addition to blood vessel mediated effects, recent studies have revealed direct actions of VEGF on nervous system development. VEGF mediates commissural axon guidance, directs granule cell migration in the cerebellum via VEGFR2 expressed in these cells [45•,46•] and modulates NMDA receptor activity before synapse formation [47]. In some contexts, VEGF can signal via Nrp1 independently of VEGFR2, and this pathway has been shown to guide commissural axon crossing at the optic chiasm [48•]. Adding to this complexity, VEGFR2 has also been implicated in non-VEGF mediated axonal guidance of subicular hippocampal neurons [49]. In addition to guidance processes, VEGF directly controls survival of subsets of neuronal populations [50] and modulates neuronal plasticity and memory functions [51•,52•], demonstrating that VEGF affects cognition processes by hippocampal circuits. Direct actions on neural cells are also seen for other VEGF family members, notably VEGF-C, which directly regulates neural stem cell development via VEGFR3 [53•]. These results show that VEGF family members function as potent regulators of neurogenesis and neural plasticity and represent therapeutic targets for neural tissue repair and neurodegenerative disease.

Blood vessel regulation of epithelial branching

Vascular branching is not only coordinated with the nervous system wiring, but also with the formation of other branched systems, including regulation of branching morphogenesis of airway and pancreatic epithelium. VEGF-dependent blood vessel growth has been recently shown to coordinate epithelial branching via production of signaling molecules that regulate epithelial branching morphogenesis.

Lung epithelial airway morphogenesis is highly stereotypic in human and mouse [54]. Blood vessels are aligned with the airways throughout all branching generations, ranging from the primary bronchi to the alveoli, where gas exchange with the capillary system occurs. While airway branching is regulated by epithelial–mesenchymal interactions [55,56], blood vessels contribute to branching morphogenesis of the mouse lung airways, as shown by reversible ablation of VEGF-dependent vessel development in mice [57]. Vessel growth inhibition inhibits distal airway branching, and this effect is independent of oxygen and nutrient supply by the vasculature, implying the existence of signaling molecules derived from EC that determine airway epithelial branching.

During adult lung regeneration following unilateral pneumectomy in mice, ‘angiocrine’ growth factors released from growing EC have been shown to sustain alveologenesis and lung regeneration. VEGF and FGF signaling in growing EC was found to increase expression of MMP14, which generated EGF-like fragments through cleavage of proteins in the extracellular space, thereby stimulating EGF signaling and proliferation in the alveolar epithelium [58]. Whether these factors also pattern embryonic airway branching is not known, but these findings open novel perspectives for regenerative therapies of lung tissue, which can be engineered *in vitro* [59] based on EC-derived factors.

In the pancreas, the vasculature also controls morphogenesis and growth, but it does so by reducing branching and differentiation of primitive epithelial cells [60]. Inducing pancreatic hypervascularization by overexpression of the angiogenic growth factor VEGF decreases pancreas size, while inhibiting blood vessel growth increases pancreas size. Signals from blood vessels sustain primitive undifferentiated epithelial cells at the expense of the formation of multipotent epithelial tip cells, thereby reducing branching, and preventing endocrine and exocrine differentiation of the embryonic pancreas. Understanding this signaling crosstalk may have practical implications for the derivation of transplantable insulin-producing pancreatic beta cells from embryonic stem cells.

Taken together, these studies show that growing blood vessels instruct growth and differentiation of the tissues they permeate, and open exciting novel perspectives for identification of the EC-derived molecules that mediate these effects during development and tissue regeneration.

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Figure 1.

VEGF binding to VEGFR2, located in close proximity to thrombospondin-2 receptor CD47, promotes VEGFR2 dimerization and activation (phosphorylation of Y¹⁰⁵⁴ and Y¹⁰⁵⁹). At the same time VEGF binding to Nrp1 promotes endocytosis of the VEGFR2/Nrp1 complex, perhaps via Ephrin-B2. Newly endocytosed VEGFR2 in Rab5 endosomes is subject to dephosphorylation of Y¹¹⁷⁵ site by PTP1b. Trafficking of this endosomes in a synectin/ myosin-VI-dependent manner away from the dephosphorylation zone results in full activation of ERK signaling. In addition, a proteolytic cleavage event may take place, releasing the cytoplasmic VEGFR2 dimer.