ELSEVIER

Guidance of vascular and neural network formation

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Blood vessels and nerves are structurally similar complex branched systems. Their guidance must be exquisitely regulated to ensure proper wiring of both networks. Recent results showed that specialized endothelial cells, resembling axonal growth cones, form the tips of growing capillaries. These endothelial tip cells guide outgrowing capillaries in response to gradients of extracellular matrix-bound vascular endothelial growth factor. Several axon guidance molecules, including Semaphorins, Netrins, Ephrins and Slits, have also been implicated in vessel pathfinding and network formation. In particular, Semaphorin3E and its receptor plexinD1 in addition to the Netrin receptor UNC5B have recently been shown to direct endothelial tip cell navigation.

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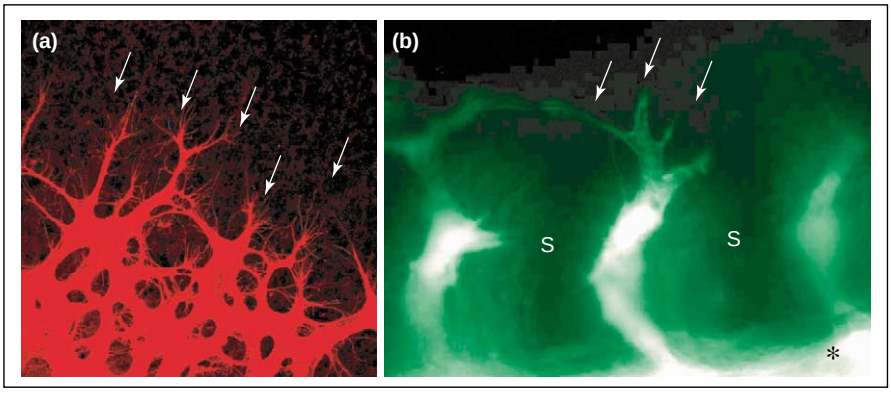
Introduction

In this review, we summarize the role of axon guidance cues in vascular network formation. Two successive processes, called vasculogenesis and angiogenesis, achieve blood vessel formation during embryonic development [1]. Vasculogenesis is the differentiation of endothelial precursor cells from the mesoderm, and their coalescence into tubes of the primary vascular plexus. This plexus consists of the central axial vessels (i.e. the dorsal aortae and the cardinal veins) in addition to a meshwork of homogenously sized capillaries, and receives the output of the first heartbeat. This primitive network subsequently expands through angiogenesis, that is, sprouting, bridging and branching by intussusception of pre-existing vessels. Angiogenesis leads to remodeling of the primary vascular plexus into a highly branched hierarchical vascular tree, composed of arteries and veins, that accommodates circulation, crucial for embryonic viability. Recruitment of mural cells (pericytes in medium-sized vessels and smooth muscle cells in large vessels) around the endothelial layer completes the formation of a functional network [1].

Branching angles, curvature of major vessels, and hierarchy along the vascular tree are highly stereotyped within and across species. Secondary sprouts, such as intersomitic vessels and the main branches penetrating different organs and the limbs, form at designated sites. The gross vascular anatomy of developing mouse, chick or zebrafish embryos is thus characterized by highly reproducible branching patterns. Embryonic vessel formation is also highly dynamic and subject to intense remodeling throughout development. Entire vessel tracts are removed or reconnected, and hemodynamic forces are crucial in shaping the final vascular pattern. For example, local alterations in perfusion produce dramatic changes in vascular patterning throughout the embryo [2•]. Oxygenation of the embryo's cells is also a determining factor for vessel patterning; regions of hypoxia constitute strong attractive signals and regions of high oxygen concentration constitute repellents [3]. In addition to these mechanisms, establishing the precise wiring of the vascular system requires an ordered series of guidance decisions, similar to those made during the precise wiring of the nervous system.

