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Guidance of Vascular Development

Lessons From the Nervous System

Bruno Larrivée, Catarina Freitas, Steven Suchting, Isabelle Brunet, Anne Eichmann

Abstract—The vascular system of vertebrates consists of an organized, branched network of arteries, veins, and capillaries that penetrates all the tissues of the body. One of the most striking features of the vascular system is that its branching pattern is highly stereotyped, with major and secondary branches forming at specific sites and developing highly conserved organ-specific vascular patterns. The factors controlling vascular patterning are not yet completely understood. Recent studies have highlighted the anatomic and structural similarities between blood vessels and nerves. The 2 networks are often aligned, with nerve fibers and blood vessels following parallel routes. Furthermore, both systems require precise control over their guidance and growth. Several molecules with attractive and repulsive properties have been found to modulate the proper guidance of both nerves and blood vessels. These include the Semaphorins, the Slits, and the Netrins and their receptors. In this review, we describe the molecular mechanisms by which blood vessels and axons achieve proper path finding and the molecular cues that are involved in their guidance. (Circ Res. 2009;104:428-441.)

Key Words: angiogenesis ■ axon guidance ■ VEGF ■ semaphorin ■ netrin

The formation of the vascular system is a key event during vertebrate embryonic development, because its function in delivering oxygen and nutrients while removing waste is crucial to ensure proper growth and differentiation of all tissues in the organism. To efficiently fulfill this role, it is essential for blood vessels to be organized into functional circuits. Blood vessels are formed through 2 distinct mechanisms: vasculogenesis, which refers to the differentiation of primitive mesodermal cells into endothelial cells, and angiogenesis, a process by which endothelial cells proliferate and migrate to colonize tissues. With the onset of the heart beat and blood flow, primitive vessels are rapidly remodeled into branched networks with a characteristic and reproducible anatomy: major axial vessels, common to developing mouse, chick and zebrafish embryos; vessel branches penetrating different organs at designated sites; and stereotyped vascular patterns specific to different organs, including brain, kidney, heart, and skeletal muscle.

Numerous growth factors are implicated in vascular development. Among these, Vascular Endothelial Growth Factor (VEGF) is a key regulator of both vasculogenesis and angiogenesis. It is critical for the emergence of endothelial cells, and promotes their subsequent proliferation and migration. VEGF is expressed by hypoxic cells and attracts vessels
toward hypoxic tissue areas. VEGF is downregulated when target cells receive appropriate oxygen supply, slowing further endothelial growth and thereby ensuring appropriate vessel coverage. Different sequestration of different VEGF isoforms in the extracellular matrix is crucial for the balance between capillary branching and enlargement of vessel size. Transgenic mice expressing only the diffusible VEGF120 isoform develop enlarged vessels with few branch points, whereas mice expressing only the matrix-bound VEGF188 isoform form thin and hyperbranched vessels. Mice expressing only VEGF164, which has intermediate matrix-binding properties, form normal vessels, indicating that this isoform alone is sufficient to ensure proper vascular patterning.

In addition to growth factor gradients, perfusion of vessels is another important factor in the regulation of pattern formation. In the mouse embryo, hemodynamic force is necessary and sufficient to induce vessel remodeling in the yolk sac. Experimental manipulation of blood flow in chick embryos by ligation of the major yolk sac artery leads to rerouting of blood flow and can transform yolk sac arteries into veins and vice versa. These findings suggest that vessels are plastic with regard to their arterial or venous identity and that both appropriate hemodynamics and appropriate growth factor gradients are together required to shape the highly stereotyped vessel anatomy of vertebrate embryos.

Such stereotyped anatomy of vessels in the human body had already been observed several hundred years ago. Moreover, anatomists such as Andreas Vesalius noted that, in peripheral tissues, blood vessels are often aligned with nerves and display similar branching patterns. The molecular mechanisms regulating common wiring of nerves and blood vessels have attracted considerable interest over the past few years. In the following sections, we discuss the similarities in nerve and vessel patterning and how common molecular pathways regulate guidance of axons and vessels to distant sites.

**Alignment of Blood Vessels and Nerves**

Alignment of blood vessels and nerves is observed in many adult peripheral tissues and can be easily visualized by whole-mount immunostaining of skin (Figure 1). In general, an artery, a vein, and a nerve course along each other, forming a neurovascular bundle. Alignment of vessels and nerves most probably reflects the mutual requirement of one for the other, i.e., larger nerves require vascularization to ensure nutrient and oxygen supply, whereas blood vessels, mainly arteries, need innervation to control vasodilatation or constriction (Figure 1). How do nerves and vessels align? Experimental evidence suggests that, depending on the context, developing axons may follow vessels, or vessels may take advantage of axons, to follow the same path.

During early mouse development, the first major arteries formed are the dorsal aorta and the carotid system, consisting of an internal and an external arterial branch that irrigate the head. Smooth muscle cells (SMCs) surrounding these vessels release diffusible factors that direct growth of sympathetic nerves along these arteries. A subset of ventrally migrating neural crest cells aggregate adjacent to and along the dorsal aorta and differentiate to form the postganglionic neurons of the sympathetic ganglion chain. The cranial-most sympathetic ganglia are the superior cervical ganglia (SCG), which come to lie adjacent to the bifurcation of the external and internal carotid arteries from the common carotid artery.

