Guidance of Vascular Development
Lessons From the Nervous System

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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
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 shows a mutual requirement of one for the other, ie, larger nerves require vascularization to ensure nutrient and oxygen supply, whereas 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.

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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 (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-
trophic 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.

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...
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 direct their migration. The next section focuses on the analogies in the mechanisms that endothelial cells and neurons use to reach their proper targets.

Evidence from the mouse and zebrafish indicates that the Notch ligand DLL4 is the key mediator of tip cell specification in vessels.27,29,35,41 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.27,29,39 Morphotypic knockdowns of Notch signaling components led to similar phenotypes in the embryonic zebrafish vasculature.38,41 Moreover, disruption of the Notch pathway by either pharmacological inhibition, DLL4-blocking antibodies or soluble DLL4-Fc recapitulated the dll44/− vascular phenotype.27,29,39,42 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.27,41

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 DLL4.20,39 In addition, expression levels of VEGFRs are altered in dll44/− retinal vessels in a way that suggests these vessels are more sensitive to VEGF signal and that Notch could regulate the VEGF response.20 Thus, a putative model for tip cell formation emerges where tip cell induction depends on the differential VEGF isofom distribution in the extracellular environment,2,3 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.43

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.44,45 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-μ/H9262) (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 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 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 Nrp-1 mutants, arterial–venous differentiation is normal in Nrp-2 knockouts. Instead, homozygous 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 Nrp-1 and Nrp-2 mutants: Nrp-1<sup>−/−</sup> mice show deficiencies in cranial nerves VII, IX, and X, which are not affected in Nrp-2<sup>−/−</sup>.
mice. Conversely, cranial nerves II and IV, which are normal in Nrp-1−/− mice, show abnormal projections in Nrp-2−/− animals.44,60,64 Dorsal root ganglion (DRG) axons expressing Nrp-1 are efficiently collapsed or repelled by Sema3A in vitro, and it was for this activity that Semaphorins were initially named collapsins.68 DRG axons from Nrp-1−/− mice lose repulsive responses to Sema3A in vitro and the guidance defects of cranial axons seen in Nrp-1−/− mice are phenocopied in sema3A mutant mice,69 indicating that Sema3A is the ligand required to normally repel Nrp-1−expressing cranial axons. Similarly, sympathetic ganglia from Nrp-2−/− animals lose response to Sema3F,44,64 and Sema3F is the primary Nrp-2 ligand in several axonal projection systems, including cranial nerves and limbic circuitry.66 spinal motor axons,67 and olfactory neurons.68 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,69 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.53,70 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 -3.81,71–73 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.53 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.74 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.75,76 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.77

Initial studies suggested that Semaphorins and VEGF family members could compete for binding to each other. Sema3A stimulation of Nrp-1–expressing endothelial cells inhibits endothelial cell migration and capillary sprouting in aortic ring assays.78 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 VEGF164.79 In contrast, Sema3A, but not VEGF164, is required for axon patterning of limb nerves.79 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.80 Furthermore, Semaphorins and VEGF induce Nrp-1 endocytosis through different pathways; VEGF binding induces clathrin-mediated endocytosis, whereas Sema3C induces lipid raft–dependent endocytosis.81 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.81

Blocking antibodies disrupting either Semaphorin binding or VEGF family member binding to Nrps were recently generated.57,77 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-2.72 Anti–Nrp-2B blocks VEGF-165 and VEGF-C binding to Nrp-2 and prevents dimerization of Nrp-2 with VEGFR-2 and VEGFR-3.57 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.57,73 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 VEGFR-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.82 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.48,83 Prahet 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.84 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 dcc-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 family homodimers or UNC5-DCC receptor heterodimers. In *Xenopus,* DCC-mediated attraction of spinal axons is converted to repulsion by ectopic expression of the 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 zonula occludens 5 domain, a DCC-binding domain and a death domain in the intracellular portion (Figure 4). Netrin-1–binding domain on UNC5 receptors has been shown to be localized in the Ig repeats. UNC5b, an adenosine receptor, has been shown to be a Netrin-1 receptor, but its function in mediating Netrin-1 events in vivo is still unclear. Finally, the epithelial integrin α6β4 has also been reported to act as a Netrin-1 receptor and to play a role in epithelial cell migration in response to Netrin-1.