In peripheral tissues, the patterning of blood vessels and nerves is often congruent. This might, at least in part, reflect both the physiological dependency of nerves on oxygen and nutrients and the requirement of blood vessels to have appropriate vasoregulation. In the skin of the embryonic limb, small arteries are aligned with nerves, whereas veins show no specific alignment. Patterning and specification of small arteries along peripheral nerves involves nerve-derived vascular endothelial growth factor (VEGF) [4]. In other situations, neuronal development and differentiation depend on blood vessels; for instance, in the adult central nervous system clusters of neural stem cells proliferate in vascular niches that are enriched in endothelial-derived growth factors that stimulate neurogenesis [5••]. The common aspects of neuro- and angio- genesis have been reviewed recently [3]. Here, we focus on the emerging evidence for common mechanisms regulating the guidance of developing blood vessels and nerves.

Figure 1



Endothelial tip cells in (a) mouse retina and (b) zebrafish ISV. **(a)** IsolectinB4 staining of postnatal day 5 mouse retina. Note numerous filopodia extending endothelial tip cells at the angiogenic front (arrows). **(b)** Transgenic (*Tg(Fli1:EGFP)*^{Y1}) zebrafish. Note tip cell extending filopodia dorsally (arrows), as ISVs migrate to form the DLAV. Dorsal aorta is shown (asterisk). Abbreviations: S, somite.

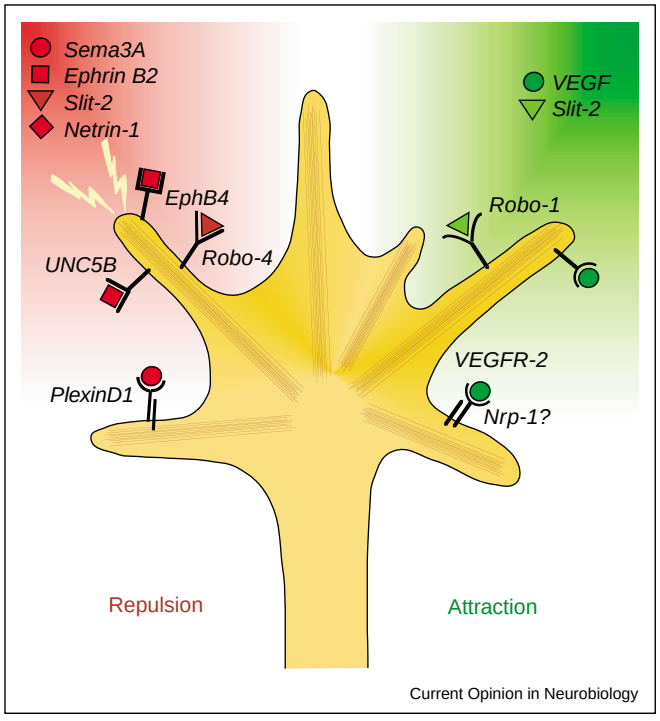
Capillary guidance by endothelial growth cones