Sympathetic SCG neurons send axonal projections either along the external carotid arteries to innervate the salivary glands or along the internal carotid arteries to the lacrimal and pineal glands, the eye, blood vessels and skin of the head, and the mucosa of the oral and nasal cavities. Makita et al recently showed that a subset of sympathetic neurons extend along the external carotid artery in response to endothelin-3, a vasoactive peptide released from the SMCs surrounding this artery. They used cDNA microarray chips to identify factors differentially expressed by SMCs of the internal carotid artery (derived from mesoderm) and the external carotid artery (derived from neural crest). Transcripts for endothelin-converting enzyme 1, the protease that generates the bioactive forms of endothelins, were selectively expressed in the developing external carotid artery SMCs. Using a series of mouse mutants deficient in different endothelins and in vitro culture experiments, they demonstrated that endothelin-3 secreted by the smooth muscle cells surrounding the external carotid artery serves as an attractant for SCG neurons expressing the EDNRA receptor. Thus, EDNRA expression may mark a subset of molecularly distinct SCG neurons destined to project toward their end organs via the external carotid artery.

Other factors regulating extension of sympathetic axons along the arterial vasculature include the glial-derived neuro-
Figure 2. Alignment of developing arteries and nerves. Immunohistochemical staining of the skin of the head of E15.5 embryos using the endothelial marker PECAM-1 and the neuronal marker Tuj-1. Note alignment of main branches of nerves (N) (green, left) with arteries (A) (red, middle and right).

Theotrophic factor (GDNF) family member Artemin and the neurotrophin NT-3. Artemin is expressed in SMCs surrounding arteries in the trunk of developing embryos, with expression progressing distally in the arteries as axons extend along them. Sympathetic chain ganglia of mice deficient for Artemin (Artn) or its coreceptors Ret or GFRα3 exhibit abnormally short and misdirected proximal axonal projections, consistent with a role for Artemin signaling in guidance of these axons. Whether Artemin signaling is selectively involved in axon guidance remains to be determined, because the initial migration of sympathetic neuroblasts forming the ganglia is also perturbed in Artn-deficient mice, leaving open the possibility that defects in axonal projection might be secondary because of defects in initial neuroblast migration.

Artemin is not the only factor mediating axon extension along blood vessels, because, despite the abnormalities seen in embryonic Artn-, Ret-, or GFRα3-deficient mice, most of these knockout mice achieve at least partial sympathetic innervation of targets in adulthood. The neurotrophin NT-3 also plays a role in sympathetic chain ganglia axon extension along vessels. Like Artemin, NT-3 is expressed by blood vessels and can attract sympathetic chain ganglion axons in vitro. As with Artn-deficient mice, mice deficient in NT-3 show reduced (but not completely abolished) axon extension from prevertebral and paravertebral ganglia along blood vessels and into peripheral targets. Assessment of axonal projections in mice lacking both factors will establish whether other blood vessel–derived growth factors contribute to proximal axon extension and whether these factors are permissive or instructive for sympathetic axon guidance in vivo. An intriguing, yet unanswered, question relates to the fact that blood vessels are not only routes for sympathetic axon guidance toward their targets but are also themselves final sympathetic neuron targets. How innervation sites for sympathetic axons on blood vessels are selected, and why some axons make connections while others continue to extend, remains to be determined.

Evidence for the converse situation, ie, nerves guiding vessels, was obtained from the skin of the embryonic limb, where sensory axons were shown to guide arterial patterning and differentiation. In the mouse embryonic skin, arteries, but not veins, preferentially coalign with peripheral nerves (Figure 2) and arterial differentiation occurs concomitantly with nerve alignment. Analysis of several mouse mutants showed that arterial differentiation did not occur in embryos lacking peripheral sensory axons and Schwann cells. Moreover, in Semaphorin 3A (Sema3A) mutant mouse embryos, in which peripheral nerve patterning is severely disorganized, arteries formed but followed the mispatterned network. The authors further demonstrated that the molecular signal secreted by peripheral nerves to promote arterial differentiation is VEGF, because ablation of nerve-derived VEGF led to failure of arterial differentiation. Intriguingly, however, alignment of vessels and nerves remained intact in this system, suggesting that other, as yet unidentified, factors are released from nerves to promote nerve–vessel alignment. Nevertheless, these data provide evidence that the peripheral developing sensory system functions as a template for the arterial branching pattern in the embryonic limb skin.

Collectively, these studies suggest that reciprocal interactions during angiogenesis and axon outgrowth provide a molecular basis for nerve–vessel alignment during vertebrate development.

Axon Guidance

Axon guidance cues present in the extracellular environment are sensed by a specialized sensory and motile structure located at the tip of an extending axon called the growth cone. Growth cones project numerous filopodia that actively extend and retract in response to extracellular cues. Guidance cues can be divided into attractive or repulsive signals that tell the growth cone where and where not to migrate, respectively. These cues can also be divided into those that are substrate- or cell membrane–bound, and so act on nearby axons, and those that are secreted from distant sources and form gradients that influence the trajectories of extending axons. Four classes of guidance cues are known: Ephrins, Semaphorins, Netrins, and Slits. In addition to these “classic” axon guidance molecules, some growth factors, including Neurotrophins, scatter factor/hepatocyte growth factor, and stem cell factor, have been implicated in axon guidance, and morphogens, including Hedgehog, Wnt, and transforming growth factor-β/bone morphogenetic protein families, contribute to providing graded positional information necessary for proper axon path finding.