Recently, Netrins have been reported to regulate developmental processes including cell adhesion, motility, differentiation, and survival in nonneuronal tissues. Roles for Netrins in these tissues have been extensively reviewed recently and are not addressed here. We focus instead on possible roles of Netrins and their receptors in developmental and pathological angiogenesis. As with axon guidance, Netrins may have bifunctional activities in angiogenesis, because different groups have reported that they can have both repulsive and attractive actions on endothelial cells.

Studies from our group have consistently revealed repulsive properties for Netrins on endothelial cells. We have found that among Netrin receptors, the repulsive type receptor Unc5b is selectively expressed in the endothelium of growing capillaries and participates in the regulation of vascular system morphogenesis, both during developmental and pathological angiogenesis (Figures 3 and 5). Except for Unc5b, expression of other Netrin receptors in Nrps are selectively deleted will show whether Nrps signals through Synectin in endothelial cells in vivo.
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 chorioallantois 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 the chick chorioallantoic membrane (CAM), there is strong upregulation and reexpression of unc5b mRNA in growing neovascular sprouts.\textsuperscript{105,108} Thus, Unc5b expression 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 unc5b deletion, the absence of 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 in vivo thus 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 mouse xenograft models and grafts on the chick CAM. In both models, Netrin-1 overexpression inhibited the initial invasion of the growing tumor nodules by Unc5b-expressing neovessels, but vessels eventually invaded the tumors and vessels density reached similar levels in Netrin-1–expressing and control-transduced tumors, suggesting that Netrin-1 selectively repels sprouting Unc5b neovessel tips but does not influence other steps of vessel growth such as proliferation.

Netrin-1 secreted by retrovirally transduced tumor cell lines is able to repel porcine aortic endothelial cells expressing Unc5b whereas this repulsion is lost in PAEC expressing a cytoplasmic deleted form of this receptor, indicating that retraction requires signaling through the Unc5b receptor. Netrin-1 treatment inhibited sprouting of microcarrier beads coated with PAEC cells expressing Unc5b, but not of nontransduced parental cells or cells expressing the cytoplasmic domain–deleted version of Unc5B, again indicating that Netrin-1 repels endothelial cells in an Unc5B-dependant manner. In contrast, Netrin-1 treatment of PAE cells or human umbilical vein or artery endothelial cells in vitro failed to induce their proliferation, suggesting that its effect is restricted to filopodial repulsion.\textsuperscript{108}

Collectively, all our data show that Unc5b activation prevents filopodia extension in endothelial cells, thereby participating in vessel patterning by negatively regulating capillary branching. Whether these effects are mediated by activation through endogenous Netrins remains to be determined, because no vascular defects have been described in mice deficient for Netrin-1,\textsuperscript{110,111} and loss-of-function genetic data for Netrin-3 and -4 are not yet available.

In support of these results, Lejmi et al recently reported an antiangiogenic role for Netrin-1 and -4 in vitro and for Netrin-4 in vivo.\textsuperscript{112} They showed that Netrin-4 is upregulated in angiogenic endothelial cells following long-term stimulation with VEGF and that Netrin-1 or Netrin-4 inhibited VEGF-induced endothelial cell migration in vitro. Recombinant Netrin-4 also inhibited pathological angiogenesis including Matrigel neovessel sprouting and laser-induced choroidal neovascularization, whereas Netrin-4–overexpressing PC3 cells showed reduced tumor neovascularization and growth compared to untransfected controls. Thus, Netrin-4 may act as a negative-feedback regulator of angiogenesis. Although one study has reported Netrin-4 binding to Unc5 receptors,\textsuperscript{113} 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 Larriveé et al\textsuperscript{108} and Lejmi et al.\textsuperscript{112} Nguyen and Caï\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 Netrin-4 protein and correlated this effect with Dec expression in neurons surrounding the ischemic area, indicating an indirect effect of Netrin-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 colocalized 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 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 microenvironement 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

