Common Factors Regulating Patterning of the Nervous and Vascular Systems*

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Abstract

The vascular and the nervous systems of vertebrates share many features with similar and often overlapping anatomy. The parallels between these two systems extend to the molecular level, where recent work has identified ever-increasing similarities between the molecular mechanisms employed in the specification, differentiation, and patterning of both systems. This review discusses some of the most recent literature on this subject, with particular emphasis on the roles that the Ephrin, Semaphorin, Netrin, and Slit signaling pathways play in vascular development.

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INTRODUCTION

The nervous and vascular systems are complex, exquisitely patterned networks that show striking similarities upon even casual observation. Both are highly branched, ramified networks extending into nearly every portion of the animal. Each is composed of largely separate efferent and afferent networks (i.e., motor and sensory nerves in the nervous system, arteries and veins in the vasculature). In many cases both systems not only are similar in their anatomical structure and form but also follow the same paths. Major vessels and nerves frequently coalign with one another (**Figure 1**), as noted for peripheral nerves and vessels in developing avian or murine skin and forelimb (Bates et al. 2003, Martin & Lewis 1989, Mukouyama et al. 2002). Both systems also show a remarkable reproducibility and conservation in their overall anatomical architecture. Furthermore, the similarities between the nervous and vascular systems extend to the molecular mechanisms important for their specification, differentiation, and patterning.

The genetic and molecular basis for the assembly and patterning of the nervous system has been extensively studied (Dickson 2002). Many proneural and neurotrophic factors have been uncovered that help direct the specification, differentiation, proliferation, and maintenance of neural and glial progenitors. The patterning of the nervous system is guided by a variety of transmembrane or secreted ligands expressed by surrounding tissues that provide cues guiding the navigation of migrating nerve axons (**Figure 2**). Ligand-receptor pairs implicated in axonal guidance include ephrins and their Eph receptors, semaphorins and their plexin and neuropilin (Nrp) receptors, netrins and their Deleted in Colorectal Cancer (DCC)/Neogenin and Uncoordinated-5 (UNC-5) receptors, and slit ligands and their roundabout (Robo) receptors. Most of these ligands act either to repel or to attract the growth cones at the tips of growing axons to steer them through other tissues toward their final targets. The ligands signal though specific receptors expressed on the growth cone surface (**Figures 2** and **3**).

In contrast to the nervous system, we still know comparatively little about the molecular mechanisms directing the assembly and patterning of blood vessels. As noted above, there is strong evidence for coordination between the vascular and nervous systems, with frequent close physical association between larger nerves and vessels. In some cases this coordination is achieved by signals directly from one tissue to the other. In other cases the coordination between nerves and vessels is achieved by each system separately utilizing the same cues and signals. In this review we explore some of the common mechanisms underlying the similarities between these two essential organ systems.

CROSS-TALK BETWEEN FACTORS PROMOTING VESSEL AND NERVE GROWTH AND MAINTENANCE

There is not only a close anatomical juxtaposition between the nervous system and the vascular system but also a strong functional coordination and interdependence. For example,

neuronal activity strongly influences local blood flow in the brain, and the extent of cerebral perfusion after stroke correlates strongly with the survival of neural tissue. Below we discuss some of the well-known neurotrophic factors that have been shown to have proangiogenic roles.

Neurotrophins are important in the nervous system, regulating cell survival, differentiation, and death as well as neurotransmitter release, synaptic strength, and connectivity (reviewed in Chao 2000). The neurotrophin family includes four related family members: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4). The neurotrophins signal through several neurotrophin-selective tropomyosin-receptor-kinase (Trk) receptors together with the pan-neurotrophin-binding receptor (p75NTR). Recent evidence has shown that these well-known neurotrophic factors have angiogenic effects in addition to their well-documented and important roles in the nervous system.

The "canonical" neurotrophin NGF has been shown also to have vascular effects (reviewed in Nico et al. 2008). Endothelial cells (EC) in culture express both the NGF TrkA receptor and NTR, and administration of NGF in vitro promotes endothelial proliferation and migration. In vivo, exogenous NGF induces a dose-dependent angiogenic response in rat cornea as well as the chick embryo and quail embryo chorioallantoic membrane assays, and NGF and TrkA are strongly upregulated in experimentally induced ischemic muscle. Although there are still few loss-of-function data available to demonstrate a critical functional role for NGF during vascular development, these and other data suggest that neuronal NGF contributes to control of both neuronal differentiation and angiogenesis. It is still unclear whether most of its angiogenic effects are direct (i.e., NGF signals received directly by ECs) or indirect (via induction of other angiogenic factors by NGF-stimulated neurons or other cell types, and/or via recruitment of proangiogenic cells).

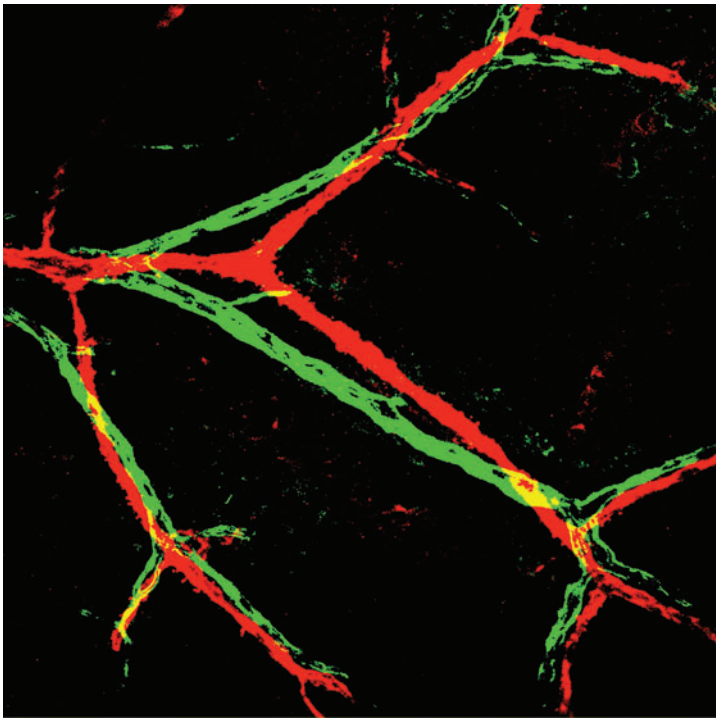

Figure 1

Coalignment of nerves and vessels in mouse skin. Whole-mount double immunofluorescence confocal image of limb skin in E15.5 *ephrinB2taulacZ/+* heterozygous embryos. Arteries (*red*, ephrinB2taulacZ) are aligned with peripheral sensory nerves (*green*, neurofilament) and follow their branching pattern in embryonic limb skin. Image courtesy of Yosuke Mukuoyama, NHLBI, NIH.

A vascular role has also been reported for the neurotrophic factor BDNF and its TrkB receptor (reviewed in Kermani & Hempstead 2007). The endothelium of arteries and cardiac and skeletal muscle vessels expresses both BDNF and TrkB, and BDNF knockout mice show impaired survival of TrkB-expressing ECs in intramyocardial arteries and capillaries in late gestation and early postnatal mice, although the vasculature of the heart does form initially during embryogenesis (Donovan et al. 2000). Mice with targeted disruption of the *TrkB* gene also show reduced numbers of intramyocardial blood vessels. Conversely, overexpression of BDNF in the developing murine heart results in increased numbers of cardiac capillaries (Wagner et al. 2005). The p75NTR receptor may have an important role in pericyte or