Of the classic axon guidance cue, Slits, Semaphorins, and Ephrins act primarily as repellents but can be attractive in some contexts, whereas Netrins can act as attractants or repellents. Thus, individual guidance molecules can have either attractive or repulsive activities under different circumstances. For example, the well-characterized chemotropic guidance cue Netrin-1 can act as a chemoattractant for dorsal commissural neurons and as a repellent for certain classes of motor neurons. For these neurons, the specific action of Netrin-1 has been shown to depend on the receptor types expressed by the responsive cells (see below). Many axons have to travel considerable distances through the body to reach their proper synaptic targets. They accomplish this task by breaking down the large distance into a series of smaller “intermediate targets.” For individual axons to navigate between intermediate targets, they must be able to switch between attraction and repulsion. The cells that form the intermediate target, which are initially attractive, must become repulsive once the axon reaches them, to ensure that the axon keeps moving onward toward the next target of its
trajectory. This switch from attraction to repulsion requires coordinated control of the expression level, as well as the activity, of guidance receptors on the axon surface, so that response to an attractant is lost, whereas response to a repellent is gained. A well-known example is the ventral midline of the central nervous system (CNS) of higher organisms, which establishes a partition between the left and right mirror-image halves. The transfer of information between the 2 sides of the nervous system occurs through commissures formed by neurons that project axons across the midline to the contralateral side of the CNS. These axons cross the midline only once. Other neurons extend axons that never cross the midline; they project exclusively on their own (ipsilateral) side of the CNS. As commissural axons approach the floor plate, receptors of the deleted in colorectal cancer (DCC) family mediate the attractive response to Netrin-1, whereas the expression level of the repellive Robo (Roundabouts), receptors for Slit proteins, is kept low to inhibit Slit-mediated repulsion. Once the axons cross the midline, however, axons will upregulate Robo and thus become responsive to repellent Slit proteins. Furthermore, Robo activity in turn contributes to switching off DCC attraction. The combination of all these steps ensures that the axon does not recross the midline by making a once-attractive environment appear repulsive.

The structural similarities between the nervous and vascular systems raise the question of whether endothelial cells can use mechanisms similar to those of axonal growth cones to be guided to their proper targets and establish a proper vascular network. Capillaries carry specialized terminal cells called tip cells, at the extremity of sprouting capillaries, which show morphological similarities to axonal growth cones. Furthermore, endothelial cells express receptors for axon guidance cues, suggesting that they may use these cues to direct their migration. The next section focuses on the analogies in the mechanisms that endothelial cells and neurons use to reach their proper targets.

**Vascular Guidance: Endothelial Tip Cells**

Sprouting capillaries, like axons, need to navigate through tissue to establish a stereotyped vascular branching pattern. Endothelial tip cells are analogous to growth cones and extend numerous filopodia, which explore the environment to guide the growth of the nascent vessel and also regulate capillary branching by detecting and connecting to neighboring sprouts. Tip cells can be found wherever there is sprouting angiogenesis, including the leading edge of developing mouse hindbrain and retina capillaries, and the intersegmental vessels of mouse and zebrafish embryos. In addition to filopodia, tip cells are distinguished by the absence of a lumen and for being mostly nonproliferative, in contrast to the lumenized and proliferative adjacent cells, called stalk cells. Tip cells also express a distinctive profile of genes, with substantially higher expression compared to stalk cells of molecular markers including platelet-derived growth factor-B, VEGF receptor (VEGFR)-2, uncoordinated (Unc)5b, Delta-like (Dll)4, and VEGFR-3 (Figure 3).

Much of our understanding of tip cell biology comes from study of the mouse retina vasculature, which develops postnatally in a reproducible spatial and temporal pattern. At birth, the avascular retina becomes hypoxic and retinal astrocytes begin to express VEGF, which stimulates endothelial cells to sprout radially from the optic nerve head into the retinal periphery. As new vessels bring oxygenated blood, VEGF expression is switched off, providing an elegant feedback loop to control angiogenesis. However, at the vascular front, there will be many endothelial cells exposed to VEGF, only a select few of which will become tip cells, with the remainder becoming stalk cells. How does the capillary select its tip cell? Recent findings suggest that the Notch signaling pathway regulates how endothelial cells respond to VEGF stimulation and so controls tip cell selection. Such a system is analogous to tracheal formation in Drosophila and thus appears to be remarkably conserved during evolution. In Drosophila, cells at the tip of a budding tracheal branch respond to a form of fibroblast growth factor to lead branch outgrowth, whereas the trailing cells receive another signal to follow these leading cells and form tubes. Selection of which cells become leaders and which become followers involves Notch-mediated lateral inhibition. A key feature of Notch action is that Notch ligands of the Delta and Jagged families are themselves transmembrane proteins, and so can signal only through adjacent cells. Thus, a general model for development of branching structures is that Notch functions by lateral inhibition, which results in determination of cell fate in a binary fashion within a given cell population.

Evidence from the mouse and zebrafish indicates that the Notch ligand Dll4 is the key mediator of tip cell selection in vessels. Analysis of sprouting vascular beds from heterozygous dll4 mouse embryos and retinas revealed ectopic filopodial extension and excessive vessel branching, accompanied by upregulation of tip cell markers, together indicating an increased number of tip cells throughout the vascular plexus. Morpholino knockdowns of Notch signaling components led to similar phenotypes in the embryonic zebrafish vasculature. Moreover, disruption of the Notch pathway by either pharmacological inhibition, DI4-blocking antibodies or soluble DI4-Fc recapitulated the dll4 vascular phenotype. Taken together, these observations imply that disruption of the Notch pathway leads to excessive tip cell specification and that Notch signaling functions to repress tip cell formation. As in Drosophila trachea, this inhibition occurs in a cell-autonomous manner, as demonstrated by mosaic experiments carried out in mouse and zebrafish embryo models.