Neuronal axons are directed to their targets with remarkable precision over large distances. Axon guidance depends on the growth cone, the motile distal tip of the axon [6,7]. In the vascular system, the extremities of capillaries carry specialized motile cells termed 'tip cells', which are similar to axonal growth cones. Using isolectinB4 endothelial cell surface labeling of developing postnatal mouse retinal vessels, Gerhardt *et al.* [8••] showed that tip cells were localized to the leading edge of the growing vascular plexus (Figure 1a). These tip cells extend numerous thin filopodia that explore their environment, suggesting that they regulate extension of capillary sprouts. Using multiphoton time-lapse imaging of transgenic zebrafish (*Tg(fli1:EGFP)*^{y1}) specifically expressing enhanced green fluorescent protein in endothelial cells, Isogai *et al.* [9•] documented the dynamic assembly of the intersegmental vessels (ISVs) in embryos. ISV formation is initiated by angioblast migration from the dorsal aorta into the intersomitic space [10]. These angioblasts form sprouts that grow dorsally between the somites and the neural tube, tracking along vertical myotomal boundaries. The sprouts grow in a saltatory fashion with numerous active filopodia extending and retracting, particularly in the dorsal-most leading extension (Figure 1b). As the growing sprout approaches the dorsolateral roof of the neural tube, it divides into two major branches, one turning caudally and one rostrally. These branches elongate and fuse together with branches from adjacent segments to form the dorsolateral anastomotic vessel (DLAV). The final ISV is composed of three endothelial cells: the dorsal-most one is T-shaped, the second constitutes a connecting cell and the third an inverted T-shape cell, with its base incorporated in the dorsal aorta and its branch directed dorsally. ISVs are formed before perfusion — indeed, filopodial movement of tip cells ceases as perfusion of these vessels is initiated. ISVs also form independent of oxygen signaling and thus constitute a prototype of genetically programmed guidance-dependent vessel patterning. ISV guidance is regulated by the combined activity of attractive and repulsive cues that not only determine selection of the appropriate branching sites along the dorsal aorta to sprout into the intersomitic boundary but also guide ISVs through the ventral somite boundaries and prevent them from straying erroneously into adjacent somites (Figures 2, 3).

Positive regulation of capillary tip cell guidance by vascular endothelial growth factor

Endothelial tip cells express VEGF receptor-2 (VEGFR-2) [8••], a high affinity receptor of VEGF [11]. VEGF exists as several alternatively spliced isoforms, VEGF120, 164 and 188 in mice (VEGF121, 165 and 189 in humans), differing in their matrix- and receptor-binding affinities. The shorter VEGF120 isoform is freely diffusible, because it lacks the heparin-binding domain necessary for interaction with the extracellular matrix, whereas the VEGF188 isoform remains bound to the extracellular matrix and the VEGF164 isoform has intermediate properties. All isoforms bind VEGFR-1 and -2, but only VEGF164 binds Neuropilin-1 (Nrp-1) [11,12]. In the postnatal mouse retina, tip cell filopodia follow a gradient of matrix-bound VEGF produced by retinal astrocytes [8••]. Alteration of the VEGF gradient by injection of soluble VEGFR-1 or by blocking antibodies against VEGFR-2 but not VEGFR-1 led to loss of tip cell filopodia; conversely, increased branching of hyaloid vessels was observed in transgenic mice overexpressing VEGF164 under the control of the αA-cristallin promoter. Endothelial tip cells primarily migrate and 'pave the path' but proliferate only minimally, in contrast to their subjacent

Figure 2



Guidance molecules implicated in endothelial tip cell attraction and repulsion. Left side: repulsive signaling. Sema3E–PlexinD1 and Netrin-1–UNC5B interactions signal repulsive endothelial tip cell guidance in mouse and zebrafish embryos *in vivo*. EphrinB2–EphB4 repulsive interaction has been demonstrated in *Xenopus* embryos. Robo-4–Slit-2 interaction has so far only been demonstrated *in vitro*. Right side: attraction. VEGF–VEGFR-2 signaling is required for tip cell extension. The precise role of Nrp-1 in this process is still unclear (signified in the schematic by?). Robo-1–Slit-2 interaction has been suggested to attract tumor vessels. See text for details.