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References

1. Coultas L, Chawengsaksophak K, Rossant J. Endothelial cells and VEGF in vascular development. Nature. 2005;438:937–945.
2. Gerhardt H, Golding M, Fruttiger M, Ruhrberg C, Lundkvist A, Abramsson A, Jeltsch M, Mitchell C, Altaloo K, Shima D, Betsholtz C. VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. J Cell Biol. 2003;161:1163–1177.
3. Ruhrberg C, Gerhardt H, Golling M, Watson R, Ioannidou S, Fujisawa H, Betsholtz C, Shima DT. Spatially restricted patterning cues provided by heparin-binding VEGF-A control blood vessel branching morphogenesis. Genes Dev. 2002;16:2684–2698.
4. Lucitti JL, Jones EA, Huang C, Chen J, Fraser SE, Dickinson ME. Vascular remodeling of the mouse yolk sac requires hemodynamic force. Development. 2007;134:3317–3326.
5. le Noble F, Mouny D, Pardanaud L, Yuan L, Djonov V, Matthijsen R, Breaat C, Fleury V, Eichmann A. Flow regulates arterial-venous differentiation in the chick embryo yolk sac. Development. 2004;131:361–375.
6. Kalcheim C, Le Douarin NM. The Neural Crest. Cambridge University Press, 1999.
7. Glebova NO, Ginty DD. Growth and survival signals controlling sympathetic nervous system development. Annu Rev Neurosci. 2005;28:191–222.
8. Makita T, Sucov HM, Gariepy CE, Yanagisawa M, Ginty DD. Endothelins are vascular-derived axonal guidance cues for developing sympathetic nerves. Nature. 2008;452:759–763.
9. Airaksinen MS, Saarma M. The GDNF family: signalling, biological functions and therapeutic value. Nat Rev Neurosci. 2002;3:383–394.
10. Baloh RH, Tansey MG, Lampe PA, Fahner TJ, Enomoto H, Simbürger KS, Leitner M, Araki T, Johnson EM Jr, Milbrandt J, Artemin, a novel member of the GDNF ligand family, supports peripheral and central neurons and signals through the GFRalpha3-RET receptor complex. Neuron. 1998;21:1291–1302.
11. Honma Y, Araki T, Gianino S, Bruce A, Heuckeroth R, Johnson E, Milbrandt J. Artemin is a vascular-derived neurotropic factor for developing sympathetic neurons. Neuron. 2002;55:267–282.
12. Enomoto H, Crawford PA, Gorodinsky A, Heuckeroth R, Johnson EM Jr, Milbrandt J. RET signaling is essential for migration, axonal growth and axon guidance of developing sympathetic neurons. Development. 2001;128:3963–3974.
13. Kuruvilla R, Zweifel LS, Glebova NO, Lonze BE, Valdez G, Ye H, Ginty DD. A neurotrophin signaling cascade coordinates sympathetic neuron development through differential control of TrkA trafficking and retrograde signaling. Cell. 2004;118:243–255.
14. Mukoyama YS, Shin D, Britsch S, Taniguchi M, Anderson DJ. Peripheral nerve-derived VEGF promotes arterial differentiation via neuropilin 1-mediated positive feedback. Development. 2005;132:941–952.
15. Carmeliet P, Tessier-Lavigne M. Common mechanisms of nerve and blood vessel wiring. Nature. 2005;436:193–200.
16. Charron F, Tessier-Lavigne M. The molecular biology of axon guidance. Science. 1996;274:1123–1133.
17. Tessier-Lavigne M, Goodman CS. Peripheral nerve guidance and growth cone mechanisms of nerve and blood vessel branching. Nature. 2005;436:193–200.
18. Dolton JG, Merz DC. Vascular development. Curr Opin Cell Biol. 1998;10:609–613.
19. Dickson BJ, Kelemen K, Nettles. Curr Biol. 2002;12;R154–R155.
20. Kidd T, Brose K, Mitchell KJ, Fetter RD, Tessier-Lavigne M, Goodman CS, Tear G. Roundabout controls axon crossing of the CNS midline and defines a novel subgroup of evolutionarily conserved guidance receptors. Cell. 1998;92:205–215.
21. Conlon H, Sabatier C, Brose K, Mitchell KJ, Fetter RD, Tessier-Lavigne M, Goodman CS, Tear G. Roundabout controls axon crossing of the CNS midline and defines a novel subgroup of evolutionarily conserved guidance receptors. Cell. 1998;92:205–215.
22. Lowry H, Murphy L, Anderson DJ. Artemin is a vascular-derived neurotrophic factor for developing sympathetic neurons. Neuron. 2002;55:267–282.
23. Carmeliet P, Tessier-Lavigne M. Common mechanisms of nerve and blood vessel wiring. Nature. 2005;436:193–200.
24. Charron F, Tessier-Lavigne M. Novel brain wiring functions for classical morphogens: a role as graded positional cues in axon guidance. Development. 2005;132:2251–2262.
25. Iruela-Arispe ML, Kalen M, Gerhardt H, Betsholtz C. Dll4 signalling...
through Notch1 regulates formation of tip cells during angiogenesis. Nature. 2007;447:776–780.