As tip cell formation depends on a balance between VEGF and Notch signaling, it is perhaps unsurprising that these pathways mutually interact. Inhibition of VEGF signaling in the retina results in downregulation of dll4 expression, whereas treatment with exogenous VEGF induces dll4, suggesting that VEGF signaling is upstream of DLL4. In addition, expression levels of VEGFRs are altered in dll4 retinal vessels in a way that suggests these vessels are more sensitive to VEGF signal and that Notch could regulate the VEGF response. Thus, a putative model for tip cell formation emerges where tip cell induction depends on the differential VEGF isoform distribution in the extracellular environment, and tip cell suppression depends on DLL4 signaling to adjacent cells, which will become stalk cells. Notch signaling
thus acts as a negative feedback mechanism downstream of VEGF to select for single endothelial tip cells at the head of sprouting capillaries.

After selection of the tip cell at the head of a new vessel sprout, this cell must be capable of responding to environmental stimuli to find its way. An increasing set of data suggests that the same cues that guide axons can also be perceived by the endothelial tip cells and so participate in blood vessel path finding.

Common Molecular Cues in Endothelial and Axon Guidance

Developing axons navigate through the embryo by responding to a number of different signals in their immediate environment. Molecules such as Semaphorins, Slits, and Netrins and their specific receptors provide key ligand–receptor interactions for this process during neuronal development. There is now clear evidence that many of these molecules can also play a role in vascular morphogenesis. In this section, we review the role of 3 families of known axon guidance molecules, the Neuropilin receptors and their Semaphorin and VEGF ligands and Netrins and Slits and their receptors, and discuss their specific roles in axon and blood vessel guidance.

Neuropilins

Neuropilin (Nrp)-1 and -2 are single-pass transmembrane proteins that share similar domain structure and 44% sequence identity. Their extracellular domain contains 2 complement-binding (CUB) domains (also known as a1 and
a2 domains), 2 coagulation factor V/VIII homology domains (b1 and b2 domains), and a MAM domain (meprin/A5 protein/phosphatase-\(\mu\)) (c domain) important for homo- and heterodimerization. The cytoplasmic domain includes approximately 42 to 44 amino acids and does not display catalytic activity but presents a binding site for the PDZ domain of Nrp-1–interacting protein (NIP), also known as Synectin (Figure 4).46–48 Both Nrps are expressed in several splice forms, including soluble forms that may function as natural inhibitors.49

Nrps were initially described as axonally expressed receptors for secreted class III Semaphorins.44,45,50 They also serve as isoform-specific VEGF coreceptors on endothelial cells and are involved in vascular development and tumorigenesis.51–54 Nrps are expressed in overlapping, but largely distinct populations of developing neurons as well as endothelial cells, with both Nrps being coexpressed during early vascular development, but segregating at later stages into Nrp-1–positive arterial endothelium and Nrp-2–positive venous endothelium.14,55,56 Still later in development, Nrp-2 is most strongly expressed by lymphatic vessels.57,58 Genetic deletion studies have shown that both Nrps serve critical and nonoverlapping roles during both vascular and neuronal development in the cells where they are expressed.

The absence of a functional Nrp-1 receptor results in embryonic lethality as a result of impaired heart and blood vessel development, which indicates that this receptor plays a central regulatory role during developmental angiogenesis.59 Embryonic lethality occurs at embryonic day (E)10.5 on a C57BL/6 background, but only at E13.5 on a CD-1 background.60 The early phenotype is attributable to impaired endothelial migration and defective arterial differentiation, but not to altered endothelial proliferation, and is also independent of blood flow patterns.61 On a CD-1 background, embryos lacking \(\text{nrp-1}\) exhibit defects in the formation of the heart outflow tract and aortic arches as well as abnormal vascular network formation in the yolk sac59 and abnormal sprouting of hindbrain vessels.62 Furthermore, in endothelial-specific \(\text{nrp-1}\) knockouts,63 certain arterial markers are missing from arterioles and arteries.15 Thus, Nrp-1 function is critical for normal vascular development and arterial differentiation. In contrast to \(\text{Nrp-1}\) mutants, arterial–venous differentiation is normal in \(\text{Nrp-2}\) knockouts. Instead, homozygous \(\text{Nrp-2}\) mutants show absence or severe reduction of small lymphatic vessels during development, suggesting that Nrp-2 is required for the formation of these vessels.58 These experiments reveal critical, yet nonoverlapping function for Nrp receptors in the vessels that express them.

In the nervous system, lack of Nrp receptors also affects nonoverlapping sets of axons. Fasciculation and guidance of distinct subsets of cranial nerves are perturbed in \(\text{Nrp-1}\) and \(\text{Nrp-2}\) mutants: \(\text{Nrp-1}^{-/-}\) mice show deficiencies in cranial nerves VII, IX, and X, which are not affected in \(\text{Nrp-2}^{-/-}\).