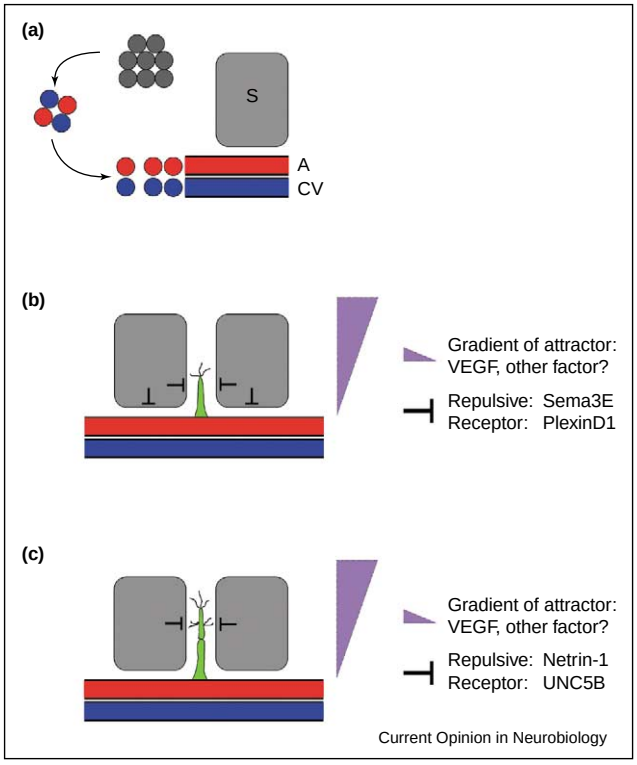
endothelial cells, termed the 'stalk cells', which do proliferate. Thus, these two types of endothelial cells interpret the VEGF signal differently: tip cells extend filopodia and stalk cells proliferate. The molecular regulation of these two distinct behaviors is currently not understood.

Evidence for a role of VEGF in tip cell guidance is also deduced from the analysis of mouse mutants selectively expressing different VEGF isoforms [13]. Vascular development is normal in mice expressing only VEGF164, indicating that this isoform alone is sufficient to ensure proper vascular patterning [14••,15]. By contrast, mice expressing only VEGF120 or VEGF188 exhibited vessel navigation defects. In VEGF120 mice, endothelial cells become incorporated into existing vessels and increase vessel size rather than forming new branches. As a result, vessels are enlarged, stunted and hypobranched. VEGF188 mice, by contrast, showed the opposite phenotype, that is, hyperbranched and thin vessels [13]. Thus, sequestration of VEGF isoforms as gradients in the matrix is crucial for the balance between capillary branching and enlargement of vessel size. Collectively, these experiments provide evidence for a positive role of VEGF in tip cell guidance (Figures 2 and 3).

Neuropilin receptors in blood vessel patterning

The VEGF164 isoform also binds Nrp-1 [12], suggesting that this interaction could be crucial for correct vessel navigation. The Nrps are a family of two related single-pass transmembrane receptors, Nrp-1 and -2. During embryonic development, Nrp-1 and -2 show overlapping but largely distinct expression patterns in the nervous system [16]. In the vascular system, both Nrps are co-expressed in yolk sac endothelial cells during vasculogenesis [17]. At later stages, Nrp-1 is preferentially expressed in arterial endothelial cells, whereas Nrp-2 labels venous and lymphatic endothelium [17–19]. Mouse knockouts for Nrp-1 are embryonic lethal, they show neural and cardiac defects in addition to defects in vessel branching in the outflow tract and the central nervous system [20,21••,22]. Knockouts of Nrp-2 lead to a distinct set of phenotypes in both the nervous system [16] and the vascular system, where small lymphatic vessels and capillaries fail to form [19]. Combined knockouts for both Nrp-1 and Nrp-2 receptors lead to defects in vasculogenesis and failure to assemble the primary vascular plexus [23], suggesting possible redundancy between Nrp-1 and -2 signaling during vascular development.

Figure 3



Schematic representation of the initial steps of vascular network formation based on zebrafish development. **(a)** Vasculogenesis. Angioblasts (grey circles) differentiate from the mesoderm, become specified to an arterial or venous fate (red and blue circles, respectively) and assemble the two major trunk vessels, dorsal aorta (A, red) and cardinal vein (CV, blue). (b,c) Angiogenic sprouting. **(b)** Gradients of attractors, including matrix-bound VEGF, and perhaps other factors, attract endothelial tip cells (green) to sprout into the intersomitic space. Repulsive Sema3E–PlexinD1 signaling participates in selection of the appropriate sprout site. Additional factors might be involved in sprout site selection, and Sema3E–PlexinD1 signaling might also act at later stages of vessel branching. **(c)** Netrin-1–UNC5B signaling does not participate in sprout site selection (b), but inhibits endothelial tip cell branching into the somite, suggesting that different repulsive signals might act at distinct vessel branching sites. Abbreviations: S, somite.

