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## Common Cues Regulate Neural and Vascular Patterning

Christopher A. Jones<sup>1,2</sup> and Dean Y. Li<sup>1,2,3</sup>

<sup>1</sup> *Department of Oncological Sciences, University of Utah, Salt Lake City, UT, 84112*

<sup>2</sup> *Program in Human Molecular Biology and Genetics, University of Utah, Salt Lake City, UT, 84112*

<sup>3</sup> *Division of Cardiology, University of Utah, Salt Lake City, UT, 84112*

### Summary

Nerves and blood vessels often follow parallel trajectories as they course through the body to their distal targets. Proteins that regulate the process of axon guidance have likewise been shown to play a critical role in blood vessel migration. With the recent description of the endothelial tip cell as an analog of the axonal growth cone, the nerve-vessel analogy seems complete. Notwithstanding these considerable similarities, one critical difference remains between neural and vascular guidance.

While a navigating axon is but a single cell, a sprouting vessel is composed of multiple cells that must be coordinately regulated. Recent studies of the Dll4-Notch1 signaling pathway have provided valuable insight into how the vasculature accomplishes this critical task.

### Keywords

angiogenesis; vascular guidance; axon guidance cues; blood vessels

### Introduction

Formation of the vertebrate vasculature is a complex process that is orchestrated by a constellation of growth factors and guidance cues [1]. During vasculogenesis, the initial phase of vascular development, endothelial cells differentiate, migrate and coalesce to form the central axial vessels, the dorsal aortae and cardinal veins. The second phase, called angiogenesis, is characterized by the sprouting of new vessels from the nascent plexus to form a mature circulatory system. Following this angiogenic remodeling, the endothelium secretes platelet-derived growth factor (PDGF), which induces the recruitment and differentiation of vascular smooth muscle cells [2]. Subsequently, the vascular smooth muscle cells secrete angiopoietins, which ensure proper interaction between endothelial and vascular smooth muscle cells [3,4]. Finally, the vascular smooth muscle cells deposit matrix proteins, such as elastin, that inhibit vascular smooth muscle cell proliferation and differentiation, thereby stabilizing the mature vessel [5,6]. Thus, to establish and maintain a mature vascular network, the endothelial and smooth muscle compartments of a vessel must interact via autocrine and paracrine signaling.

Significant strides have been made in deciphering the molecular mechanisms underlying vasculogenesis and angiogenesis. However, we are only beginning to appreciate the guidance

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Correspondence should be addressed to D.Y.L. E-mail: dean.li@hmbg.utah.edu, phone: 801-585-1694, FAX: 801-585-0701.

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programs utilized by vertebrates to generate the highly stereotypical pattern of the mature vascular network.

## Neural guidance pathways

Our knowledge of the way attractive and repulsive cues mediate organ system patterning has emerged from studies of the nervous system. At the cellular level, growing axons must navigate through a complex microenvironment to enervate a distal target tissue. The ability of the axon to reach this destination is predicated upon its ability to sense and respond to an array of guidance cues, both long-range diffusible factors and short-range membrane-bound proteins [7]. It is difficult to strictly classify these factors as attractive or repulsive due to the fact that a given molecule can elicit either response under different circumstances. The ultimate choice is dependent upon the complement of receptors expressed by the axon, the intracellular state of the growth cone, and the presence of other guidance molecules in the extracellular milieu. Thus, the context in which an axon encounters this relatively small number of factors can define the path choices required to establish complex neural connections [8,9].

There are four major classes of guidance cues, which include the secreted Semaphorins, Netrins, Slits, and the membrane-bound Ephrins. Each of these ligands activates cognate transmembrane receptors to affect attraction or repulsion of growing axons. Semaphorins primarily function as short-range inhibitory cues by stimulating receptors from the plexin and neuropilin families [10]. Netrins interact with Unc5, Neogenin, and DCC (deleted in colorectal cancer) receptor families, or combinations thereof, and may repel or attract axons depending on the type of neuron and complement of receptors that are expressed on the surface of the growth cone. For example, Unc5 signaling exclusively specifies repulsion, while DCC can mediate either repulsion or attraction to netrin [11,12]. The Slit proteins activate the Roundabout (Robo) family of transmembrane receptors to predominantly mediate chemorepulsion of growing axons [13]. The Ephrins are membrane-bound ligands for the Eph family of receptor tyrosine kinases and mediate short-range repulsive juxtacrine signaling [14,15,16]. In addition, Notch signaling, although principally associated with cell-fate determination, also regulates neurogenesis through inhibition of neurite outgrowth [17,18,19]. Together, these ligand-receptor pairs establish the precise pattern of synapses that are required for a functional neural network.

## Neural and vascular guidance pathways share common signaling mechanisms

Over the past decade it has become apparent that molecular mechanisms underlying development of the nervous system have been co-opted by the vasculature. In fact, each of the aforementioned classes of axon guidance molecules has been shown to regulate some facet of vascular patterning, although the precise role of some of these proteins remains controversial.