Figure 4. Ligand–receptor pairs that regulate both axon guidance and blood vessel development. Ligands (Semaphorins class 3, VEGF, Netrins, Slits) are depicted at the top, and receptors (PlexinD1, Neuropilins, VEGFRs, UNC5, DCC, Robo1, Robo4) are depicted at the bottom. CC indicates Robo-conserved motifs; CUB, complement-binding domain; DB, DCC binding; DD, death domain; FNIII, fibronectin type 3–like; Ig, immunoglobulin domain; IPT, Ig-like fold/plexin; Lam, laminin domain; LRR, leucine-rich repeat; MAM, meprin/A5 protein/phosphatase-\(\mu\)–related; P1/2/3, conserved regions in the cytoplasmic domain of DCC; PSI, plexin–semaphorin–integrin; SP1/2, serine/threonine protein kinase catalytic domain 1/2; TSP1, thrombospondin-1–like; TyK, tyrosine kinase; ZU5, zona occludens-1–like.
mice. Conversely, cranial nerves II and IV, which are normal in Nrp-1/–/– mice, show abnormal projections in Nrp-2/–/– animals. Dorsal root ganglion (DRG) axons expressing Nrp-1 are efficiently collapsed or repelled by Sem3A in vitro, and it was for this activity that Semaphorins were initially named collapsins. DRG axons from Nrp-1/–/– mice lose repulsive responses to Sem3A in vitro and the guidance defects of cranial axons seen in Nrp-1/–/– mice are phenocopied in sema3A mutant mice, indicating that Sem3A is the ligand required to normally repel Nrp-1–expressing cranial axons. Similarly, sympathetic ganglia from Nrp-2/–/– animals lose response to Sem3F, and Sem3F is the primary Nrp-2 ligand in several axonal projection systems, including cranial nerves and limbic circuitry. Spinal motor axons, and olfactory neurons, although these and other studies have convincingly demonstrated a role for Semaphorins and Nrp receptors in axon guidance and fasciculation, this ligand–receptor system also plays a role in the migration of neural crest cells, indicating that Nrps play multiple roles in the development of the peripheral nervous system.

Because of their short intracellular domains, it is unclear whether Neuropilins can transduce biological signals on their own. In the nervous system, Nrps have been shown to act as Semaphorin ligand-binding moieties of a receptor complex comprising the signal transducing Plexin receptors. In the vascular system, Nrps form complexes with VEGFRs, with Nrp-1 partnering with VEGFR-2, whereas Nrp-2 can be communoprecipitated with VEGFR-2 and -3. In vitro, all antibodies coimmunoprecipitated with VEGFR-2 and -3. It has thus been proposed that the presence of Nrp receptors could enhance signal transduction through VEGFRs in the presence of VEGF ligands, which can bind to Nrps. Kawamura et al recently demonstrated that Nrp-1 is required for VEGF-induced activation of p38 mitogen-activated protein kinase and that this pathway would be critical for the association of endothelial cells with pericytes. Among the 9 mammalian plexins, PlexinD1 is selectively expressed in developing blood vessels and is required for proper vascular patterning in both zebrafish and mouse. The coexpression of Nrps, VEGFRs and Plexins suggests multiple possible modes of Semaphorin and/or VEGF signaling through these receptors in endothelial cells. In addition, a recent study reported that both Nrp-1 and Nrp-2 can bind hepatocyte growth factor, and potentiate c-met signaling in endothelial cells in vitro and in vivo.

Initial studies suggested that Semaphorins and VEGF family members could compete for binding to each other. However, Sema3A-deficient mice and mice expressing a Nrp-1 variant that cannot bind Sema3A show normal vascular development, arguing that Sema3A is not required for angiogenesis in the mouse, which instead would be controlled by VEGF164. In contrast, Sema3A, but not VEGF164, is required for axon patterning of limb nerves. Thus, these data suggest that Nrp-1 contributes to both neuronal and vascular patterning by preferentially relaying Sema3A signals in peripheral axons and VEGF164 signals in blood vessels. Crystal structures of Nrp-1 and Nrp2 extracellular domain fragments suggest that VEGF and Semaphorins do not directly compete for Nrp binding, providing a structural explanation for the absence of competition observed in the in vivo studies. Furthermore, Semaphorins and VEGF induce Nrp-1 endocytosis through different pathways; VEGF binding induces clathrin-mediated endocytosis, whereas Sema3C induces lipid raft–dependent endocytosis. Therefore, each of the 2 disparate endocytic pathways used by Nrp-1 could contribute to its signaling specificity by coupling it to a different set of downstream effectors, depending on which ligand it binds. Blocking antibodies disrupting either Semaphorin binding or VEGF family member binding to Nrps were recently generated. Anti–Nrp-1A antibody blocks Sema3A binding and anti–Nrp-1B is specific for the VEGF-binding domain of Nrp-1, although both prevent receptor dimerization with VEGFR-2. Anti–Nrp-2B blocks VEGF-165 and VEGF-C binding to Nrp-2 and prevents dimerization of Nrp-2 with VEGFR-2 and VEGFR-3. In vitro, all antibodies reduce VEGF-driven endothelial cell migration and sprouting angiogenesis, with anti-Nrp-1 antibodies active on human umbilical vein endothelial cells and anti–Nrp-2B active on VEGF-C–treated lymphatic endothelial cells. In vivo administration of these antibodies results in reduced tumor angiogenesis (Nrp-1) and lymphangiogenesis (Nrp-2), respectively, indicating that blocking of Nrp receptors may be a useful strategy for reducing tumor angiogenesis and metastasis in a clinical setting.

