Role of neural guidance signals in blood vessel navigation

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Abstract

Despite the tremendous progress achieved in both vasculogenesis and angiogenesis in the last decade, little is still known about the molecular mechanisms underlying the pathfinding of blood vessels during their formation. However, emerging evidence shows that different axonal guidance cues, including members of the Slit and semaphorin families, are also involved in the blood vessel guidance, suggesting that blood vessels and nerves share common mechanisms in choosing and following specific paths to reach their respective targets. These promising findings open novel avenues not only in vascular biology but also in therapeutic angiogenesis. Indeed, the identification of new molecules involved in the guidance of blood vessels may be helpful in designing angiogenic strategies, which would insure both the formation of new blood vessels and their guidance into an organized and coordinated network.

Keywords: Neural; Signals; Blood vessels

1. Introduction

In vertebrates, blood vessels form an extensive and elaborate network, which is spatially organized to optimize delivery of oxygen and nutrients to cells throughout the body and removal of metabolites and waste products. During embryonic development, endothelial precursors differentiate and coalesce into tubes to form the central axial vessels (i.e., the dorsal aortae and the cardinal veins), a process called ‘vasculogenesis’ [1]. Subsequently, this primitive network expands via sprouting, bridging and branching by intussusception (angiogenesis) and undergoes remodeling and pruning of excess vessels to finally give rise to the complex and hierarchical circuit, which is highly conserved among vertebrate species [1,2]. In order to obtain a functional network, mural cells [pericytes in medium-sized and smooth muscle cells (SMC), in large vessels] are recruited around the endothelial layer, stabilizing the endothelium by producing an extracellular matrix (ECM; arteriogenesis) [2].

Although a wealth of knowledge has been generated in the past decade about the molecular mechanisms underlying both vasculogenesis and angiogenesis, several critical questions on how the vessels choose and follow specific paths to reach their correct targets remain unanswered. Understanding this process may have implications, not only for vascular biology but also in the development of future strategies for therapeutic angiogenesis. Indeed, for therapeutic angiogenesis to be successful in establishing a mature and functional vascular network, two complementary processes should be ensured: (i) the induction of new vessel growth and (ii) the guidance of these vessels into a correctly organized network.

Given the anatomical resemblance in the patterning of nerves and blood vessels, much is likely to be learnt about vessel patterning by studying the mechanisms of axon guidance—a process, which has been more extensively investigated in the past decade. Indeed, rapidly emerging genetic studies in the mouse and zebrafish suggest that blood vessels and nerves share common pathways for their navigation towards their targets [3]. Due to space restrictions, the purpose of this review is not to provide an all-encompassing overview, but to highlight the emerging links...
between the patterning of blood vessels and nerves, giving particular attention to the netrins, slits, semaphorins and vascular endothelial growth factor (VEGF). The role of ephrins in vessel morphogenesis has been recently overviewed [4,5].

2. Blood vessels and nerves: different tasks, common features

The vascular and nervous systems have several striking anatomical similarities. They both consist of a highly complex and precisely branched network, reaching the most distant cells in the organism. The specialization of the arterio-venous blood vessel system to transport oxygen and remove carbon dioxide resembles the organization of the nervous system to transmit, via afferent sensory and efferent motor neurons, electrical impulses bidirectionally. Moreover, both blood vessels and nerves consist of two cell types, endothelial/mural cells, and neurons/glia, respectively.

In addition to sharing morphological features, both the vascular and nervous systems develop by following similar sequences of developmental events. As in the vascular system, the first step in the nervous system development involves the differentiation of progenitors into neurons with the subsequent formation of central structures, such as the neural tube and dorsal root ganglia. From these, the neurons sprout axons to reach distal targets in a highly ordered and stereotyped manner, thus establishing an intricately interconnected peripheral neural network [6–8]. Certain nerves follow the path of blood vessels and are closely aligned with branching vessels [9], raising the question whether the molecular signals, governing branching of these two systems, are related and interdependent. This hypothesis has recently been supported by genetic mouse studies, for instance, revealing that peripheral sensory nerves are required both for the differentiation of small skin arteries and the pattern of blood vessel branching [10]. In addition, the neurotrophic factor artemin (ARTN), produced by SMC, drives sympathetic nerves to follow vessels projecting towards their targets [11].