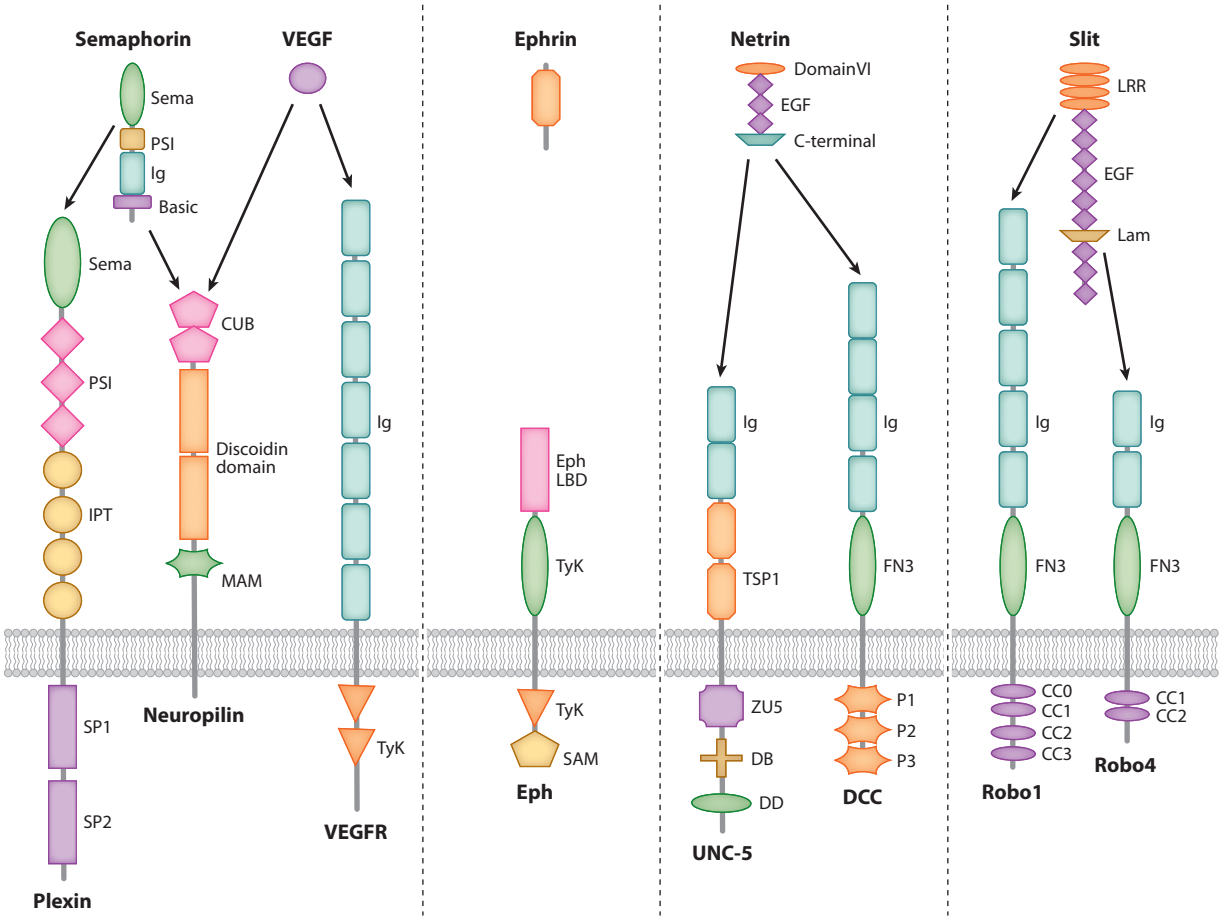

Figure 2

Receptors and ligands known to steer axons and blood vessel sprouts during development. Membrane-bound (Sema and Ephrin) and soluble [Vascular endothelial growth factor (VEGF), Netrin, and Slit] ligands are depicted on top. Membrane-bound receptors Plexin, Neuropilin, Vascular endothelial growth factor receptor (VEGFR), Eph, Uncoordinated-5 (UNC-5), Deleted in Colorectal Cancer (DCC), and Roundabout (Robo) 1 and 4 are depicted on the bottom. Conserved domains are shown: CC, Robo-conserved domain; CUB, complement-binding domain; DB, Dcc-binding domain; DD, death domain; Eph LBD, Ephrin ligand-binding domain; FN3, fibronectin type 3-like; Ig, immunoglobulin domain; IPT, Ig-like fold/plexin; Lam, laminin domain; LRR, leucine-rich repeat; MAM, meprin/A5 protein/phosphatase-μ-related; P1/2/3, Dcc conserved domains; PSI, Plexin-Semaphorin-Integrin; SP1/2, serine/threonine protein kinase catalytic domain; TSP1, thrombospondin-1-like; TyK, tyrosine kinase; ZU5, zona occludens-1-like.

smooth muscle recruitment and survival. Mice with a truncated NTR allele have enlarged, leaky aortae with defective smooth muscle investment, although other data suggest this allele might be a gain-of-function mutation (Paul et al. 2004, von Schack et al. 2001). Smooth muscle cells (SMCs) also undergo neurotrophin- and NTR-dependent apoptosis in atherosclerotic plaques and after vascular injury (Kraemer 2002, Wang et al. 2000). The signaling pathways downstream of neurotrophins in ECs are not clear at this point, although the PI3K/AKT and MEK/ERK pathways have been implicated.

Other neuropeptides have been implicated in the regulation of vessel growth and survival as well, including the neuropeptide secretoneurin (reviewed in Fischer-Colbrie et al. 2005).

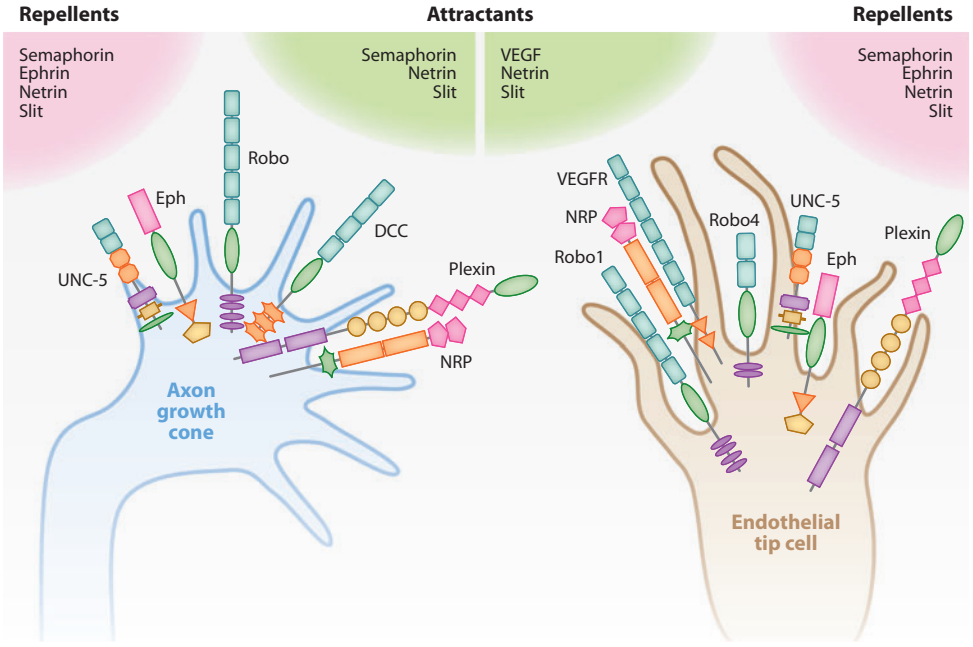

Figure 3

Similar ligand-receptor pairs help guide axonal growth cones and endothelial tip cells. Eph-Ephrin signaling is mostly repulsive for both growth cone and endothelial tip cell path finding. Semaphorins act mostly as repellent cues in growth cones and also in endothelial tip cells. Netrins can be both attractants and repellents depending on the cellular context and on the receptor to which they bind. DCC, Deleted in Colorectal Cancer; NRP, Neuropilin; Robo, Roundabout; UNC-5, Uncoordinated-5; VEGF, Vascular endothelial growth factor; VEGFR, Vascular endothelial growth factor receptor.

Secretoneurin promotes neurite outgrowth in the cerebellum and has been implicated in neuronal inflammation and/or neuroprotection. Secretoneurin is highly expressed in nerve fibers closely apposed to blood vessels, and recent evidence has shown that it also acts as a potent angiogenic cytokine, stimulating migration, proliferation, and tube formation in ECs in vitro and neovascularization in the cornea in vivo. Furthermore, secretoneurin is upregulated in ischemic brain tissue and promotes survival and growth of both neurons and vessels when administered in a rat experimental stroke model (Shyu et al. 2008). Secretoneurin is also upregulated in a HIF-1α-dependent manner in experimentally induced ischemic muscle, and antisecretoneurin-blocking antibodies inhibit angiogenesis in this model (Egger et al. 2007). As for the neurotrophins described above, however, it is not clear how many of the in vivo proangiogenic and neuroprotective effects are due to the direct effects of secretoneurin on ECs as opposed to the recruitment of other proangiogenic cells and/or factors, or direct effects on neurons.

COMMON FACTORS DIRECTING PATTERNING OF THE NERVOUS AND VASCULAR SYSTEMS

In the nervous system, patterning of nerve tracts is accomplished by a variety of cues and signals from the nearby environment directing axon navigation. Four major classes of axonal guidance molecules have been identified: Ephrins, Semaphorins, Netrins, and Slits (**Figure 2**). These molecules mediate either repulsive or attractive interactions with migrating growth

cones via class-specific receptors expressed on the growth cone surface (**Figures 2** and **3**). As described in detail below, recent studies have provided conclusive evidence that these same guidance factors also play a role in guiding the growth and patterning of the developing vasculature.

The Eph and Ephrin Proteins

The Eph-ephrin system has been shown to have important roles in regulating axonal guidance, tissue boundaries, and topographic mapping of neural connections in the nervous system (reviewed extensively in Poliakov et al. 2004). The Eph receptor tyrosine kinases are a large family of transmembrane receptors activated by members of an equally large family of cell surface–bound ligands, the ephrins. There are two subclasses of ephrin proteins. The ephrin-A subclass proteins are attached to the cell surface via a glycosylphosphatidylinositol anchor, whereas ephrin-B ligands possess a transmembrane domain. The Eph receptors are also divided into EphA and EphB receptor subclasses on the basis of their ephrin class binding preference. Eph-ephrin binding appears to be fairly promiscuous within subclasses, with some limited interaction also reported between the subclasses. In addition to their ability to bind to and activate their cognate Eph receptor tyrosine kinases (forward signaling), ephrins also can transduce signals themselves via reverse signaling. The signaling pathways downstream from both forward and reverse ephrin-Eph signaling are complex and still not well understood in many cases, and they regulate several different cellular responses.