Surprisingly, biochemical data obtained with these antibodies indicate that they do not act primarily on reducing VEGF-mediated signal transduction. Treatment of cultured endothelial cells with VEGF ligands in the presence or absence of anti–Nrp-1B or –Nrp-2B antibodies had little effect on VEGFR phosphorylation or phosphorylation of known downstream VEGFR signaling targets extracellular signal-regulated receptor kinase (ERK)-1/2 or AKT. Thus, rather than acting through canonical VEGFR signaling, Nrps may convey additional downstream signaling molecules to the VEGFR complex. Evidence that Nrps may signal independently of VEGFRs has also been obtained by fusing the extracellular domain of the epidermal growth factor (EGF) receptor to the intracellular and transmembrane domains of Nrp-1. This chimeric receptor can promote cell migration in response to EGF, which indicates that the intracellular domain of Nrps can indeed transduce biological signals by themselves, without a coreceptor. So far, the only cytoplasmic protein known to bind to the intracellular domain of Nrp-1 is Synectin, also known as GIPC and Neuropilin-interacting protein (NIP). Synectin is a PDZ adaptor protein that couples uncoated endocytic vesicles to the molecular motor myosin VI and is required for the trafficking of endocytosed membrane receptors. Synectin-deficient mice and zebrafish knockdowns show defective arterial branching morphogenesis and inhibition of Nrp-1–mediated endothelial cell migration. Prahl et al have shown a reduction of Nrp-1/VEGFR-2 interaction in Synectin-deficient endothelial cells, suggesting a role for Synectin in intracellular bridging of Nrp-1–VEGFR-2 receptor complexes and signaling. Knock-in experiments where the intracellular domains of
Netrins and Their Receptors

The Netrins are a family of evolutionarily conserved and structurally related secreted molecules, which display homology to the short arms of laminin γ chains (Netrin-1 and Netrin-3) or laminin β chains (Netrin-4) (Figure 4). The first Netrin to be identified was UNC-6 in Caenorhabditis elegans, followed by vertebrate Netrins and NetrinA and -B in Drosophila. Three members of the Netrin gene family (Netrin-1, Netrin-3 and β-Netrin/Netrin-4) have been identified in mammals. Netrins contain a laminin VI domain, 3 EGF-like repeats similar to the laminin V domain, and a heparin-binding carboxyl-terminal domain (domain C).

In all species in which they were identified, Netrins were demonstrated to regulate axon guidance, giving rise to their name after the Sanskrit word netr: one who guides. Netrins act as bifunctional guidance cues; they can attract some axons, while repelling others. During embryogenesis, Netrin-1 is secreted from cells at the ventral midline of the central nervous system and attracts commissural axons toward the midline. Netrins have also been shown to repel other types of axons, including the trochlear motor axons in vertebrates. Attraction to Netrin-1 is mediated via activation of receptors of the DCC family, as shown by genetic loss-of-function experiments in mice: in both netrin-1- and ddc-deficient mice, commissural axons stall and fail to approach the midline. The DSCAM receptor has also recently been shown to bind Netrin-1 and cooperate with DCC in mediating Netrin-1 attraction and turning of commissural axons. In response to activation by Netrin-1, DSCAM can mediate turning responses of commissural axons in mammalian models. Furthermore, overexpression of DSCAM is capable of mediating a turning response in Xenopus neurons, even though DSCAM is not normally expressed by these neurons, and a dominant negative form of the receptor can selectively block Netrin-1 responses in these cells.

Repulsion in response to Netrin-1 requires signaling through UNC5 receptor homodimers or UNC5-DCC receptor heterodimers. In Xenopus, DCC-mediated attraction of spinal axons is converted to repulsion by ectopic expression of UNC5 family receptors, a mechanism dependent on the interaction of the cytoplasmic parts of both receptors. UNC5 can also mediate repulsion in the absence of DCC, albeit at a shorter range. Netrin-mediated attraction can also be converted to repulsion by altering the level of intracellular cyclic nucleotides.

The DCC family consists of DCC and Neogenin, whereas the UNC5 family comprises 4 members, UNC5A to -D. The DCC receptor is composed of an extracellular domain containing 6 fibronectin type 3 repeats (FN3) and 4 immunoglobulin repeats (Ig), a transmembrane domain, and an intracellular domain, which contains 3 domains coined P1, P2, and P3 (Figure 4). The intracellular domain of DCC contains several putative protein-binding and phosphorylation sites. The Netrin-1–binding domain is localized in one of the fibronectin type 3 repeats, although it is unclear which repeat binds Netrin-1. The UNC5 family are transmembrane receptors composed of 2 immunoglobulin and 2 thrombospondin-like domains in the extracellular region, and a zona occludens 5 domain, a DCC-binding domain and a death domain in the intracellular portion (Figure 4). Repulsion in response to Netrin-1 requires signaling through UNC5 receptor homodimers or UNC5-DCC receptor heterodimers. In Xenopus, DCC-mediated attraction of spinal axons is converted to repulsion by ectopic expression of UNC5 family receptors, a mechanism dependent on the interaction of the cytoplasmic parts of both receptors. UNC5 can also mediate repulsion in the absence of DCC, albeit at a shorter range. Netrin-mediated attraction can also be converted to repulsion by altering the level of intracellular cyclic nucleotides.

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primary endothelial cells and neovessels invading Matrigel plugs was undetectable, apart for low levels of Neogenin and Unc5A.\textsuperscript{28,108} During embryonic development, Unc5b is expressed in arterial endothelial cells and sprouting capillaries including endothelial tip cells in both mouse and chick. Interestingly, expression of this receptor is almost undetectable in the nervous system in both species, except for the developing retina, ear, and cerebellum in mice, suggesting that during evolution and diversification of the Netrin receptor family, expression of Unc5b has been coopted by the vascular system. Unc5b mRNA expression is downregulated in quiescent vasculature of adult mice and in quiescent vessels of the chorioallantoic membrane\textsuperscript{109} of the chick embryo. However, when sprouting angiogenesis is reinduced using various models, including Matrigel implants, tumor xenografts, and oxygen-induced retinopathy in mice, or on using various models, including Matrigel implants, tumor embryo. However, when sprouting angiogenesis is reinduced perhaps reflecting a less important contribution of sprouting ligation, where endothelial reexpression was not observed, this pattern is conserved between chick and mice. An exception is associated with active sprouting angiogenesis (Figure 5), and this pattern is conserved between chick and mice. An exception is angiogenesis in the mouse hindlimb following femoral artery ligation, where endothelial reexpression was not observed, perhaps reflecting a less important contribution of sprouting angiogenesis to this form of neovascularization.\textsuperscript{108}