28. Lu X, Le Noble F, Yuan L, Jiang Q, De Lafarge B, Sugiyama D, Breant C, Claes F, De Smet F, Thomas JL, Autiero M, Carmeliet P, Tessier-Lavigne M. The netrin receptor UNC5B mediates guidance events controlling morphogenesis of the vascular system. Nature. 2004;432:179–186.

29. Suchting S, Freitas C, le Noble F, Benedito R, Breant C, Duarte A, Eichmann A. The Notch ligand Delta-like 4 negatively regulates endothelial tip cell formation and vessel branching. Proc Natl Acad Sci U S A. 2007;104:3225–3230.

30. Tammela T, Zarkada G, Wallgard E, Murtomaki A, Suchting S, Wirzenius M, Roca C, Adams RH. Regulation of vascular morphogenesis by Notch signaling. Genes Dev. 2004;18:2469–2473.

31. Fruttiger M. Development of the retinal vasculature. Angiogenesis. 2007;10:77–88.

32. Roca C, Adams RH. Regulation of vascular morphogenesis by Notch signaling. Genes Dev. 2007;21:2511–2524.

33. Ghabrial AS, Krasnow MA. Social interactions among epithelial cells-interacting protein: a PSD-95/Dlg/ZO-1 domain-containing protein that interacts with the cytoplasmic domain of neuropilin-1. J Neurosci. 1999;19:6519–6527.

34. Takagi S, Hirta T, Agata K, Mochii M, Eguchi G, Fujisawa H. The A5 antigen, a candidate for the neuronal recognition molecule, has homologies to complement components and coagulation factors. Neuron. 1991;7:295–307.

35. Duarte A, Hirashima M, Benedito R, Trindade A, Diniz P, Bekman E, Murphy AJ, Adams NC, Lin HC, Walsh FS, Kolodkin AL, Ginty DD. Neuropilin is a semaphorin III receptor. Neuron. 1997;19:547–559.

36. Walz A, Feinstein P, Khan M, Mombaerts P. Axonal wiring of guanylate cyclase-D-expressing olfactory neurons is dependent on neuropilin 2. Neuron. 2003;35:2479–2488.

37. Krebs LT, Shutter JR, Tanigaki K, Honjo T, Stark KL, Gridley T. Neuropilin-2, a novel member of the neuropilin family, is a high affinity receptor for vascular endothelial growth factor. Exp Cell Res. 2006;312:668–675.

38. Soker S, Takahashi S, Miao HQ, Neufeld G, Klagsbrun M. Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. Cell. 1999;98:735–745.

39. Herzog Y, Kalcheim C, Kahane N, Reshef R, Neufeld G. Differential expression of neuropilin-1 and neuropilin-2 in arteries and veins. Mech Dev. 2001;109:115–119.

40. Moyon D, Pardanaud L, Yuan L, Breant C, Eichmann A. Plasticity of endothelial cells during arterial-venous differentiation in the avian embryo. Development. 2001;128:3359–3370.

41. Siekmann AF, Lawson ND. Notch signalling limits angiogenic cell progression and tumour angiogenesis. Nat Rev Cancer. 2008;8:623–645.

42. Ridgway J, Zhang G, Wu Y, Stawicki S, Liang WC, Chanthery Y, Murphy AJ, Adams NC, Lin HC, Walsh FS, Kolodkin AL, Ginty DD, Carano R, Koch AW, Wu Y, Watts RJ, Tessier-Lavigne M, Bagri A. Blocking neuropilin-2 function inhibits tumor cell metastasis. Cancer Cell. 2008;13:331–342.

43. Yuan L, Moyon D, Pardanaud L, Breant C, Karkkainen MJ, Alitalo K, Eichmann A. Abnormal lymphatic vessel development in neuropilin 2 mutant mice. Development. 2002;129:4977–4806.

44. Kowalski T, Kitsukawa T, Bekku Y, Matsuda Y, Sanbo M, Yagi T, Fujisawa H. A requirement for neuropilin-1 in embryonic vessel formation. Development. 1999;126:4895–4902.

45. Jones EA, Yuan L, Breant C, Watts RJ, Eichmann A. Separating genetic and hemodynamic defects in neuropilin 1 knockout embryos. Development. 2008;135:2479–2488.