Forward signaling in most contexts leads to repulsion of Eph-expressing cells (Poliakov et al. 2004). Subsets of migrating neural crest cells or the growth cones of migrating axons that express particular Eph receptors retract away from tissues with which they come into contact that express their cognate ephrins. Similarly, ephrin-Eph signaling can help to separate cell populations and tissues and to establish tissue boundaries. However, forward signaling can also promote adhesion or proliferation in some circumstances, particularly in the presence of low concentrations of ephrin ligand. Indeed, the differential responses of Eph-expressing cells to low versus high concentrations of ephrins may be important in helping to simultaneously promote both interaction and separation between different tissues and/or cell types; this is likely the case for arterial and venous endothelial populations, as described below. Whereas adhesive interactions appear to require binding but not signaling, repulsive interactions require signaling through the Eph receptor (Holmberg et al. 2000). The switch from adhesion to repulsion can involve metalloproteinase cleavage (Hattori et al. 2000, Janes et al. 2005) and/or endocytic internalization of the Eph receptor (Marston et al. 2003, Zimmer et al. 2003), both activated by ephrin ligand binding. Although Eph-ephrin signaling functions primarily in the context of cell-to-cell surface *trans*-interactions, recent studies suggest that Ephs and ephrins can also interact in *cis*. For example, ephrin-A5 binds in *cis* to the extracellular fibronectin type III-like domains in EphA3, blocking forward signaling by *trans*-receptor activation (Carvalho et al. 2006). Furthermore, as described in more detail below, ephrinB2 appears to have contact-independent functions in SMCs (Foo et al. 2006).

Eph-ephrin signaling in the vasculature. Some of the first evidence for Eph-ephrin function in the vasculature came from studies indicating an important role in arterial-venous differentiation. Although the existence of two separate and functionally distinct networks of arterial and venous blood vessels has been appreciated for thousands of years, only in the past several decades has evidence shown that arterial and venous ECs are functionally and molecularly distinct cell types. One of the first such molecular distinctions to be uncovered was that ephrinB2 and EphB4 are differentially expressed in arterial and venous endothelium, respectively (Wang et al. 1998). EphrinB2 is specifically expressed in arteries but not veins, whereas EphB4 is expressed in veins but

not arteries. The artery-specific expression of ephrinB2 persists into adulthood and is found in even the smallest capillaries midway between terminal arterioles and postcapillary venules, contradicting the classical view that capillaries have neither arterial nor venous identity (Gale et al. 2001, Shin et al. 2001). EphrinB2 is also strongly expressed at sites of adult angiogenesis such as during wound healing, follicular maturation and corpus luteum formation in the ovary, tumor angiogenesis, and experimentally induced VEGF-driven corneal neovascularization, suggesting that these new vessels may possess arterial characteristics. The expression of both ephrinB2 and EphB4 in endothelium is initiated early in murine embryonic development, well before circulatory flow begins, indicating that their initial expression is genetically determined. As described below, however, arterial-venous identity and the later expression of these genes can be modified or directed by hemodynamics and/or local environmental factors.

Targeted disruption of the *ephrinB2* and *EphB4* genes in mice has shown that the differential expression of these genes plays an important role in the development of properly patterned arterial-venous vascular networks. EphrinB2/EphB4 signaling plays a critical role in arterial-venous differentiation, promoting proper arterial-venous distinction, morphogenesis, and angiogenic remodeling of arterial-venous vascular networks (Adams et al. 1999, Gerety et al. 1999, Wang et al. 1998). Mice homozygous for a *tau-LacZ* knock-in allele of *ephrinB2* knockout mice display both arterial and venous defects in angiogenesis of capillary networks in the head and yolk sac as well as defects in myocardial trabeculation (Adams et al. 1999, Wang et al. 1998). Mice with targeted disruption of the *EphB4* gene show a phenotype nearly identical to mice lacking ephrinB2. The symmetrical phenotypes of targeted inactivation of ephrinB2 and EphB4 suggest that these proteins function as a ligand-receptor pair (Gerety et al. 1999). An endothelium-specific knockout of ephrinB2 exhibits the same embryonic lethal phenotype as the complete knockout, indicating that its function is required within the endothelium for angiogenesis (Gerety & Anderson 2002). Interestingly, initial arterial- or venous-restricted expression of *tau-lacZ* knocked in to either the *ephrinB2* or *EphB4* locus is not affected in animals homozygous null for either gene. This indicates that whereas these genes are required for proper functional remodeling of arterial-venous networks, they are not needed for initial arterial-venous endothelial specification. The *ephrinB2* gene is also required in the lymphatic endothelium. EphrinB2 is expressed in ECs of collecting lymphatic vessels, whereas EphB4 is found throughout the lymphatic vasculature. Mutant mice lacking the C-terminal PDZ interaction domain of ephrinB2 (ephrinB2DeltaV mice) exhibit major lymphatic defects, including a failure to remodel their primary lymphatic capillary plexus into a hierarchical vessel network, hyperplasia, and lack of luminal valve formation (Makinen et al. 2005). Furthermore, mice expressing only an ephrinB2 in which the five conserved tyrosine residues in the C-terminal domain have been replaced by phenylalanine to disrupt phosphotyrosine-dependent signaling events (ephrinB2[5F] mice) also display a lymphatic phenotype, although in this case it is relatively mild (Makinen et al. 2005).

Somewhat contradictory and confusing data have been obtained regarding the respective functional roles of forward versus reverse ephrinB2-EphB4 signaling in the blood vasculature. Expression of ephrinB2 full-length and cytoplasmic domain deletion mutants in *Xenopus* results in identical defects in the formation of trunk intersegmental vessels (ISVs), as is observed with dominant negative EphB4 mutants, indicating that remodeling occurs through forward, not reverse, signaling (Helbling et al. 2000). However, in one study mice designed to be specifically impaired in ephrinB2 reverse signaling owing to lack of the entire cytoplasmic domain (a deletion of approximately 85 C-terminal amino acids) exhibited similar vascular remodeling defects to those displayed by mice completely deficient for ephrinB2 or EphB4, suggesting an essential requirement for

bidirectional communication during murine vascular development (Adams et al. 2001). These findings are supported by a recent study showing that increased EphB4 expression in B16 melanoma cells impairs the survival of arterial ECs in mouse tumor xenograft model systems by reverse signaling via ephrinB2 (Huang et al. 2007). However, the results of Adams et al. are contradicted by the Makinen et al. study noted above, which failed to find a blood vascular phenotype in a similar ephrinB2 C-terminal domain truncation mutant. The reason for the discrepancy between these findings is not clear but differences in the precise ephrinB2 deletions generated or in the genetic backgrounds of the targeted mice may be factors. In any case, additional studies will be needed to fully parse out the respective roles of forward and reverse ephrinB2-EphB4 signaling in the vasculature.

Vascular expression of ephrinB2 is also not restricted to the endothelium. Although it is initially present only in the endothelial layer of blood vessels, as development proceeds ephrinB2 expression progressively extends from the arterial endothelium to surrounding SMCs and to pericytes (Gale et al. 2001, Shin et al. 2001). Mural cell–specific inactivation of ephrinB2 results in perinatal lethality, with vascular defects in the skin, lungs, gastrointestinal tract, and kidney glomeruli as well as abnormal migration of SMCs to lymphatic capillaries. Thus, this gene is required in arterial smooth muscle in addition to arterial endothelium (Foo et al. 2006). In vitro, ephrinB2-deficient SMCs are defective in spreading, focal-adhesion formation, and polarized migration and show increased motility. Interestingly, ephrinB2 null cells exhibit this behavior even in low-density cultures in the absence of cell-cell contact, suggesting that ephrinB2 plays a cell-autonomous role in SMCs. It is unclear at this point whether the cell-autonomous effects of ephrinB2 loss reflect a loss of *cis*-interactions with the EphB4 receptor or a more direct ligand-independent role for ephrinB2 signaling.

Although it is clear that the initial acquisition of arterial-venous identity and differential ephrinB2-EphB4 expression in the embryo is genetically determined, recent evidence demonstrates that ECs can be reprogrammed and that there is a high degree of plasticity and hemodynamic and/or environmental control over arterial-venous identity. This issue is of critical medical importance in clinical settings where vessels of different identity are grafted together, such as during dialysis treatment or bypass surgery. Changes in the transplanted vessels after grafting (Cahill et al. 1987, Henderson et al. 1986, Stark et al. 1997, Wallner et al. 1999) and the significant risk of graft failure involved in these therapies (Hoch et al. 1999) do suggest a limited degree of plasticity in EC arterial-venous identity.

Two separate groups performed quail-chick grafting experiments to test the plasticity of arterial-venous EC fate during early development (Moyon et al. 2001, Othman-Hassan et al. 2001). Portions of embryonic arteries or veins were grafted from quail donors at various stages of development into chick hosts, and the arterial-venous identity of donor cells contributing to different host vessels was assessed using artery- or vein-specific molecular markers. Using expression of Nrp-1 and ephrinB2 as arterial markers and Tie2 as a vein-specific probe, both groups found that arterial or venous ECs from young donors can populate both types of vessels in host embryos and assume the appropriate molecular identity in their new locales, but this plasticity becomes progressively lost in ECs grafted from donors older than E7. However, when isolated ECs or dissected endothelial epithelia were grafted from these older donors instead of intact vascular segments, the older ECs were able to colonize both types of vessels as well as younger ECs. These results indicate that initial specification of arterial or venous cell fate is reversible and that additional inputs may influence or be required to maintain a specific arterial-venous identity. It is likely that ephrinB/EphB-mediated communication between arterial and venous cells is important for maintaining arterial-venous identity. This has been demonstrated in patent vein grafts in both humans and aged rats, in which EphB4 transcripts and

immunodetectable proteins are downregulated in ECs and SMCs. This loss of EphB4 is associated with intima-media thickening during vein graft adaptation to the arterial environment. Interestingly, neither ephrinB2 transcripts nor arterial markers such as dll4 and notch are strongly induced (Kudo et al. 2007). Whereas it does not exactly duplicate the genetic regulation of EC determination in embryogenesis, vascular adaptation as viewed through vein grafts in adult mammals maintains an adequate subset of those genes to mediate plasticity.