In addition to VEGF isoforms, Nrps bind to secreted class III semaphorins (Sema3s) [16]. Nrps associate with two different types of receptors to mediate signal transduction: in the nervous system, Sema3 binding to a complex of Nrp and plexin receptors leads to axonal growth cone collapse; in endothelial cells, signal transduction is mediated by formation of a complex of Nrp-1 with VEGFR-2 [24]. *In vitro*, the migratory response of endothelial cells to VEGF165 is enhanced in the presence of Nrp-1, but Sema3A and VEGF165 compete with each other to bind to Nrp-1, and Sema3A can inhibit VEGF-dependent angiogenesis [25]. Several recent studies have shown a role for Sema3 signaling in angiogenesis. For instance, Sema3A is expressed by endothelial cells of developing blood vessels in chicks and mice and inhibits endothelial migration by interfering with integrin function [26,27•]. Sema3F inhibits tumor angiogenesis and metastasis in a mouse model [28,29]. These results are consistent with several possible modes of action: Sema3s could influence endothelial cell migration directly by binding to Nrp–plexin complexes, indirectly by competing with VEGF for Nrp-binding, or by acting through other signaling pathways.

Gu *et al.* [30] identified 7 amino acids in the Nrp-1 extracellular domain that were crucial for binding to Sema3 but not to VEGF. Substitution of these residues by genetic manipulation in mice selectively disrupted the interactions of Nrp-1 with Sema3, but its ability to bind VEGFs was retained [21••]. Although neural development was severely affected, overall vascular development was normal in these mice, indicating that Sema3–Nrp-1 signaling is dispensable for vascular development. By contrast, endothelial-specific ablation of the *Nrp-1* gene led to severe malformation of the vascular system, consistent with the idea that VEGF binding to Nrp-1 is responsible for its effects on vascular development [21••]. Because Nrp-1 selectively binds VEGF164, it might be expected that mouse mutants lacking this isoform would show similar branching defects to *Nrp-1* mutants. Ruhrberg *et al.* [13], however, reported distinct patterning defects, suggesting a more selective requirement of Nrp-1 during vessel branching. Analysis of the formation of hindbrain vessels in *Nrp-1*$^{-/-}$ mice revealed selective deficiencies in the lateral branching of tip cell filopodia in the subventricular zone [22]. Although these results suggest a role for Nrp-1 in tip cell guidance, the ligand binding requirements and cell-type specificity remain to be determined.

PlexinD1-semaphorin signaling in blood vessel formation

PlexinD1 belongs to the family of nine mammalian plexins and is expressed in developing blood vessel endothelial cells [31,32]. Loss-of-function of plexinD1 in zebrafish and mouse embryos leads to perturbed vessel pathfinding [33••,34••,35••]. In zebrafish, loss-of-function mutations of plexinD1 are responsible for the out-of-bounds (obd) mutation [10,33••]. Assembly of the dorsal aorta and cardinal vein, artery–vein specification and endothelial cell proliferation are normal in *obd* mutant fish. However, selection of the appropriate site of sprouting of the ISVs from the dorsal aorta is perturbed in *obd* mutants; ISVs do not respect anterior–posterior intersomitic boundaries and erroneously branch throughout the somites, particularly in the ventral trunk. Thus, obd selectively perturbs ISV sprout site selection and initial dorsal extension of the forefront migrating endothelial cell.