46. Gerhardt H, Ruhrberg C, Abramsson A, Fujisawa H, Shim a D, Betsholtz C. Neuropilin-1 is required for endothelial tip cell guidance in the developing central nervous system. Dev Dyn. 2004;231:503–509.

47. Gu C, Rodriguez ER, Reimert DV, Shu T, Fritzsche B, Richards LJ, Kolodkin AL, Ginty DD. Neuropilin-1 conveys semaphorin and VEGF signaling during neural and cardiovascular development. Dev Cell. 2003;5:45–57.

48. Giger RJ, Cloutier JF, Sahay A, Prinjha RK, Leve ngood DV, Moore SE, Pickering S, Simmons D, Rastan S, Walsh FS, Kolodkin AL, Ginty DD, Geppert M. Neuropilin-2 is required in vivo for selective axon guidance responses to secreted semaphorins. Neuron. 2000;25:29–41.

49. Luo Y, Raible D, Raper JA, Collapsin: a protein in brain that induces the collapse and paralysis of neuronal growth cones. Cell. 1993;75:217–227.

50. Sahay A, Molliver ME, Ginty DD, Kolodkin AL. Semaphorin 3F is critical for development of limbic system circuitry and is required in neurons for selective CNS axon guidance events. J Neurosci. 2003;23:6671–6680.

51. Huber AB, Kania A, Tran TS, Gu C, De Marco Garcia N, Lieberman L, Johnson D, Jessell TM, Ginty DD, Kolodkin AL. Distinct roles for secreted semaphorin signaling in spinal motor axon guidance. Neuron. 2005;48:949–964.

52. Walz A, Feinstein PK, Khan M, Mombaerts P. Axonal wiring of guanylate cyclase-D-expressing olfactory neurons is dependent on neuropilin 2 and semaphorin 3F. Development. 2007;134:4063–4072.

53. Schwarz Q, Vieira JM, Howard B, Eckhost BJ, Ruhrberg C. Neur onephilin-1 and -2 control cerebral angiogenesis and axon guidance through neural crest cells. Development. 2008;135:1605–1613.

54. Fujisawa H. Discovery of semaphorin receptors, neuropilin and plexin, and their functions in neural development. J Neurobiol. 2004;59:24–33.

55. Favier B, Alam A, Barron P, Bonnin J, Labodie P, Fons P, Mandron M, Herva uit JP, Neufeld G, Sai vi P, Herbert JM, Bono F. Neuropilin-2 interacts with VEGFR-2 and VEGFR-3 and promotes human endothelial cell survival and migration. Blood. 2006;108:1243–1250.
72. Karpenan T, Heckman CA, Keskitalo S, Jeltsch M, Ollila H, Neufeld G, Tamagnone L, Altalito K. Functional interaction of VEGF-C and VEGF-D with neuropilin receptors. FASEB J. 2006;20:1462–1472.

73. Pan Q, Chantry H, Liang WC, Stawicki S, Mak J, Rathore N, Tong QC, Schwarz Q, Ruhrberg C. Selective requirements for NRP1 and neuropilin-2 in angiogenesis. Development. 2007;134:1833–1843.

74. Appleton BA, Wang P, Maloney J, Yin J, Liang WC, Stawicki S, Mortara A, Wang L, Zeng H, Wang P, Soker S, Mukhopadhyay D. Neuropilin-1–antibody complexes provide insights into semaphorin and VEGF binding. Circ Res. 2008;103:e71–e79.

75. Serafini T, Kennedy TE, de la Torre JR, Tessier-Lavigne M. The netrins define a family of axon outgrowth-promoting ligands during nervous system development. Neuron. 1999;19:4938–4947.

76. Geisbrecht BV, Dowd KA, Barfield RW, Longo PA, Leahy DJ. Netrin binds discrete subdomains of DCC and UNC5 and mediates interactions between DCC and hematopoietic cell-specific transcription factor. J Biol Chem. 2003;278:32561–32568.

77. Skarnes WC, Tessier-Lavigne M. Netrin-1 is required for commissural axon guidance in the developing vertebrate nervous system. Cell. 1995;86:175–185.

78. Ly A, Nikolova A, Suresh G, Zheng Y, Tessier-Lavigne M, Stein E. DSCAM is a netrin receptor that collaborates with DCC in mediating turning responses to netrin-1. Cell. 2008;133:1241–1254.