Although we have focused thus far on ephrin-Eph signaling in arterial-venous differentiation, there is also evidence that such signaling is required for proper morphogenesis of vascular networks. Although the data are limited thus far, some evidence also suggests that ephrin-Eph signaling can regulate guidance and patterning of vessels. In particular, one study in *Xenopus* embryos has reported that ephrin-EphB4 signaling plays a role in the guidance of trunk ISVs (Helbling et al. 2000). Several Ephrin-B ligands are expressed in trunk somitic tissue adjacent to the migratory pathways taken by intersomitic veins during their angiogenic growth. Injection of *Xenopus* embryos with RNAs encoding dominant negative EphB4 receptors or misexpressing ephrin-B ligands to disrupt EphB4 signaling resulted in abnormal growth of intersomitic veins into the adjacent somitic tissue. These results suggest that EphB4 and B-class ephrins might provide repulsive guidance cues to migrating ECs. Proper somite patterning has been shown to be required for ISV guidance in zebrafish, although the specific role of somitic Eph/ephrin in vascular patterning was not studied in this model (Shaw et al. 2006).

As described in the section below, stronger evidence for a specific role in guidance and patterning has been obtained for another family of neuronal guidance molecules, the semaphorins.

Semaphorins and Their Receptors

The semaphorins are a large family of membrane-bound and secreted proteins defined by the presence of a basic amino-terminal 500–amino acid "sema" domain containing 17 conserved cysteines that modulates the proteins' signaling activity. Eight classes of semaphorins have been defined based on their structural features, with classes 3–7 found in vertebrates. Class 1, 4, 5, 6 and 7 semaphorins are transmembrane or glycosylphosphatidylinositol (GPI)-linked proteins, whereas class 2, 3 and 8 (V) semaphorins are secreted (although generally matrix-associated). Semaphorins signal primarily through transmembrane plexin receptors, which function together with a variety of coreceptors, most notably the Nrps, as well as many different accessory proteins and effectors (reviewed in Zhou et al. 2008).

Semaphorins. The role of semaphorins as neuronal guidance factors is well established. The first semaphorin discovered, the transmembrane semaphorin-1a (Sema-1a, originally named Fasciclin IV), was identified in a screen for molecules found in interesting patterns in the developing grasshopper nervous system (Kolodkin et al. 1992). Shortly thereafter, a neuronal growth cone collapsing activity was purified from chicken brain and found to be a secreted semaphorin [Sema3A, originally named "Collapsin" (Luo et al. 1993)]. Although semaphorins function mainly as repulsive guidance factors during axonal pathfinding, semaphorins can also sometimes act as attractive or context-dependent bifunctional factors. At least some transmembrane semaphorins also have the capacity to transduce reverse signals in a fashion analogous to the ephrin reverse signaling described above. In additional to their canonical roles as axon guidance factors, semaphorins have recently been shown to play important functional roles in many other tissues and contexts, including in the vasculature.

The secreted class III semaphorins have been most extensively studied, and it is mainly the members of this subfamily that have been shown to have roles in the vasculature. Type 3 semaphorins inhibit EC motility and survival in vitro, opposing the effects of VEGF. Studies of nerve and vessel patterning in the

developing avian forelimb suggest that both independently employ semaphorin signals for repulsive guidance (Bates et al. 2003). Larger vessels and nerves are spatially juxtaposed in the forelimb, but alterations in the pattern of one do not affect the other. However, Sema3A is expressed in a complementary pattern in both vessels and nerves, and ectopic expression of Sema3A from implanted beads causes hypovascularity and nerve repulsion in adjacent tissue, whereas local inhibition of Sema3A via Sema3A antibody or soluble Nrp-1 soaked beads results in increased local capillary formation. A variety of other reports, too numerous to review here, have confirmed the angiogenic effects of Sema3A and reported angiogenic effects of other semaphorins, including Sema3B, Sema3F, Sema4D, and Sema6D (reviewed in Capparuccia & Tamagnone 2009). Although angiogenic properties have been demonstrated for these and other semaphorins in both in vitro and in vivo assays, in vivo loss-of-function studies in mice and zebrafish generally have not yielded dramatic defects in vascular development, which most likely reflects redundant functions of semaphorins in vivo. However, analysis of semaphorin receptors has clearly demonstrated essential roles for semaphorin signaling in vascular development.

Neuropilins. Some of the first evidence for a vascular role for semaphorins and their receptors came from studies on the Nrps, type 3 semaphorin receptors. Nrp was first identified as a molecular target for a monoclonal antibody (A5) generated against the optic tectum of *Xenopus* tadpoles (Takagi et al. 1987). Based on its expression in neuropil in specific regions of the nervous system, the A5 antigen was renamed Nrp. Nrp-1 (He & Tessier-Lavigne 1997, Kolodkin et al. 1997) and closely related Nrp-2 (Chen et al. 1997) were subsequently shown to be receptors for the semaphorins and to be required for their role in axon pathfinding. In addition to expression in the nervous system, both *Nrp* genes are expressed in the developing vasculature. Nrp-1 and -2 are initially coexpressed in the endothelium, but they become segregated as development proceeds, with Nrp-1 becoming restricted to arteries and Nrp-2 to veins and lymphatic vessels (Herzog et al. 2001, Moyon et al. 2001, Mukouyama et al. 2002, Yuan et al. 2002). The first evidence of a vascular function came from biochemical purification of a new VEGF receptor that revealed that it was identical to Nrp-1 (Soker et al. 1998). Further work showed that Nrp could function as an isoform-specific coreceptor for VEGFR2 on ECs (Gluzman-Poltorak et al. 2000a,b; Soker et al. 1998). Coexpression of Nrp-1 with VEGFR2 in vitro enhances VEGF165 binding to VEGFR2 and promotes VEGF165-mediated migration of ECs (Makinen et al. 1999). The short VEGF121 isoform is not able to bridge the Nrp-1/VEGFR2 complex, although recent work with Nrp function-blocking antibodies shows that VEGF121 does bind Nrp-1 and that Nrp-1 is required for full responsiveness to VEGF121 by an as-yet undetermined mechanism (Pan et al. 2007), possibly involving signaling directly through Nrp (Wang et al. 2006, 2007).

Knockout studies in mice have confirmed that Nrp-1 and Nrp-2 play important roles in both axon guidance and neural development as well as in angiogenesis in vivo. Targeted inactivation of Nrp-1 in mice causes defects in axon pathfinding and disorganization of nerve fibers (Kitsukawa et al. 1997), but Nrp-1 null mice also exhibit vascular defects including impairment in neural vascularization, agenesis and transposition of great vessels, insufficient aorticopulmonary truncus (persistent truncus arteriosus), disorganized and insufficient development of vascular networks in the yolk sac, and abnormal sprouting and growth of hindbrain vessels (Gerhardt et al. 2004, Kawasaki et al. 1999). Mice with endothelium-specific targeted disruption of Nrp-1 also show defects in arterial differentiation (Gu et al. 2003). The vascular morphogenesis phenotypes reflect a direct consequence of Nrp-1 loss on vessels rather than a secondary consequence of disruption of circulatory flow, as shown by a recent reexamination of the Nrp-1 null versus wild-type sibling phenotypes of murine embryos cultured in

vitro (E.A. Jones et al. 2008). Although Nrp-2 null mice were initially reported to have defects only in the nervous system (Chen et al. 2000, Giger et al. 2000), a subsequent study showed that Nrp-2 knockout mice also have vascular defects, although in this case the defects are restricted to the lymphatic vasculature (Yuan et al. 2002). Homozygous Nrp-2 null embryos show the absence or severe reduction of small lymphatic vessels and capillaries with reduced proliferation of lymphatic ECs. The specific requirement for Nrp-2 function during development appears to be confined to lymphatic capillaries because arteries, veins, and larger, collecting lymphatic vessels develop normally. Generation of compound mutant animals with much more severe vascular defects has shown that Nrp-1 and Nrp-2 are at least partially functionally redundant during early development (Takashima et al. 2002). Mice homozygous null for both Nrp-1 and Nrp-2 die in utero at E8.5 with totally avascular yolk sacs. Mice homozygous null for Nrp-2 but heterozygous for Nrp-1, or homozygous null for Nrp-1 but heterozygous for Nrp-2, are also embryonic lethal, but they survive to E10–E10.5. Although these embryos are not completely avascular, they possess highly abnormal vessels in both the yolk sac and embryo proper, including less dense, less branched, and less connected vascular networks with avascular spaces and hemorrhage. The vascular role of these proteins has also been examined in zebrafish, which have four Nrps, Nrp-1a, Nrp-1b, Nrp-2a, and Nrp-2b, expressed in the vasculature (Bovenkamp et al. 2004, Lee et al. 2002, Martyn & Schulte-Merker 2004, Yu et al. 2004). Knocking down individual *Nrp* genes in zebrafish by injecting morpholine antisense oligonucleotides ("morpholinos") targeting these genes causes vascular defects. However, the defects resulting from knockdown of any one zebrafish Nrp are less severe than in the mouse knockouts, and single or multiple morpholino knockdown also causes pleiotropic morphological and other defects that complicate interpretation of vascular phenotypes and the degree to which these reflect endothelium-autonomous Nrp functions.