In mouse embryos, knockout of *plexinD1* also results in intersomitic vessel patterning defects [34••,35••]. PlexinD1-deficient mouse mutants show exuberant branching of intersomitic blood vessels and loss of the normal segmented blood vessel pattern. This misguidance was initially proposed to result from disrupted Sema3A–plexinD1 repulsion. Given the expression of *sema3a1*, *sema3a2* and *semaZ8* within the zebrafish somites [33••], a possible ligand for plexinD1-induced repulsive guidance of intersomitic blood vessels is Sema3A. However, the results on Sema3A are controversial; whereas morpholino-mediated knockdown of *sema3a1* or *sema3a2* resulted in vascular defects that did not phenocopy the *plexinD1* mutation [33••,36], local overexpression of *sema3a2* in zebrafish embryos inhibited ISV extension adjacent to the overexpressing cells [33••]. In mouse embryos, *Sema3A* mutations lead to subtle vascular defects [27•] that are not observed on all genetic backgrounds. However, because neither Sema3A nor Sema3C bind plexinD1 directly [34••], and Sema binding to Nrp-1 is not necessary for vascular development in mice [21••], the endogenous ligand mediating repulsive guidance might be another Sema.

A very recent study has identified Sema3E as the ligand involved in plexinD1 signaling [35••]. Sema3E is expressed in the caudal region of the somite, immediately adjacent to the intersomitic blood vessels expressing plexinD1. *Sema3E* and *plexinD1* mouse mutant embryos exhibit highly similar vascular phenotypes [35••]. Interestingly, this phenotype does not depend on Nrps; double-mutant mice deficient in *Nrp-2* and in the Nrp-1 Sema3 binding site show normal segmental blood vessel patterns. *In vitro* cell binding- and collapse-assays showed that Sema3E directly signals through plexinD1, independently of the presence of Nrps. Blood vessels avoid chick embryo somites that overexpress *Sema3E*, suggesting that this molecule mediates endothelial cell repulsion. Thus, Sema3E directly signals through plexinD1 to restrict blood vessel growth to the intersomitic boundaries [35••]. Taken together, the data support a model in which Sema3E–plexinD1 exerts a repulsive action on endothelial tip cells, whereas VEGF–VEGFR-2 signaling promotes attraction and VEGF–Nrp-1 signaling regulates as yet ill-defined aspects of branching (Figures 2, 3).

Sema3E homozygous mutant animals are viable and fertile, suggesting that they develop a functional vasculature despite their embryonic vessel patterning defects. Early patterning defects might thus be corrected as the vasculature becomes perfused. Plexin D1 homozygous mutants develop to term, but die shortly after birth because of deficiencies in the patterning of the outflow tract of the heart. Interestingly, patterning defects of the outflow tract are observed in knockouts of other members of this signaling complex [14••,20,21••,34••,37,38]. Correct patterning of the cardiac outflow tract was proposed to require the combined action of VEGF165 signaling through VEGFR-2–Nrp-1 complexes and of Sema3A and -3C signaling through complexes of plexinD1 and Nrp-1 and -2, respectively [34••]. Sema6D, signaling through plexinA1–VEGFR-2 receptor complexes, also contributes to this process [39].

Repulsive signaling regulates intersegmental vessel formation: don't stray into the somite

After having entered the intersegmental boundary, ISVs must be prevented from going into the somitic tissue. Additional guidance factors, including Ephrin–Eph, Slit–Robo and Netrin–Netrin receptors are candidate signaling pathways to mediate this repulsive guidance. EphrinB2 is expressed in the caudal region of somites and creates a repulsive corridor for neural crest cells [40]. In *Xenopus*, ISVs express the EphrinB2 receptor EphB4, and disruption of EphrinB2–EphB4 interaction leads to aberrant ISV invasion into somitic tissue [41]. In mouse, EphrinB2 is expressed in embryonic arteries, including intersomitic arteries, whereas its receptor EphB4 is expressed in veins [42–44]. In mouse mutants, aberrant growth of ISV into somites has been observed in some ephrinB2 mutant strains [43], but not in others [45]. Mouse knockout experiments suggest that Ephrin–Eph signaling appears to be involved in boundary formation between arteries and veins, restricting inappropriate mixing [42–46].