Which are the molecular events taking place when nerves and blood vessels navigate towards their respective targets? At present, most of the currently available information is deduced from studies on axon guidance, although insights about vessel navigation are being increasingly generated. When projecting along their trajectory, axons have to make pathfinding decisions at the level of the so-called ‘intermediate targets’ or ‘choice points’ [12]. The key player in driving this pathfinding is the growth cone—a highly motile structure located at the tip of the axon, which explores the microenvironment, by extending finger-like filopodia and veil-like lamellipodia, to detect and subsequently respond to a variety of guidance cues [13]. Both diffusible and cell surface-associated molecules act as long- or short-range pathfinding signals, respectively (see below) [12]. As the growth cone extends in the microenvironment, various molecular cues activate their respective receptors on the growth cone surface and induce attractive and repulsive signals. These signaling pathways are eventually integrated and transmitted into changes of the cytoskeleton and growth cone movement [14–17]. Assembly of the cytoskeleton components results in growth cone advancement, its disassembly leads to axon retraction, while asymmetric signaling at one side of the growth cone induces turning and a change in the direction of growth. Members of the Rho family of small GTPases—primarily, Rac, Cdc42 and Rho—play critical roles in this process by regulating the dynamic changes of the actin cytoskeleton [18–21]. More specifically, attractive guidance cues activate Cdc42 and Rac while inhibiting RhoA, resulting in actin polymerization at the leading edge of the growth cone; conversely, repulsive cues, through activation of RhoA and inhibition of Rac and Cdc42, induce actinomyosin contraction. Relative levels of the cyclic nucleotides cAMP and cGMP are also known to regulate the growth cone response from repulsion to attraction [22,23].

Similar mechanisms of pathfinding likely govern blood vessel patterning—although they remain less well characterized. Recent findings characterized a class of specialized endothelial cells at the leading edge of growing blood vessels, i.e., the “tip cells”, which are reminiscent of the axonal growth cone. These tip cells act as sensors, transducers and motility devices, by extending their filopodia to sample the microenvironment and by regulating the extension of the capillary sprouts [24,25]. Given this striking similarity between axon and vessel guidance, it might be anticipated that both systems share common guidance cues for their pathfinding. In the following sections, we will focus on some of the families of such guidance molecules. While some axon guidance signals clearly also regulate vessel pathfinding, the evidence for others is still circumstantial and being uncovered rapidly.

3. Guidance cues in axon pathfinding: do they have a role in vessel guidance?

Considerable insight about the molecular basis of axon pathfinding has been deduced from genetic studies in invertebrates and the characterization of axon guidance in vertebrate models, such as the projections of axons from retinal ganglion cells and the commissural axons in the spinal cord [26,27]. Overall, these studies have led to the identification of different families of guidance cues, which are highly conserved throughout the vertebrates: the Netrins, Slits and Semaphorins will be discussed below.

3.1. Netrins

Three members of the netrin gene family have been identified in mammals: netrin-1, netrin-3 and netrin4/β-
netrin [28–32]. Netrins are secreted, diffusible proteins related to laminin, which also bind to extracellular matrix (ECM) components [33–35]. Their function has been conserved throughout evolution, from worms to flies and vertebrates. Genetic screens in C. elegans [36,37], loss-of-function studies in Drosophila [38,39] and mouse [40], together with in vitro experiments with chicken neural explants [28,33], revealed that netrins, secreted by midline cells, attract commissural axons towards the ventral midline of the central nervous system. However, these molecules have also been demonstrated to repel some axons, such as the trochlear motor axons in vertebrates [41–44]. Interestingly, this dual activity to attract or repel axons depends on the receptor type to which the netrins bind. Two families of netrin transmembrane receptors have been identified in vertebrates: (i) the Deleted in Colorectal Cancer (DCC) family, consisting of DCC and neogenin [45] which are homologues of the C. elegans UNC-40 [36,46] and the Drosophila Frazzled protein [47]; and (ii) the UNC5 family, consisting of UNC5H-1, -2, -3 and -4 [48–51], which share homology with the C. elegans UNC-5 [52]. Recently, the A2b receptor, a member of the adenosine receptor family, has been identified as a novel netrin-1 receptor in mammals [53], but its function remains controversial and largely unknown [53,54].