In mice homozygous for an \textit{unc5b} deletion, the absence of \textit{unc5b} results in aberrant filopodial extension in sprouting capillaries, which in turn gives rise to increased capillary branching.\textsuperscript{28} Unc5b activation using Netrin-1 as an agonist results in retraction of filopodia in endothelial tip cells of the retina and in aortic ring sprouting assays. Adult neovessel sprouting processes, including basic fibroblast growth factor–induced Matrigel plug invasion were also inhibited by recombinant Netrin-1. Retroviral overexpression of Netrin-1 in tumor cell lines led to reduced tumor neovessel sprouting compared to control vector–transduced cells in vivo and in vitro.\textsuperscript{28,108,112,114} These results from other groups have found that Netrin-4 does not bind Unc5b directly\textsuperscript{108,112,114} but may rather exerts its antiangiogenic effects by binding to Neogenin, which then recruits Unc5b to mediate signaling of the antiangiogenic activities of Netrin-4.\textsuperscript{112} Analysis of Netrin-4–deficient mice will show whether loss of this molecule leads to excessive angiogenesis and vessel branching.

In contrast to the results described above, other studies report that Netrin-1 and Netrin-4 can induce proliferation and migration of endothelial cells in vitro.\textsuperscript{114–117} In 2 of these studies,\textsuperscript{114,116} the receptors implicated in mediating Netrin effects could not be detected on the endothelial cells used, although they were of the same origin (human umbilical vein and artery) as the ones used by Lariève et al\textsuperscript{108} and Lejmi et al.\textsuperscript{112} Nguyen and Cal\textsuperscript{115} have reported DCC expression in bovine aortic endothelial cells using antibody staining, and they suggest that the promigratory effects of Netrin-1 they observe in vitro were mediated through DCC signaling and an ERK-1/2–endothelial nitric oxide synthase feed-forward mechanism. However, PCR analysis of endothelial cells is consistently negative for DCC expression in other studies.\textsuperscript{28,108,112,114,116} raising doubts about potential roles of DCC as an “attractive” receptor for Netrin-1 on endothelial cells. The receptor(s) mediating the attractive effects by Netrins on endothelial cells in vitro thus remain to be identified conclusively.

Wilson et al reported that in vivo injection of plasmids encoding Netrin-1 and Netrin-4 both accelerated neovascularization in a model of hindlimb ischemia by increasing smooth muscle cell recruitment and could reverse neuropathy and vasculopathy in a diabetic mouse model.\textsuperscript{114} However, the mechanisms by which Netrins can revert these hallmarks of diabetes in the presence of a persistent diabetic milieu remain unclear. The Netrin receptor responsible for these effects has not been identified in those studies, because the authors could not detect expression of any known Netrin receptors in ischemic tissue in vivo. The possibility that Netrin overexpression in ischemic tissues might target and influence the
function of nonvascular inflammatory cells such as macrophages and/or monocytes remains to be determined. Hoang et al report enhanced angiogenesis in a mouse cerebral ischemia model following administration of Ntn-4 protein and correlated this effect with Dcc expression in neurons surrounding the ischemic area, indicating an indirect effect of Ntn-4 stimulation of angiogenesis.\(^\text{118}\)

Finally, Navankasattusas et al, recently reported that conditional deletion of a floxed unc5b mutant allele using Tie-2-cre led to defects in the placental vasculature and loss of placental arterioles, resulting in flow reversal in the umbilical artery and embryonic death.\(^\text{119}\) This study suggests that Unc5b activation could promote angiogenesis in specific vascular beds. However, it remains to be determined whether this allele represents a true null mutation, because CRE-mediated deletion of loxP sites inserted within introns 3 and 13 may generate a truncated protein containing part of the ligand-binding and the cytoplasmic domains.

Together, these data suggest that Netrins may have dual activity during angiogenesis, just like they do in axon guidance. However, the receptor(s) that mediate attraction/proliferation in endothelial cells in response to Netrins remain to be identified.

**Slits and Roundabouts**

Slits are large secreted glycoproteins, initially described as repulsive guidance cues in neural development.\(^\text{120,121}\) Slits have also been shown to play a role in embryonic kidney induction,\(^\text{122}\) leukocyte migration,\(^\text{123}\) and angiogenesis.\(^\text{124}\)

Structurally, Slits comprise a long stretch of 4 leucine-rich repeats, 7 to 9 EGF repeats, and a LamG domain (Figure 4).\(^\text{125}\) There are also Slit cleavage fragments, which appear to have different cellular association characteristics, with the smaller C-terminal fragment being more diffusible and the larger N-terminal and full-length fragments being more tightly cell associated.