Early in vitro studies suggested that semaphorins and VEGF family members compete for Nrp binding, with VEGF promoting and Sema3A inhibiting endothelial sprouting and migration (Miao et al. 1999). Experiments using a neuroectodermal progenitor cell line in vitro supported this idea, although these experiments implicated the VEGFR1 rather than VEGFR2 receptor for regulating migration, survival, and proliferation (Bagnard et al. 2001). Sema3A selectively interferes with VEGF-induced angiogenesis in vitro and in vivo, although interestingly Sema3A appears to promote vascular permeability (Acevedo et al. 2008). However, blood vessels develop normally in mice lacking Sema3A or expressing an Nrp-1 isoform incapable of binding Sema3A, although axon patterning of limb nerves is defective (Gu et al. 2003, Vieira et al. 2007). Conversely, VEGF164 is required for proper limb vascular patterning but does not have an effect on limb nerves (Vieira et al. 2007), and arteriogenesis in the skin also requires Nrp-1-mediated positive feedback of VEGF signaling (Mukouyama et al. 2005). These and other data suggest that Nrp-1 is required independently for neural responses to Sema3A and vascular responses to VEGF164 in the limb and elsewhere. Interestingly, the facial nerve is regulated by both VEGF164 and Sema3A binding to Nrp-1, but in this case the two ligands are independently regulating the behavior of the cell bodies and axons of the nerve cells, respectively (Schwarz et al. 2004). Structural and biochemical studies have shown that semaphorin and VEGF likely bind to separate domains of Nrp-1 (Appleton et al. 2007) and that they induce Nrp endocytosis via distinct pathways (Salikhova et al. 2008), supporting the idea that they do not directly compete for Nrp-1 binding. Furthermore, Nrp function-blocking antibodies with distinct effects have been generated that selectively prevent Nrp-1 or Nrp-2 binding to either VEGF or semaphorin proteins (Liang et al. 2007). As discussed in more detail below, recent evidence also suggests that the endothelium responds to at least some semaphorin ligands in an Nrp-independent fashion.

In addition to their roles as coreceptors for semaphorin and VEGF ligands, there is evidence that Nrps can partner with additional signaling proteins in different cell types (reviewed in Uniewicz & Fernig 2008). In ECs, Nrps also act as coreceptors for hepatocyte growth factor (HGF) (Sulpice et al. 2008), fibroblast growth factor (FGF) (Janes et al. 2005), and placenta growth factor (PlGF) (Migdal et al. 1998), which suggests that Nrps may also help to mediate cellular responses to these ligands. Association with other proteins also appears to be necessary for Nrp-VEGFR2 complex formation and/or signaling. The PDZ domain in the cytoplasmic tail of Nrp is bound by Synectin, also called GIPC or Nrp-interacting protein (NIP), and this binding facilitates interaction of Nrp-1 with VEGFR2 (Prahst et al. 2008, Wang et al. 2006). Targeted deletion or knockdown of Synectin in mice or zebrafish causes defects in vascular morphogenesis and endothelial migration (Chittenden et al. 2006). Nrp-1 association with a novel ligand, Galectin-1, has also been reported to promote VEGFR2 signaling (Hsieh et al. 2008).

Plexins. Nrps are important for vascular and neural VEGF- and semaphorin-dependent signaling, but most evidence indicates that transduction of semaphorin signals, especially that of type 3 semaphorins, occurs through plexin proteins (Raper 2000). A variety of reports have suggested a vascular role for the plexin-B family of receptors. Sema4D is angiogenic in vitro and in vivo, and its effects are mediated by its high-affinity receptor, PlexinB1. Some reports have suggested that the effects elicited by PlexinB1 require coupling and activation of the Met tyrosine kinase (Conrotto et al. 2005), whereas others suggest that they are Met-independent (Basile et al. 2004), utilizing RhoA and Rho kinase to promote the integrin-dependent activation of Akt and ERK as well as EC motility (Basile et al. 2007). Sema4D knockdown reduces the size and vascularity of Sema4D-expressing tumor xenografts; tumor-associated macrophages are apparently the main cells producing Sema4D (Basile et al. 2004, Sierra et al. 2008). However, PlexinB1 knockout mice are viable and fertile, with few if any defects, and they can mount a normal angiogenic response to at least some orthotopically implanted tumors (Fazzari et al. 2007), suggesting that the in vivo function of PlexinB1 is redundant.

Vascular roles have also been ascribed to other B-type plexins such as PlexinB3 (Artigiani et al. 2004) as well as A-type plexins, but some of the best evidence for a role for semaphorin signaling in repulsive guidance of vessel patterning in vivo has come from recent studies on the PlexinD1 receptor. PlexinD1 is required for proper guidance and patterning of growing angiogenic vessels in both mice and zebrafish. PlexinD1 is a divergent member of the plexin receptor family expressed in vascular ECs, in addition to its expression in the central nervous system (van der Zwaag et al. 2002). During early murine and zebrafish development PlexinD1 expression is restricted to the vasculature (Gitler et al. 2004, Gu et al. 2004, Torres-Vazquez et al. 2004). Loss-of-function experiments carried out in developing mice and zebrafish show that this receptor is required for guidance and patterning of trunk ISVs. ISV network formation has been described in detail in the zebrafish using two-photon time-lapse imaging of transgenic animals expressing green fluorescent protein (GFP) in endothelium (Isogai et al. 2003). As ISVs grow along the somite boundaries they extend (and retract) numerous long filopodia in all directions, although the net growth of the vascular tip tracks fairly closely along the vertical myoseptum. The tips of these growing angiogenic sprouts are highly reminiscent of neuronal growth cones in both appearance and behavior. ISV sprouts grow closely along the boundaries between somites before interconnecting dorsally (Isogai et al. 2003). Loss of PlexinD1 function in mice (Gitler et al. 2004, Gu et al. 2004) or zebrafish (Torres-Vazquez et al. 2004) causes dramatic mispatterning of these vessels, which sprout and grow without regard

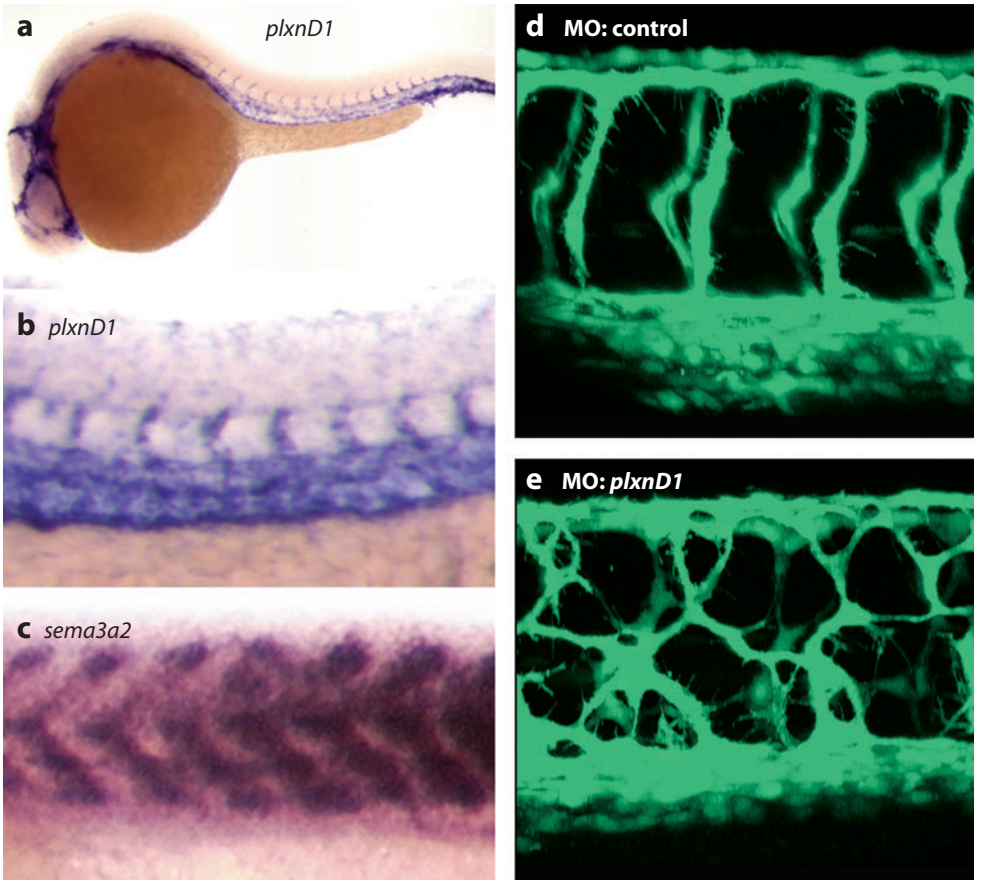

Figure 4

Semaphorin-PlexinD1 signaling directs the patterning of growing intersegmental vessels (ISVs) in the zebrafish trunk. In situ hybridization of 27 hours postfertilization (hpf) zebrafish shows complementary expression of *plxnD1* in the vasculature (*a*,*b*) and *sema3a2* in the medial region of developing somites (*c*). The *plxnD1*-expressing ISV sprouts grow along the *sema3a2*-free corridor along the intersomitic boundaries. (*d*,*e*) Confocal imaging of trunk vessels in control (*d*) or *plxnD1* (*e*) morpholino (MO)-injected 48 hpf *TG(fli1:EGFP)*^{y1} embryos. In control embryos (*d*) the ISVs track closely along intersomitic boundaries, but in animals lacking PlexinD1 function (*e*), vessels are able to grow into the semaphorin-rich central regions of the somites. Images are from Torres-Vazquez et al. (2004).

to intersomitic boundaries (**Figure 4**). Type 3 semaphorins are expressed in zebrafish somites in a complementary fashion to growing vessels (Shaw et al. 2006), and reducing individual trunk semaphorins in zebrafish also causes vascular defects. Furthermore, *sema3a2* misexpression inhibits the growth of ISVs in zebrafish and sema3A reduces EC migration in vitro, both in a PlexinD1-dependent manner (Gitler et al. 2004, Torres-Vazquez et al. 2004). These results suggest that somitic semaphorins act as repulsive guidance cues for growing ISVs expressing PlexinD1, guiding their growth along intersomitic boundaries.