Biochemical and genetic studies in different species demonstrated that axon attraction is mediated by the DCC/UNC40 receptors [45,46,55], while repulsion requires signaling by the UNC5 family receptors, acting either alone or in combination with DCC family receptors [36,43,56]. UNC5 is capable of mediating short-range repulsion by netrin, while both UNC5 and DCC are required to mediate long-range repulsion by netrin, when its concentrations are low [43,56]. This cooperation between UNC5 and DCC involves a direct interaction of their cytoplasmic domains, as demonstrated by ectopic expression of rat UNC5H2 in Xenopus commissural neurons [56]. Thus, netrins are key guidance molecules for different axons, acting over a short range (close to the surface of the cells producing them) or over a long range (up to a few millimeters distant from their source; reviewed in Ref. [57]).

Although very little is known about a possible role of netrins in the morphogenesis of organs outside the nervous system, their wide developmental expression pattern suggests that these molecules may have such a role [30,31,58]. Indeed, netrin-1/neogenin regulate mammary gland morphogenesis by assuring close apposition of cap cells and preluminal cells at the leading edge of the terminal end buds [59]. In the context of vessel patterning, it is interesting to note that, in addition to its expression in the neural tube, netrin-1b is also expressed in the zebrafish hypochord [60], a transient structure ventral to the notochord in the amphibian and fish embryo, which is known to be involved in the patterning of the dorsal aorta [61,62]. Our recent studies (in collaboration with A. Eichmann and M. Tessier-Lavigne) indeed indicate that loss or knockdown of UNC5H2 or netrin in mice and zebrafish cause abnormal vessel guidance and excessive vessel branching, indicating that netrin-1 and UNC5H2 provide critical repulsive guidance cues for navigating vessels [156] (Table 1).

### 3.2. Slits

Another intriguing class of guidance cues is the Slit family, which includes large, secreted proteins, highly conserved from C. elegans to vertebrates [63–66]. In mammals, three members, i.e., Slit-1, -2 and -3, are all expressed in the nervous system, with Slit-2 and -3 being also expressed elsewhere [65,67–69]. Slit proteins typically have multiple binding domains, including four leucine-rich repeats (LRRs), that are critical for biological functions, nine (seven in Drosophila) EGF-like repeats, which are thought to control Slit diffusion, and a C-terminal cystein knot [70–73]. Although Slit proteins were identified originally in Drosophila almost 20 years ago [74], their role has been elucidated only recently. Indeed, in vitro and genetic studies in Drosophila and mouse demonstrated that midline-derived Slits act as chemo-repellents, by preventing...

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### Table 1

Overview of the vascular phenotypes (excluding defects involving only the heart) observed during development of mice or zebrafish morphants in which either a guidance molecule or its receptor has been knocked down.

<table>
<thead>
<tr>
<th>Targeted gene</th>
<th>Vascular phenotype in mouse</th>
<th>Vascular phenotype in zebrafish</th>
</tr>
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<tbody>
<tr>
<td>Netrin-1</td>
<td>Not reported</td>
<td>Abnormal vessel patterning and excessive branching [156]</td>
</tr>
<tr>
<td>UNC5H2</td>
<td>Abnormal vessel patterning [156]</td>
<td>Abnormal vessel patterning and excessive branching [156]</td>
</tr>
<tr>
<td>Robo-4</td>
<td>Not reported</td>
<td>Intersomitic blood vessel defects [92]</td>
</tr>
<tr>
<td>Sema3A</td>
<td>Abnormal development of the anterior cardinal vein and of the intersomitic vessels [120]</td>
<td>Delayed development of the dorsal aorta [119]</td>
</tr>
<tr>
<td>PlexinD1</td>
<td>Intersomitic blood vessel defects and pharyngeal arch patterning defects [122]</td>
<td>Interssegmental vessel patterning defects [123]</td>
</tr>
<tr>
<td>Npn-1</td>
<td>Impairment of neural vascularization, abnormal and highly variable patterns of great vessels and vascular defects in the yolk sac [124]</td>
<td>Impaired circulation in the intersomitic vessels [125]</td>
</tr>
<tr>
<td>Npn-2</td>
<td>Severe reduction of small lymphatic and capillaries [126]</td>
<td>Not reported</td>
</tr>
<tr>
<td>Npn-1/Npn-2</td>
<td>Lack of blood vessel formation in the yolk sac, avascular regions in the head and the trunk and lack of capillary plexus [127]</td>
<td>Not reported</td>
</tr>
</tbody>
</table>
ipsilateral axons from crossing the midline and commissural axons from recrossing it [63,64,75,76]. This repulsion is mediated by Slit interaction with receptors of the Roundabout (Robo) family, which have been identified in organisms ranging from Drosophila, C. elegans to vertebrates [75,77–80]. Members of this family are single-pass transmembrane proteins with an extracellular region containing five immunoglobulin (Ig) domains and three fibronectin type III repeats [77,80]. In mammals, four Robo receptors, Robo-1, -2, -3 and -4, have been identified, with Robo-4 (also referred to as Magic Roundabout) being structurally divergent from the other proteins because it lacks two Ig domains and one fibronectin repeat [77,81–83].