79. Vieira JM, Schwarz Q, Ruhrberg C. Selective regulation of arterial endothelial cell phenotype by VEGF-A and VEGF-C. Circ Res. 2007;105:621–629.

80. Keino-Masu K, Masu M, Hinck L, Leonardo ED, Chan SS, Culotti JG, Tessier-Lavigne M. Deleted in colorectal cancer (DCC) encodes a netrin receptor. Cell. 1996;87:175–185.

81. Colamarino SA, Tessier-Lavigne M. The axonal chemorepellent netrin-1 is also a chemorepellent for trockholar motor axons. Cell. 1995;81:621–629.

82. Miao HQ, Soker S, Feiner L, Alonso JL, Raper JA, Klagsbrun M. Neuropilin-1 and neuropilin-2 act as coreceptors, potentiating proangiogenic activity. Blood. 2008;111:2036–2045.

83. Sennlaub F, Plouet J. Netrin-4 inhibits angiogenesis via binding to neuropilin 2. EMBO J. 2007;26:4902–4912.

84. Klagsbrun M, Peale F, Koch AW, Wu Y, Bagri A, Tessier-Lavigne M, Watts RJ. Blocking neuropilin-1 function has an additive effect with anti-VEGF to inhibit tumor growth. Cancer Cell. 2007;11:53–67.

85. Vida MC, Childs S, Epstein JA, Weinstein BM. Semaphorin-plexin signaling patterns the developing vasculature. Dev Cell. 2004;7:117–123.

86. Vieira JM, Schwarz Q, Ruhberg C. Selective requirements for NRP1 ligands during neurovascular patterning. Development. 2007;134:1833–1843.

87. Serafini T, Colamarino SA, Leonardo ED, Pan Q, Chan SS, Culotti JG, Tessier-Lavigne M. Functional interaction of VEGF-C and VEGF-D with neuropilin receptors. FASEB J. 2006;20:1462–1472.

88. Kennedy TE, Serafini T, Galko MJ, Mirzayan C, Jessell TM, Tessier-Lavigne M. The UNC-5 netrin receptor guides pioneer axon migrations in C. elegans. Science. 2003;299:1065–1069.

89. Ly A, Nikolova A, Suresh G, Zheng Y, Tessier-Lavigne M, Stein E. DSCAM is a netrin receptor that collaborates with DCC in mediating turning responses to netrin-1. Cell. 2008;133:1241–1254.

90. Chittenden TW, Claes F, Lanahan AA, Autiero M, Palac RT, Tkachenko I, Vassar R, Fruttiger M, LeCouter J, Carmeliet P, Tessier-Lavigne M. Selective regulation of arterial endothelial cell phenotype by VEGF-A and VEGF-C. Circ Res. 2007;105:621–629.

91. Bennett KL, Bradshaw J, Youngman T, Rodgers J, Greenfield B, Aruffo A, Linsley PS. Deleted in colorectal carcinoma (DCC) binds heparin via its fifth fibronectin type III domain. J Biol Chem. 1997;272:26940–26946.

92. Geisbrecht BV, Dowd KA, Barfield RW, Longo PA, Leahy DJ. Netrin binds discrete subdomains of DCC and UNC5 and mediates interactions between DCC and hematopoietic cell-specific transcription factor. J Biol Chem. 2003;278:32561–32568.

93. Corset V, Nguyen-By-Charvet KT, Forcet C, Moyse E, Chedotal A, Stein E. A ligand-gated association between cytoplasmic domains of UNC5 and DCC family receptors converts netrin-induced growth cone attraction to repulsion. Cell. 1999;97:927–941.

94. Bennett KL, Bradshaw J, Youngman T, Rodgers J, Greenfield B, Aruffo A, Linsley PS. Deleted in colorectal carcinoma (DCC) binds heparin via its fifth fibronectin type III domain. J Biol Chem. 1997;272:26940–26946.

95. Vida MC, Childs S, Epstein JA, Weinstein BM. Semaphorin-plexin signaling patterns the developing vasculature. Dev Cell. 2004;7:117–123.

96. Vieira JM, Schwarz Q, Ruhberg C. Selective requirements for NRP1 ligands during neurovascular patterning. Development. 2007;134:1833–1843.

97. Serafini T, Colamarino SA, Leonardo ED, Pan Q, Chan SS, Culotti JG, Tessier-Lavigne M. Functional interaction of VEGF-C and VEGF-D with neuropilin receptors. FASEB J. 2006;20:1462–1472.