The Ephrin/Eph signaling pathway appears to play an important role in regulating endothelial cell migration, analogous to its task of guiding axons in the nervous system. Studies in both mice and frogs have revealed that intersomitic blood vessels express Eph receptors, while the somites express Ephrin ligands. Juxtacrine interactions between this ligand-receptor pair mediate repulsive guidance signaling that prevents the intersomitic vessel from invading the somitic compartment [20,21].

Like the Ephrins, the Semaphorins and their cognate receptors have been implicated in the regulation of vascular development. It was reported that knockdown of *Sema3a1* in zebrafish [22] or targeted ablation in mice [23] resulted in defective formation of the intersomitic vessels. More recently, positional cloning of the zebrafish *out-of-bounds* (*OBD*) mutation, which causes

highly arborized intersomitic vessels, identified the semaphorin receptor Plexin-D1 [24]. In a companion report, gene targeting of the *Plexin-D1* locus in mice resulted in blood vessel and cardiovascular defects [25]. Interestingly, independent knockdown of the presumptive Plexin-D1 ligands, *Sema3a1* and *Sema3a2*, caused only moderate intersomitic vessel phenotypes when compared to *OBD* mutants or *Plexin-D1* morphants, suggesting either functional redundancy or an alternative ligand [24]. Indeed, a subsequent study showed that another Semaphorin family member, *Sema3e*, but not *Sema3a*, formed a high affinity complex with Plexin-D1, and targeted inactivation of *Sema3e* caused intersomitic vessels defects that phenocopied a null allele of *Plexin-D1* [26]. These data suggest that *Sema3e*-Plexin-D1 signaling is critical for mammalian vascular patterning.

There is ample, albeit contradictory evidence that the prototypical axonal attractant Netrin is involved in vascular development. Our laboratory has demonstrated that, similar to their role in the nervous system, Netrins stimulate migration of endothelial cells [27,28]. Furthermore, we have shown that knockdown of *Netrin1a* in zebrafish embryos prevents formation of the parachordal vessel, and overexpression of Netrin1 ameliorates defects in a murine model of ischemia [27]. However, Lu et al. [29] has reported that deletion of the Netrin receptor *Unc5b*, which is expressed in the vasculature of the mid-gestational mouse, causes excessive branching in multiple arterial vascular beds. They argue that this increased peripheral resistance in the arterial system leads to heart failure and lethality in *Unc5b*<sup>-/-</sup> animals [29]. These data suggest that Netrin-*Unc5b* signaling specifies a repulsive cue to the endothelium. Future studies will be needed to precisely define the role of Netrin and *Unc5b* in regulating vascular development.

The most recent ligand-receptor pair to be implicated in regulating endothelial cell behavior is Slit-Robo. Three Robo family members have been described in the nervous system, and we and others, identified a fourth member of the family, named Magic Roundabout or Robo4 [30,31]. Unlike *Robo1-3*, *Robo4* is expressed in the endothelium of the embryonic and adult mouse, and appears to be up regulated in the vessels of human neoplastic lesions [32]. Studies from our laboratory showed that Robo4 interacts with Slit2 to suppress migration of HEK cells ectopically expressing the receptor, and primary human endothelial cells, which express endogenous Robo4 [31]. These data have been corroborated by a recent report showing that recombinant Slit2, or overexpression of Robo4 is sufficient to inhibit the migration of endothelial cells [32]. Cumulatively, these observations suggest that Slit-Robo signaling mediates chemorepulsion in the vascular endothelium. There are, however, several studies that imply an alternate function for Robo4. Suchting et al. [33] showed that Robo4 was unable to interact with recombinant Slit1-3 using BiaCore analysis and immunoprecipitation, indicating that Robo4 is not a receptor for Slit proteins. They also provided evidence that excess soluble Robo4 ectodomain could inhibit the migration and tube formation of endothelial cells, suggesting that Robo4 promotes angiogenesis via an unknown or unappreciated ligand. Along these lines, Ramchandran and colleagues have proposed that Robo4 induces angiogenesis in the zebrafish through activation of the Rho GTPases [34]. These contradictory findings illuminate the need for additional analysis of Robo4 signaling in the vascular system. Perhaps characterization of *Robo4* null mice will provide necessary insight into this interesting paradox.

## Dll4-Notch1 signaling and the tip cell

Although functionally distinct, the nervous and vascular systems are remarkably similar at the anatomical level. The information presented thus far has elaborated upon this likeness to include the molecular mechanisms underlying neural and vascular guidance. These similarities can be further extended based on the recent characterization of the endothelial tip cell [36]. Analogous to the axonal growth cone, the tip cell is a highly dynamic structure that uses filipodial protrusions to sample the extracellular environment and dictate the direction in which

the vascular plexus will expand [36]. Unlike the growth cone, which must initiate extension of a single axon, the tip cell must coordinate the expansion of the proliferating stalk cells that comprise the vascular plexus. A series of elegant experiments led to a model where heparin generated gradients of immobilized vascular endothelial growth factor (VEGF-A) induce tip cell formation and direct cell migration, whereas local concentrations of VEGF-A control stalk cell proliferation and growth of the plexus [35,36].