Slits bind to transmembrane receptors of the Roundabout family and additionally bind heparan sulfate proteoglycans, which may help in stabilizing the Slit/Robo complex or function as coreceptors presenting Slits to Robos or to alternative receptors.\(^\text{125}\) So far, 3 Slit proteins (Slit1 to -3) and 4 Robo proteins (Robo1, Robo2, Robo3/Rig-1, and Robo4/magic Roundabout) have been found in mammals. Robos 1 to 3 are prominently expressed in the nervous system and are characterized by the presence of 5 Ig-like domains followed by 3 fibronectin type 3 (FNIII) repeats, a transmembrane portion and a cytoplasmic tail containing up to 4 conserved Robo-specific motifs termed CC0 to CC3. Robo was named because of the neuronal phenotype arising from its deletion in Drosophila, where ipsilateral axons that normally avoid the midline cross it, and commissural axons cross and recross it repeatedly.\(^\text{126}\) In addition to the firmly established role for Slit/Robo signaling in commissural midline crossing, they are implicated in additional axon guidance processes, including formation of longitudinal CNS tracts, projection of vomeronasal axons to the accessory olfactory bulb, and branching of central trigeminal sensory axons, and they regulate differentiation and migration of diverse neuronal cell populations.\(^\text{127}\)

In contrast to Robo1-3, Robo4 was identified as an endothelial-specific Robo receptor using bioinformatics tools.\(^\text{128}\) Although structurally related to the other Robo receptors, Robo4 contains only 2 Ig-like domains and 2 FNIII repeats in the extracellular portion and lacks the CC1 and CC3 motifs found in most other Robo proteins. In situ hybridization with robo-4 antisense riboprobes detects endothelial cells in developing mouse embryos and tumor vessels.\(^\text{129}\) The 3-kb 5'-flanking region of human Robo4, containing SP1- and Ets-binding sites, directs endothelial cell–specific expression in vitro.\(^\text{130,131}\) This promoter was coupled to β-galactosidase and introduced into the Hprt locus of mice by homologous recombination. Reporter gene activity was observed in the vasculature of adult organs (particularly in microvessels), tumor xenografts, and embryos, where it localized with the endothelial cell-specific marker CD31, indicating that this upstream promoter contains information for cell type-specific expression in the intact endothelium.

The zebrafish Robo4 homolog is expressed in both neuronal tissue and in blood vessels, including dorsal aorta, cardinal vein, and sprouting intersomitic vessel tip cells.\(^\text{132}\) Morpholino knockdown of Robo4 in zebrafish resulted in temporal and spatial disruption of intersomitic vessel development, indicating a requirement for Robo4 in directing blood vessel growth to the correct path.\(^\text{132}\) Angioblasts isolated from these embryos and cultured ex vivo showed more active and extensive movement when compared to angioblasts from control morpholino-injected fish and display lower amounts of active Cdc42 and Rac, consistent with loss of attractive function of Robo4 in mutant.\(^\text{133}\)

There has been some controversy regarding the function of Robo4 as an attractive or a repulsive guidance receptor, and whether it is a receptor for Slits. Park et al showed that Robo4 can provide a repulsive cue to migrating endothelial cells during murine vascular development by binding to Slit2 and inhibiting cellular migration.\(^\text{129}\) However, this contrasts with results obtained by Wang et al, who showed that purified Slit2 acted as a chemoattractant to endothelial cells and to transformed cells overexpressing Robo1.\(^\text{134}\) They also showed that tumor blood vessels express Robo1 and that overexpression of Slit2 in tumor xenografts results in increased tumor angiogenesis. A role for Robo1 during developmental angiogenesis has not been described so far. However, Robo1 expression and heterodimerization with Robo4 in cultured endothelial cells in vitro has been described recently, indicating that Slit2 binding to endothelial Robo1 could activate Robo4 and affect endothelial cell migration.\(^\text{135}\)

A recent study has suggested that Robo4, instead of having a guidance function, is required to maintain blood vessel integrity.\(^\text{136}\) Robo4 expression was observed in endothelial stalk cells, as opposed to tip cells, and Slit2 was shown to inhibit endothelial migration, tube formation, and permeability induced by VEGF. Therefore, Robo4 could help to maintain vascular integrity by preventing stalk cells from being activated by VEGF. Binding of Slit2 to Robo4 has, however, never been convincingly demonstrated, even when using the sensitive Biacore detection method.\(^\text{137}\) These findings are likewise contradicted by a different study that showed inhibition of angiogenesis and endothelial cell mi-
gration using a soluble Robo4 extracellular domain. In summary, solid evidence that links Robo4 function with ligand binding has so far remained elusive. Because there is no developmental vascular phenotype reported for any of the Slit or Robo mutants, elucidating the precise role of this pathway in vessel morphogenesis or guidance may await identification of a vessel-specific ligand.

Conclusions
It has become apparent in recent years that there are extensive similarities between the development of nerves and blood vessels. Both are branched structures that require guidance to reach their proper targets. A variety of molecules that were previously thought to be restricted to axonal guidance processes have now been shown to modulate blood vessel guidance as well. These insights are of significance as they could have potential therapeutic applications. The fact that molecules such as the Slits, Semaphorins, and Netrins may modulate physiological, as well as pathological, angiogenesis is likely to lead to the development of novel strategies to promote or inhibit angiogenesis. The design of agonists that can activate specific axon guidance molecules could especially be significant in the future development of angiogenesis-targeting therapies. In addition, crosstalk of blood vessels and nerves is not restricted to a role of axonal guidance cues in blood vessels, but receptors for vascular endothelial growth factors have been found on neuronal cell bodies and are implicated in the regulation of neuronal survival. Furthermore, neurogenesis in the central nervous system occurs in sites where blood vessels lie, and it was proposed that endothelial cells provide a microenvironment or “vascular niche” regulating the self-renewal and differentiation of neural progenitor cells. Thus, endothelial cell–neuronal crosstalk appears to regulate multiple aspects of the development of both systems and promises many exciting discoveries in the years to come.

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Disclosures
None.

References


128. Humniecei K, Gorn M, Suchting S, Poulsom R, Bicknell R. Magic roundabout is a new member of the roundabout receptor family that is endothelial specific and expressed at sites of active angiogenesis. *Genomics.* 2002;79:547–552.