As noted above, subsequent studies have suggested that, at least in the mouse, a different type 3 semaphorin expressed in the trunk, Sema3E, may signal through PlexinD1 independently of Nrps (Gu et al. 2004). Sema3E

is expressed in somites and binds to ISVs in vivo. Unlike other type 3 semaphorins, Sema3E binds PlexinD1-expressing cells in vitro in the absence of Nrp, promoting their collapse. Knockout of Sema3E gives a trunk vascular phenotype reminiscent of PlexinD1 nulls, whereas mice doubly homozygous for *Nrp1*$^{Sema-}$ and *Nrp2* null (retaining VEGF-Nrp-1 signaling but presumably lacking Sema-Nrp signaling) form apparently normal ISVs. However, Sema3E knockout mice are viable and fertile (Gu et al. 2004), unlike PlexinD1 null mice, which die shortly after birth (Gitler et al. 2004), which suggests that Nrp-independent Sema3E is not the exclusive ligand for PlexinD1 in mice. In vitro, Sema3A binds efficiently to PlexinD1 (Gitler et al. 2004) and PlexinD1 is required for EC migration in response to Sema3A, supporting the idea that PlexinD1 is an important signaling receptor for Sema3A in endothelium. Recent reexamination of the phenotypes of PlexinD1 knockout mice (Kanda et al. 2007) and description of newly generated mice with an endothelium-specific knockout of PlexinD1 (Zhang et al. 2009) have shown that these mice have skeletal abnormalities that strongly resemble those found in Sema3A null mice (Behar et al. 1996). These results suggest that the skeletal abnormalities are secondary to vascular defects and that Sema3A signaling via PlexinD1 is critical for this. Interestingly, a report on PlexinD1 signaling in the nervous system suggests that PlexinD1 can utilize both Nrp-dependent and Nrp-independent modes of signaling (Chauvet et al. 2007). On corticofugal and striatonigral neurons expressing PlexinD1 but not Nrp-1, Sema3E acts as a repellent. In contrast, on subiculo-mammillary neurons coexpressing PlexinD1 and Nrp-1, Sema3E acts as an attractant. Thus, Nrp-1 switches the activity of PlexinD1 from repulsion to attraction. PlexinD1 also appears to bind to other semaphorin ligands, including Sema4A (Toyofuku et al. 2007). As recent data from the zebrafish suggest (Lamont et al. 2009), ECs likely respond to, and integrate, inputs from multiple semaphorin-plexin signals, each of which may play a distinctive role.

Netrins and Their Receptors

The netrins are secreted molecules with homology to laminin γ- or β-chains that guide neuronal axon and migrating cells. They were first identified in genetic screens for motility mutants in nematodes (Hedgecock et al. 1990, Ishii et al. 1992). Vertebrate netrin isolated from brain extracts was subsequently found to promote outgrowth of axons from spinal cord explants (Kennedy et al. 1994, Serafini et al. 1994). Mammals have three netrins, Netrin-1, -3 (displaying homology to the γ-chain of laminin), and -4 (β-Netrin, displaying homology to the β-chain of laminin). Netrin-1 binds to two main families of transmembrane receptors, the DCC/Neogenin family (with two members, DCC and Neogenin) and the UNC-5 family (with four mammalian members: UNC-5A, -B, -C, and -D). Unlike many other axonal guidance factors, Netrin-1 has been conclusively shown to be able to act as both an attractant and a repulsive cue. Studies in nematodes, flies, and mice suggest that the DCC receptor mediates attractive responses to netrins (Chan et al. 1996, Fazeli et al. 1997, Kolodziej et al. 1996), whereas UNC-5 receptors are responsible for their repulsive effects (Ackerman et al. 1997, Hedgecock et al. 1990, Keleman & Dickson 2001, Przyborski et al. 1998). Studies in *Xenopus* have reported that the different receptors can also interact to coordinate differential responses to netrin ligand, with the netrin-mediated association between the DCC and UNC-5 cytoplasmic domains converting the attractive responses of the DCC receptor to repulsive responses (Hong et al. 1999). It is perhaps this duality in the function of netrin signaling that has led to recent controversy over its role in the vasculature.

One series of studies has provided convincing evidence for an antiangiogenic role for netrins (Bouvree et al. 2008, Larrivee et al. 2007, Lu et al. 2004). The Netrin-1 receptor UNC-5B is expressed in the endothelium of developing murine and avian blood vessels. The expression of UNC-5B is enriched on arteries and sprouting tip cells. Targeted inactivation of

the *UNC-5* gene in mice leads to aberrant extension of endothelial tip cell filopodia, excessive vessel branching, and abnormal navigation (Lu et al. 2004) Similarly, morpholino knockdown of zebrafish *unc-5b* was reported to cause aberrant vessel branching. Netrin-1 applied to ECs in vitro causes filopodia retraction, but only when UNC-5B is present. Follow-up studies reported that Netrin-1 inhibits angiogenesis in an UNC-5B-dependent manner in a variety of experimental settings, including matrigel implants, aortic ring assays, 3D endothelial culture, and implanted tumors (Larrivee et al. 2007). UNC-5B is minimally expressed in the quiescent adult vasculature but upregulated at sites of neoangiogenesis, suggesting the potential usefulness of Netrin-1 as an antiangiogenic agent for use in adults (Bouvree et al. 2008, Larrivee et al. 2007). Netrin-1 was also shown to have antiangiogenic effects in avian embryos by grafting cell lines expressing recombinant chick or human Netrin-1 into avian embryos at different stages of development and showing that Netrin-1-expressing cells appeared to inhibit angiogenesis at all stages examined (Bouvree et al. 2008). Netrin-4 has also been reported to inhibit angiogenesis in vitro via binding to Neogenin and recruitment of UNC-5B (Lejmi et al. 2008). Netrin-4 is overexpressed in VEGF-stimulated ECs, and Netrin-4 knockdown increases the ability of these cells to form tubular structures in matrigel. Netrin-4 binds to Neogenin and promotes association between UNC-5B and Neogenin, and silencing of either receptor abolishes the inhibitory effect of Netrin-4 on EC migration. Netrin-4 also delays tumor angiogenesis in a tumor xenograft model, suggesting that it can act as an antiangiogenic factor in vivo through its binding to Neogenin and recruitment of UNC-5B.

The reports described above have provided strong evidence for an antiangiogenic role for netrins, but another set of compelling studies has suggested that netrins display a proangiogenic role. In one such report, Netrin-1 was shown to stimulate proliferation and migration of vascular ECs (VECs) and vascular smooth muscle cells (VSMCs) as well as promote VMSC adhesion in vitro and angiogenesis in the chorioallantoic membrane and corneal micropocket assays in vivo (Park et al. 2004). Neogenin is expressed in VSMCs, and Neogenin-blocking antibodies inhibited the migration of VSMCs to Netrin-1, suggesting that this receptor mediates netrin signaling in VSMCs. The netrin receptor in VECs was not identified in this study, nor in a follow-up report (Wilson et al. 2006) showing that netrins stimulate proliferation, migration, and tube formation of human ECs in vitro and that this stimulation is independent of known netrin receptors. This study also reported that local delivery of netrins promoted revascularization in the mouse hindlimb ischemia model (Wilson et al. 2006). Another report reexamined the phenotypes of UNC-5B knockout mice using endothelium-specific knockouts, describing results different from those in the previous publication (Navankasattusas et al. 2008). These authors reported that the only site of vascular abnormality found in mutant embryos was in the arterioles of the placental labyrinth, where structural and functional vascular deficiency led initially to flow reversal in the umbilical artery and ultimately to embryonic death. Surprisingly, they did not find excessive vascular sprouting or branching as described by Lu et al. (2004). On the contrary, they found that instead of promoting increased branching, knockdown of *netrin1a* in zebrafish inhibited formation of a specific trunk vessel, the parachordal vessel.