The role of the Slit family and their Roundabout (Robo) receptors in vascular guidance remains to be clarified. In the nervous system, Slits act as chemorepellents, preventing ipsilateral axons from crossing the midline and commissural axons from re-crossing it [6]. In mammals, three Slit family members, that is, Slit-1, -2 and -3, are expressed in the nervous system midline. Four Robo receptors, Robo-1 to -4, are known in mammals, with Robo-4 (also referred to as Magic Roundabout) [47] being structurally divergent from the other proteins. Robo4 is expressed in developing ISVs in mouse embryos and human microvascular endothelial cells (HMVECs). Exposure of HMVEC to Slit-2 inhibited endothelial migration (Figure 2; [48]). Conversely, exposure of human umbilical vein endothelial cells (HUVECs), which express Robo1, to Slit-2 stimulates endothelial cell chemotaxis *in vitro* and tumor angiogenesis *in vivo* (Figure 2; [49]). Robo-1 knockout mice are viable and fertile, and vascular defects have (thus far) not been reported [50]. Robo-4 knockout mice have not been reported yet.

Genetic evidence of a role for Netrins in vessel guidance has been provided recently. Netrins are laminin-related secreted bifunctional guidance cues, attracting some axons and repelling others. Attraction and repulsion are mediated by binding to deleted in colorectal cancer (DCC) and uncoordinated 5 (UNC5) family receptors, respectively [6,51]. Lu *et al.* [52••] have recently

demonstrated that among the Netrin receptors, the UNC5B receptor is selectively expressed in the vascular system by arteries, a subset of capillaries and endothelial tip cells. Inactivation of the *Unc5b* gene in mice led to increased capillary branching and embryonic lethality. Treatment of endothelial cells with the ligand Netrin-1 resulted in tip cell filopodial retraction, and this effect was abolished in *Unc5b*-deficient mice, suggesting that Netrin-1 mediates repulsive guidance of capillary tip cells through UNC5B signaling (Figures 2 and 3). Morpholino knockdown of the zebrafish ortholog of *Unc5b* or its ligand *Netrin-1a* led to aberrant pathfinding of ISV. Interestingly, the phenotype appeared strikingly different from the *obd* phenotype. ISV sprouting into the intersegmental space and initial dorsal migration were unaffected. Aberrant pathfinding occurred at the level of the horizontal myoseptum (which normally expresses Netrin-1a), where instead of extending dorsally, ISVs in both *Netrin-1a* and *Unc5b* morphants mainly deviated laterally. As a result, capillary branching was increased and ISVs were misguided, phenocopying the mouse mutation [52••]. These results suggest that different repulsive cues, including Semaphorins and Netrins, might act at specific guide-posts for developing vessels (Figure 3), much like axon growth to a distant target is regulated by intermediate guide posts.

Conclusions

Insight from studies on axon guidance is rapidly increasing our understanding of the molecular biology of vessel guidance. It is now clear that at least some of the molecular mechanisms are conserved in both systems. Studies on vessel guidance also hold some promise for neuroscientists, as the repertoire of guidance molecules expressed on vessels appears reduced compared with that of neurons. Future challenges in vascular biology include the identification of the full panoply of guidance molecules involved in directing growing vessels, as well as the downstream signaling mechanisms operating in endothelial tip cells. Axonal growth cone motility is ultimately mediated by cytoskeletal changes in actin filaments and microtubules, both have been shown to mediate downstream effects of cell-surface guidance receptors in the nervous, but not in the vascular, system. Moreover, it will be very interesting to determine whether the guidance cues are also involved in instructing congruent development of vessels and nerves. Finally, there is hope for the future that our knowledge will expand to be of use therapeutically to guide vessels and nerves in the many diseases that affect both systems.

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