98. Kennedy TE, Serafini T, de la Torre JR, Tessier-Lavigne M. The netrins define a family of axon outgrowth-promoting ligands during nervous system development. Neuron. 1999;19:4938–4947.

99. Harris R, Sabatelli LM, Seeger MA. Guidance cues at the Drosophila CNS midline: identification and characterization of two Drosophila Ntrin/UNC-6 homologues. Neuron. 1996;17:217–228.

100. Moore SW, Tessier-Lavigne M, Kennedy TE. Netrins and their receptors. Adv Exp Med Biol. 2007;621:17–31.

101. Bennett KL, Bradshaw J, Youngman T, Rodgers J, Greenfield B, Aruffo A, Linsley PS. Deleted in colorectal carcinoma (DCC) binds heparin via its fifth fibronectin type III domain. J Biol Chem. 1997;272:26940–26946.

102. Geisbrecht BV, Dowd KA, Barfield RW, Longo PA, Leahy DJ. Netrin binds discrete subdomains of DCC and UNC5 and mediates interactions between DCC and hematopoietic cell-specific transcription factor. J Biol Chem. 2003;278:32561–32568.

103. Corset V, Nguyen-By-Charvet KT, Forcet C, Moyse E, Chedotal A, Stein E. A ligand-gated association between cytoplasmic domains of UNC5 and DCC family receptors converts netrin-induced growth cone attraction to repulsion. Cell. 1999;97:927–941.

104. Stein E, Zou Y, Poo M, Tessier-Lavigne M. Binding of DCC by netrin-1 to mediate axon guidance independent of adesinone A2B receptor activation. Science. 2001;291:1976–1980.

105. Vida MC, Childs S, Epstein JA, Weinstein BM. Semaphorin-plexin signaling patterns the developing vasculature. Dev Cell. 2004;7:117–123.
neogenin and recruitment of Unc5B. *Proc Natl Acad Sci U S A.* 2008; 105:12491–12496.

113. Qin S, Yu L, Gao Y, Zhou R, Zhang C. Characterization of the receptors for axon guidance factor netrin-4 and identification of the binding domains. *Mol Cell Neurosci.* 2007;34:243–250.

114. Wilson BD, Li M, Park KW, Suli A, Sorensen LK, Larriue-Lahargue F, Urness LD, Suh W, Asai J, Kock GA, Thorne T, Silver M, Thomas KR, Chien CB, Losordo DW, Li DY. Netrins promote developmental and therapeutic angiogenesis. *Science.* 2006;313:640–644.

115. Nguyen A, Cai H. Netrin-1 induces angiogenesis via a DCC-dependent ERK1/2-eNOS feed-forward mechanism. *Proc Natl Acad Sci U S A.* 2006;103:6530–6535.

116. Park KW, Crouse D, Lee M, Karnik SK, Sorensen LK, Murphy KJ, Kuo LI, Dy LI. The axonal attractant Netrin-1 is an angiogenic factor. *Proc Natl Acad Sci U S A.* 2004;101:16210–16215.

117. Yang Y, Zou L, Wang Y, Xu KS, Zhang JX, Zhang JH. Axon guidance cue Netrin-1 has dual function in angiogenesis. *Cancer Biol Ther.* 2007;6:743–748.

118. Hoang S, Lianuw J, Choi M, Choi M, Guzman RG, Steinberg GK. Netrin-4 enhances angiogenesis and neurologic outcome after cerebral ischemia. *J Cereb Blood Flow Metab.* In press.

119. Navankasuttas S, Whitehead KJ, Suli A, Sorensen LK, Lim AH, Zhao J, Park KW, Wythe JD, Thomas KR, Chien CB, Li DY. The netrin receptor UNC5B promotes angiogenesis in specific vascular beds. *Development.* 2008;135:659–667.

120. Brose K, Bland KS, Wang KH, Arnott D, Henzel W, Goodman CS, Tessler-Lavigne M, Kidd T. Slit proteins bind Robo receptors and have an evolutionarily conserved role in repulsive axon guidance. *Cell.* 1999; 96:795–806.

121. Kidd T, Bland KS, Goodman CS. Slit is the midline repellent for the robo receptor in Drosophila. *Cell.* 1999;96:785–794.

122. Grieshammer U, Le M, Plump AS, Wang F, Tessler-Lavigne M, Martin GR. SLIT2-mediated ROBO2 signaling restricts kidney induction to a single site. *Dev Cell.* 2004;6:709–717.