Another report has suggested that Netrin-1 promotes angiogenesis by increasing endothelial nitric oxide synthase (eNOS)-dependent production of nitric oxide (NO) (Nguyen & Cai 2006). Exposure of bovine aortic ECs to Netrin-1 resulted in increased NO production, whereas Netrin-1-stimulated angiogenesis was abolished by NO scavengers. DCC antibody or DCC small interfering RNA (siRNA) inhibited Netrin-1-stimulated NO production and angiogenesis, indicating that this response depends on the DCC receptor. Additional data suggested that this response involved MEK/ERK signaling and eNOS. Several studies have shown that exogenously added

Netrin-1 also has a proangiogenic effect in the adult brain. Overexpression via an adeno-associated viral Netrin-1 vector (AAV-NT-1) stimulates proliferation and migration of human cerebral ECs and human aortic SMCs in vitro and promotes focal neovascularization in the adult brain in vivo (Fan et al. 2008). In addition, intracerebroventricular administration of Netrin-4 protein after distal middle cerebral artery occlusion in mice (an ischemic stroke model) leads to increased blood vessel density, endothelial proliferation, and improved behavioral recovery at 1 week after stroke (Hoang et al. 2008). Endogenous Netrin-4 is upregulated in the ischemic core, whereas expression of DCC, but not UNC-5A or UNC-5B, increases on neuronal processes in the peri-infarct cortex.

Given the strong and sometimes contradictory evidence for pro- versus antiangiogenic effects of netrins, it is difficult to reconcile all of the results obtained to date. However, as noted above, netrin has been shown to act as a bifunctional guidance cue in the nervous system as well, and this dual role may explain how at least some of these apparently opposed results could have been obtained. UNC-6, the *C. elegans* netrin homolog, is expressed in the ventral portion of the nematode, where it attracts ventrally directed axons and repels dorsally directed axons (Hedgecock et al. 1990, Ishii et al. 1992). Similarly, vertebrate Netrin-1 simultaneously attracts some axons to the floor plate while steering others away (Colamarino & Tessier-Lavigne 1995). The dual function of netrins probably depends at least in part on differential responses to netrin concentration, with low concentrations promoting attraction and high concentrations repulsion. One recent publication has reported a concentration-dependent dual role for Netrin-1 in angiogenesis (Yang et al. 2007). They found that Netrin-1 could either stimulate or inhibit proliferation and migration of human umbilical vein endothelial cells (HUVECs) in vitro, with lower Netrin-1 concentrations leading to stimulation and higher concentrations to inhibition. Similar Netrin-1 concentration-dependent enhancement or inhibition was also observed during corneal neoangiogenesis in vivo. Loss of UNC-5B prevents the inhibitory effects and some but not all of the stimulatory effects. This might be expected if, as described above, DCC or other netrin receptors engage in stimulatory or attractive effects. Unfortunately, these authors were unable to detect expression of other netrin receptors. Differential expression of netrin receptors or associated proteins could, however, account for some of the observed distinct responses to netrins. Another recent report focusing on the potential role of netrin as a survival factor has also attempted to reconcile some of the contradictory findings in this area (Castets et al. 2009). These authors' results suggest that Netrin-1 normally acts as a survival factor for ECs by blocking the proapoptotic effects of UNC-5B and its downstream death signaling effector, the serine/threonine kinase DAPK. This would explain why loss of UNC-5B (loss of a proapoptotic signal) leads to EC survival and enhanced angiogenesis (Lu et al. 2004), whereas loss of Netrin-1 (loss of an antiapoptotic signal or survival factor) leads to exacerbated EC death and reduced vasculature (Wilson et al. 2006). Each of these reports suggests potential models for dual pro- and antiangiogenic activities of netrin that potentially explain some, but not all, of the discrepancies in the results obtained to date. Whereas it seems likely that netrin signaling does have the capacity to play a dual role in the vasculature, further work is needed to unravel the complexities of how this is actually achieved.

Slit-Robo Signaling

The fourth major signaling molecule-receptor pair implicated in vascular development and vessel guidance is composed of the Slit ligands and their receptors, the *Robo* genes. This pair of signaling molecules has long been implicated in the wiring of the nervous system. The first group member identified was the Robo1 receptor, which was uncovered in a screen for *Drosophila melanogaster* mutants with defects in

commissural axon midline guidance (Seeger et al. 1993). Follow-up studies showed that Robo is a receptor involved in axon growth cone repulsion away from the midline, functioning as a "gatekeeper" controlling midline crossing (Kidd et al. 1998). The Slit protein was subsequently identified as a repellent guidance cue that acts on the Robo receptor during commissural axon pathfinding (Battye et al. 1999, Kidd et al. 1999). This repellent role was later found to be conserved during the wiring of the nervous system in vertebrates (Brose et al. 1999, Li et al. 1999). Recent work has shown that Slit-Robo signaling is not restricted to patterning of the central nervous system, nor it is always repulsive. In the immune system Slit limits the migration of chemokine-activated leukocytes (Wu et al. 2001). Also, concomitant with its discovery as a repulsive cue, an amino-terminal fragment of mammalian Slit2 was biochemically purified as a positive regulator of sensory axon elongation and branching (Wang et al. 1999).

Slit and Robo are both multidomain proteins (**Figure 2**). This raised the question of which Slit and Robo domains are used during different signaling events. Based on several studies, the idea of a "Robo code" was proposed. This code postulates that after crossing the midline, the final lateral positions of commissural axons depend on the particular set of Robo receptors expressed by each axon, which are activated in response to a gradient of Slit released by the midline using different Robo receptors. In the nervous system, the Slit-induced inhibitory effect on axonal migration is due to the direct interaction between the cytoplasmic domain of Robo and single transmembrane DCC, the receptor for the attractant molecule netrin (Bashaw & Goodman 1999). However, in the immune system, the Slit-Robo complex inhibits migration stimulated by the chemokine receptor CXCR4, a seven-transmembrane G protein-coupled receptor (Wu et al. 2001). Like the other three neural guidance families discussed above, the Slit-Robo family has also been implicated in the patterning and organization of the cardiovascular system. As described below, strong evidence has accumulated for a role for Slit-Robo signaling in heart development in fruit flies, but evidence for a specific vertebrate vascular role for Slit-Robo signaling has been more limited and somewhat contradictory.

Recent evidence points to an important role for Slit-Robo signaling in heart development in *D. melanogaster* (Santiago-Martinez et al. 2006, 2008). Although invertebrates do not have an endothelium-lined vascular system, most do possess hearts, and the initial steps of heart tube formation are very similar in vertebrates and invertebrates. The fruit fly heart is formed by the parallel alignment of two rows of cells that surround a single lumen. These cell populations arise in bilateral fields in the lateral mesoderm and then migrate toward the midline of the embryo, similar to the early stages of vertebrate heart formation. The cardioblasts (CBs) form a row proximal to the midline and are in direct contact with the lumen of the heart. The pericardiocytes (PCs) have a more distal position to the midline and are juxtaposed to the CBs. The Robo code has been proposed to have a role in the positioning of both CBs and PCs with respect to the midline (Santiago-Martinez et al. 2006). The two cell populations express different combinations of Robo receptors. During the initial phases of heart tube formation, the inner row of CBs expresses Robo whereas the outer row of PCs expresses both Robo and Robo2. The CBs express the repellent *slit*, as detected by in situ hybridization. The protein is later secreted, and it localizes to the membranes of both CBs and PCs, where it exerts its effects. Mutant analysis reveals differential roles for the Robo receptors during heart tube formation. In *slit* mutants or *robo*, *robo2* double mutants, CBs and PCs fail to reach the midline of the embryo, suggesting that the role of this guidance system is not necessarily repulsive but promigratory. However, ectopic expression of *robo2* in CBs results in a lateral shift of the row of CBs away from the midline, and *robo2* mutants show PCs migrating beyond the row of CBs. Moreover, *robo* mutant embryos do not show significant misalignment phenotypes. From these results it is unclear whether Slit-Robo signaling provides attractive or repulsive cues for the

migration of CBs and PCs toward the midline. One interesting possibility is that it does both, and that the effect of the Slit/Robo interaction is time-dependent. Slit signaling through both Robo and Robo2 might have permissive and redundant promigratory effects early, during the initial migration of CBs and PCs away from the lateral mesoderm. Then, at a slightly later stage, Slit may have a repulsive role on the migration of PCs, stopping them from migrating beyond the row of CBs. Finally, at an even later stage, during the fusion of the CBs to form the heart tube lumen, Slit secreted by the CBs might exert a autocrine effect on the localization of adhesion molecules guiding the fusion of the correct membrane domains and the formation of a heart lumen of the correct size and shape (Medioni et al. 2008, Santiago-Martinez et al. 2008). This model suggests that Slit-Robo signaling is used dynamically during the formation of the *Drosophila* heart to achieve different purposes at different times. It is not known which other molecular components of the system would allow such impressive versatility of Slit-Robo signaling in a very narrow window of time.