If a gradient of VEGF-A is all that is required to form a tip cell, what prevents all endothelial cells from adopting this phenotype? The answer to this question has emerged from analysis of Delta-like ligand 4 (Dll4)-Notch1 signaling in the murine retina and zebrafish embryo [37–41]. Dll4 is an endothelial-specific ligand that interacts with its cognate receptor Notch1 on the surface of endothelial cells. The outcome is the  $\gamma$ -secretase-dependent proteolysis of the Notch1 intracellular domain (NICD), resulting in translocation of the NICD to the nucleus and subsequent changes in gene expression [42]. The fundamental significance of Dll4 function *in vivo* was established by gene targeting experiments, which showed that ablation of a single copy of *Dll4* resulted in severe vascular defects and embryonic lethality [43,44]. Additionally, global or vascular-specific deletion of the Dll4 receptor Notch1 also resulted in vascular anomalies and lethality [45,46], indicating the fundamental requirement for Dll4-Notch1 signaling during vascular development.

The haploinsufficient phenotype of *Dll4* is reminiscent of the extreme dosage sensitivity for VEGF-A during murine embryogenesis [47,48], and studies have determined that VEGF is epistatic to Notch signaling in a genetic pathway that regulates arterial identity [49]. In agreement with this idea, Lobov et al. [39] found that Dll4 is up regulated in murine retinas following intraocular injection of VEGF-A. Using an ICR mouse strain to decrease embryonic lethality associated with *Dll4* haploinsufficiency, they and others demonstrated that *Dll4*<sup>+/-</sup> animals exhibit increased numbers of filipodial protrusions at the sprouting front of the retinal vascular plexus, and in the peripheral vascular plexus, which is normally devoid of such structures [37,39,41]. As filipodial protrusions are a unique characteristic of the endothelial tip cell, these data indicate that loss of *Dll4* leads to an increased number of tip cells. Accordingly, Siekmann and Lawson [40] reported that loss of an essential Notch signaling component, recombining binding protein suppressor of hairless (Rbpsuh) in zebrafish, caused all of the endothelial cells in the segmental arteries to adopt tip cell behavior. The consequence, in both the murine retina and embryonic zebrafish, is an abnormally patterned vascular bed that is characterized by excessive branching [37–41]. Intraocular injection of  $\gamma$ -secretase inhibitors, which suppress activation of the Dll4 receptor Notch1, caused a similar increase in filipodial extensions [37,41], suggesting that a principal function of the Dll4-Notch1 pathway is to restrict the tip cell phenotype to a precise number of cells at the sprouting front of the vascular plexus. This role of Notch1 in the vasculature is similar to its role in limiting axon sprouting in the nervous system, providing further evidence that the neural and vascular networks utilize similar molecular mechanisms.

## Conclusions

The identification of distinct endothelial cell populations (tip and stalk cell) within the retinal vascular plexus, along with the demonstration that the Dll4-Notch1 pathway acts to prevent the stalk cell from adopting a tip cell fate heralds a new beginning in our ongoing quest to understand endothelial biology. These seminal discoveries suggest that signal transduction specifically within the stalk cells has an important role in regulating the patterning, and perhaps function of the vascular system. In the future it will be important to identify and characterize signaling networks that operate within the stalk cells. Of particular interest will be determining whether the neural/vascular guidance molecules and their cognate receptors can regulate stalk cell-specific signaling.

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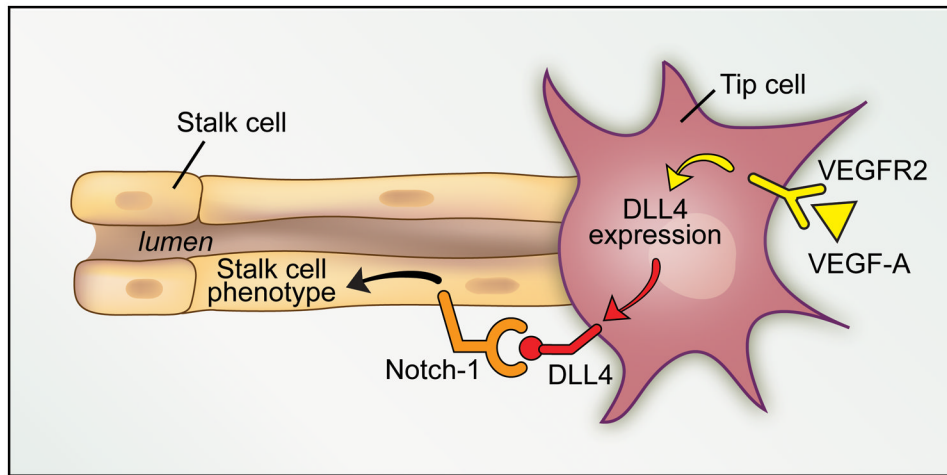
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**Figure 1.** DLL4-Notch1 signaling enforces the stalk cell phenotype. In the developing murine retina, activation of VEGFR2 by VEGF-A initiates expression of DLL4 in the tip cell. As DLL4 is membrane restricted, juxtacrine signaling between DLL4 and Notch1 on an adjacent cell leads to induction of gene expression programs that enforce the stalk cell phenotype. The consequence is precise control of the number of tip cells at the sprouting front of the vascular plexus.