123. Wu JY, Feng L, Park HT, Havioglu N, Wen L, Tang H, Bacon KB, Jiang Z, Zhang X, Rao Y. The neuronal repellent Slit inhibits leukocyte chemotaxis induced by chemotactic factors. *Nature.* 2001;410:948–952.

124. Legg JA, Herbert JM, Clissold P, Bicknell R. Slits and Roundabouts in cancer, tumour angiogenesis and endothelial cell migration. *Angiogenesis.* 2008;11:13–21.

125. Dickson BJ, Gilestro GF. Regulation of commissural axon pathfinding by slit and its Robo receptors. *Annu Rev Cell Dev Biol.* 2006;22:651–675.

126. Seeger M, Tear G, Ferres-Marco D, Goodman CS. Mutations affecting growth cone guidance in Drosophila: genes necessary for guidance toward or away from the midline. *Neuron.* 1993;10:409–426.

127. Chedotal A. Slits and their receptors. *Adv Exp Med Biol.* 2007;621:65–80.

128. Hummlnecki L, 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.

129. Park KW, Morrison CM, Sorensen LK, Jones CA, Rao Y, Chien CB, Wu JY, Urness LD, Li DY. Robo4 is a vascular-specific receptor that inhibits endothelial migration. *Dev Biol.* 2003;261:251–267.

130. Okada Y, Jin E, Nikolova-Krotevski V, Yano K, Liu J, Beeler D, Spokes K, Kityayama M, Funahashi N, Doi T, Junes L, Minami T, Oettgen P, Aird WC. A GABP-binding element in the Robo4 promoter is necessary for endothelial expression in vivo. *Blood.* 2008;112:2336–2339.

131. Okada Y, Yano K, Jin E, Funahashi N, Kityayama M, Doi T, Spokes K, Beeler DL, Shih SC, Okada H, Danilov TA, Maynard E, Minami T, Oettgen P, Aird WC. A three-kilobase fragment of the human Robo4 promoter directs cell type-specific expression in endothelium. *Circ Res.* 2007;100:1712–1722.

132. Bedell VM, Yeo SY, Park KW, Chung J, Seth P, Shivalingappa V, Zhao J, Obara T, Sukhatme VP, Drummond IA, Li DY, Ramchandran R. Roundabout4 is essential for angiogenesis in vivo. *Proc Natl Acad Sci U S A.* 2005;102:6373–6378.

133. Kaur S, Castellone MD, Bedell VM, Konar M, Gutkind JS, Ramchandran R. Robo4 signaling in endothelial cells implies attraction guidance mechanisms. *J Biol Chem.* 2006;281:11347–11356.

134. Suchting S, Heal P, Tahtis K, Stewart LM, Bicknell R. Soluble Robo4 receptor inhibits in vivo angiogenesis and endothelial cell motility mediated via WASP and other actin nucleation-promoting factors. *FASEB J.* In press.

135. Bedell VM, Li DY. Robo4 stabilizes the vascular network by inhibiting pathologic angiogenesis and endothelial hyperpermeability. *Nat Med.* 2008;14:448–453.

136. Suchting S, Heal P, Tahtis K, Stewart LM, Bicknell R. Soluble Robo4 receptor inhibits in vivo angiogenesis and endothelial cell migration. *FASEB J.* 2005;19:121–123.

137. Le Bras B, Barallobre MJ, Homman-Ludjye J, Ny A, Wyna S, Tammela T, Haiko P, Karkainen MJ, Yuan L, Muriel MP, Chatzopoulou E, Birent C, Zalc B, Carmeliet P, Alitalo K, Eichmann A, Thomas JL. VEGF-C is a trophic factor for neural progenitors in the vertebrate embryonic brain. *Nat Neurosci.* 2006;9:340–348.

138. Shen Q, Goderie SK, Jin L, Karanth N, Sun Y, Abramova N, Vincent P, Pumiglia K, Temple S. Endothelial cells stimulate self-renewal and expand neurogenesis of neural stem cells. *Science.* 2004;304:1338–1340.

139. Tavazoie M, Van der Veken L, Silva-Vargas V, Louissant M, Colonna L, Zaidi B, Garcia-Verdugo JM, Doetsch F. A specialized vascular niche for adult neural stem cells. *Cell Stem Cell.* 2008;3:279–288.
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