Some of the first evidence in support of a vertebrate vascular role for Slit-Robo signaling came with the identification of a new Robo receptor family member, named Robo4 (Park et al. 2003). Robo4 is the most divergent of the four Robo receptors. It lacks two of the four cytoplasmic conserved domains (CC0–CC3) used for intracellular signaling, and its extracellular ligand-binding domains also differ significantly from those of the three founding members of the Robo family. Mammalian Robo4 has only two immunoglobulin (IgG) and two fibronectin (FN) domains, instead of the typical five IgG and three FN domains found in Robo1–3. Unlike the other Robo genes, Robo4 is specifically expressed in the vascular endothelium during murine embryonic development. As has been shown for neuronal Robos, Robo4 binds Slit and inhibits cellular migration in a heterologous expression system. Robo4 is also expressed in primary ECs, and application of Slit inhibits migration of these cells (Park et al. 2003). Expression of Robo4 is also upregulated on tumor-associated ECs, which is suggestive of a role during tumor angiogenesis (Gorn et al. 2005, Seth et al. 2005). Zebrafish in which the *robo4* gene has been either knocked down using morpholinos or overexpressed by mRNA injection fail to properly form trunk ISVs, suggesting a possible role in angiogenesis in vivo (Bedell et al. 2005). However, unlike the murine gene, zebrafish *robo4* expression is not restricted to the vasculature, complicating interpretation of the results of gene knockdown experiments in which animals show evidence of potentially confounding nonvascular general defects. Furthermore, inhibition of vessel sprouting is reported in these experiments both when Robo4 is knocked down as well as when it is overexpressed, making it difficult to develop a model for its action.

In contrast to the zebrafish results, a recent report on targeted disruption of the *Robo4* gene in mice did not reveal a role for this gene in vascular development. Mice were generated with the *Robo4* allele replaced by an *alkaline phosphatase* (AP) gene (*robo4*^{AP}) (C.A. Jones et al. 2008). *Robo4*^{AP} homozygous mice develop a normal vascular system, are born in normal numbers, and are viable and fertile, showing that Robo4 signaling is not required for developmental angiogenesis. However, Slit-Robo4 signaling does have effects on pathological angiogenesis and vascular integrity. Activation of Robo4 by exogenous Slit2 inhibits VEGF165-induced EC migration, tube formation, and permeability in cultured lung ECs in vitro obtained from knockout mice and their siblings. Exogenous Slit also inhibits VEGF165-stimulated vascular leakage in the retina and blocks oxygen-induced retinopathy and choroidal neoangiogenesis in vivo. All of these responses occur in a Robo4-dependent manner. However, assaying for the effects of Robo4 loss in vivo in the absence of exogenously added Slit ligand reveals minimal or no increase in dermal and retinal permeability or choroidal neoangiogenesis, suggesting that endogenous

Slit signaling does not play a major role in limiting these pathological vascular responses. In the oxygen-induced retinopathy model, loss of Robo4 does elicit increased neovascularization when assayed in the absence of exogenous Slit2, indicating that endogenous signaling via Robo4 does play a role in limiting pathological vessel overgrowth in the retina. A recent report has shown that the Robo4 intracellular domain interacts with the adaptor protein paxillin in a Slit2-dependent manner (Jones et al. 2009). Robo4-paxillin interaction blocks activation of small GTPase Arf-6 and Rac (a signaling pathway activated downstream of VEGF). In vivo, inhibition of Arf6 activity mimics activation of Robo4 by reducing VEGF165-induced retinal hyperpermeability. The insights provided by this paper open new avenues in the search for therapies for diseases characterized by enhanced angiogenesis and vascular permeability.

Some evidence also suggests that signaling via the Robo1 receptor plays a role in tumor angiogenesis, although in contrast to the above results a proangiogenic role is proposed. In one report the Slit2 protein attracted ECs and promoted tube formation in a Robo1- and phosphatidylinositol kinase-dependent manner in vitro, whereas neutralization of Robo1 reduced the microvessel density and tumor mass of human malignant melanoma A375 cells in vivo (Wang et al. 2003). Another study using a model of chemically induced squamous cell carcinoma in the hamster buccal pouch showed that Slit2 expression was correlated with increased tumor vascularity and that administration of a Robo1-blocking antibody inhibited tumor angiogenesis and tumor growth (Wang et al. 2008).

In sum, although a variety of studies have thus far provided intriguing hints that Slit-Robo signaling may play a role in regulating vessel growth and patterning, the effects of loss-of-function experiments performed to date have been relatively modest. This may reflect redundant functions within the Slit-Robo signaling family, or even between Slit-Robo signaling and some of the other neural/vascular guidance factors described above. Vascular phenotypes have not been reported for Slit ligand knockouts, although extensive redundancy between the Slit ligands has made it difficult to even detect neuronal phenotypes of Slit knockouts, with relatively mild neural misguidance phenotypes recently noted in a triple knockout (Long et al. 2004). Ventral midline neural expression of Slit ligands in the trunk hints that this signaling pathway may be involved in attraction or repulsion of vessels to or from the vicinity of the neural keel (as for commissural neurons), but further study will obviously be required to draw firm conclusions about the in vivo role of Slit-Robo signaling during vascular development.

CONCLUSIONS

The similarities between the circulatory and the nervous system are apparent at three levels. At the macroscopic, anatomical level, both systems are characterized by highly branched and ramified layouts. Moreover, their branches run with an impressive degree of coalignment even though the systems have different anatomical origins (**Figure 1**). Close coordination between the assembling neuronal and vascular systems makes sense given the high metabolic demands of the nervous system, which require that neurons be well-supplied with blood-borne nutrients and oxygen. Many similarities can also be found at the cellular level. The growing or most distal ends of both extending axons and angiogenic sprouts, the growth cone and the tip cell respectively, are organized into highly specialized structures that can be regarded as proficient environmental sensors. Growth cones and tip cells both have expanded cellular membranes, organized as filopodia, loaded with a myriad of membrane receptors that allow them to rapidly respond to gradients of trophic and guidance factors (**Figure 5**). Taking into consideration all of these anatomical and cellular similarities, it should come as no surprise that the vasculature and the nervous system are revealing an ever-growing number of common

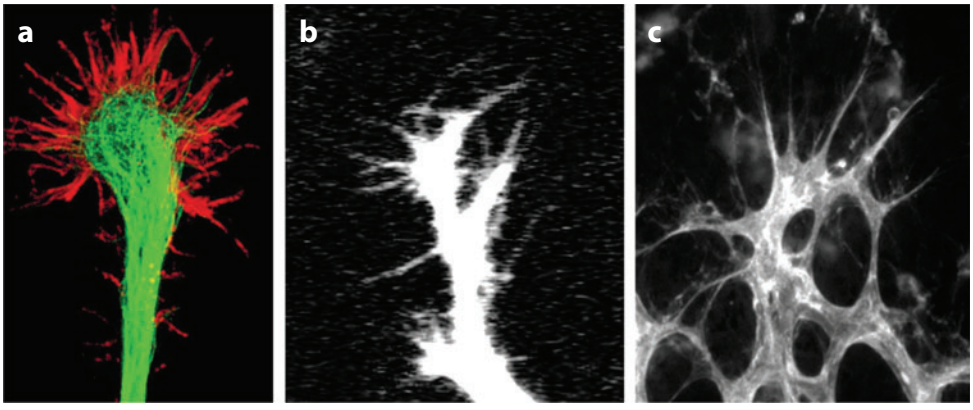

Figure 5

Cellular resemblances between axonal growth cones and endothelial tip cells. (*a*) Axonal growth cone in culture with tubulin-rich filopodia visualized at the distal-most tip of the axon (*red*). (*b*,*c*) Endothelial tip cells (*b*) at the distal migrating end of a growing intersegmental vessel in the zebrafish trunk and (*c*) at the periphery of the extending superficial retinal vascular plexus. Like neuronal growth cones, endothelial tip cells show evidence of extensive protrusive activity with numerous long filopodia. Panel *a* is reproduced from NIH Publication No. 97-4038, "Mind Over Matter" (1997, National Institute on Drug Abuse), panel *b* is from Isogai et al. (2003), and panel *c* is courtesy of Dr. Xuri Li (National Eye Institute, NIH).

molecular mechanisms regulating their development, patterning, and postnatal reorganization. In this review we have discussed a few of the most recent findings regarding these common molecular mechanisms, the major highlights of which are summarized below.

SUMMARY POINTS

Neuronal growth factors such as neurotrophins are now known to have important roles in the survival of ECs, angiogenesis, and vascular integrity.

As during axon guidance in the nervous system, repulsive and attractive guidance molecules steer ECs and pattern the vascular tree. The four major families of guidance cues needed to establish the pattern of the nervous system (Ephrins, Semaphorins, Netrins, and Slits) are now all known to be important for the correct patterning of the vascular system.

Ephrins are major determinants of arterial-venous fate and patterning of the vascular network.

Semaphorins provide repulsive cues that help guide migrating ECs and pattern the vasculature. Nrp, a Semaphorin coreceptor in the nervous system, functions in the vasculature as a coreceptor for VEGFR2. Semaphorin binding to Nrp can inhibit VEGF signaling through VEGFR2 and thereby interfere with angiogenic signaling.

Semaphorins in the vascular system can also signal through activation of Plexin receptors, as in the nervous system, although at least some of this signaling is Nrp independent. Different Plexins have been reported to have anti- or proangiogenic functions.

The role of Netrins in the vasculature is controversial. As in the nervous system, Netrin might act as a bifunctional guidance cue, mediating either repulsive or attractive guidance depending on the cellular and environmental context.

Data on a vascular role for Slit-Robo signaling are limited. The apparent lack of a requirement for Slit-Robo signaling may be due to redundancy among Robo receptors.

We are now aware of a variety of different guidance cues and receptors that pattern developing vessels, and we have a limited understanding of their functional consequences. However, it is still far from clear how different guidance systems interact with each other, what molecular mechanism ECs employ to transduce guidance information, and how a cellular response is mounted downstream of the guidance information.

DISCLOSURE STATEMENT

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