Studies in Drosophila clarified the mechanisms by which the commissural axons are first attracted by netrins towards the midline and, once they have crossed it, are prevented to recross the midline by becoming responsive to the repulsive activity of the Slit proteins. Prior to reaching the midline, expression of Robo on axonal growth cones is repressed by Commissureless (Comm) [78], which secures that Robo is retained in intracellular compartments [84]. Upon crossing the midline, Comm loses this suppressive activity, so that Robo becomes exposed onto the cell surface, thereby making the growth cone responsive to Slit. Activation of Robo also silences the attractive effect of netrin, via a direct interaction of the cytoplasmic domain of Robo to that of the netrin receptor DCC (as shown by a study on embryonic Xenopus spinal axons [85]), thereby facilitating expulsion of the commissural axons away from the midline. Various lines of evidence suggest that a similar system is also operational in mammals. Similar to Comm in Drosophila, Robo-3 in mammals prevents precrossing spinal commissural axons from responding to floor plate Slit repellents, thereby allowing commissural axons to become attracted to the midline [86]. Upon crossing the midline, commissural axons become responsive to Slit [87], explaining why these axons either do not leave or recross the midline irregularly in triple Slit knockout mice [76].

In addition to the well-characterized role of Slit proteins in axon guidance, a growing body of evidence shows that they are involved in the migration of different cell types, including neuronal cells [88], chemotactic leukocytes [89], neural crest cells [90] and myoblasts [91]. Notably, Robo-4 is specifically expressed on endothelial cells in both the embryo and adult, and significantly upregulated in tumor vessels and in mice with vascular sprouting and patterning defects (i.e., mice lacking the activin receptor-like kinase 1 gene) [83,92]. In vitro, Slit-2 inhibits the migration of Robo-4-expressing endothelial cells, consistent with a repulsive activity [92]. Another study suggested, however, that Slit-2, via Robo-1, attracts endothelial cells via Robo-1 activation [93]. Slit-2 is expressed in various solid tumors, whereas Robo-1 is upregulated in tumor vessels and neutralization of Robo-1 inhibits tumor growth in vivo [93]. Furthermore, knockdown of Robo-4 expression induces abnormal patterning of intersomitic vessels in zebrafish [94]. Thus, Slit-2/Robo-4 provides repulsive cues for endothelial cells in vitro, whereas Slit-2/Robo-1 signals chemotacttract endothelial cells in tumors in vivo (Fig. 1). These puzzling findings raise the question whether vascular Slit may act as an attractive or a repulsive cue, depending on the receptor to which it binds.

### 3.3. Semaphorins

Semaphorins belong to a large family of phylogenetically conserved secreted and membrane-associated proteins, characterized by the presence of a highly conserved ~500 amino acid extracellular semaphorin domain (‘sema’) at their amino termini, which mediates binding to its receptor (reviewed in Refs. [95,96]). To date, more than 20 different semaphorins have been identified, which are divided into eight classes on the basis of sequence similarities and structural features (Semaphorin Nomenclature Committee, 1999). The first two classes consist of invertebrate semaphorins, while classes 3 to 7 include vertebrate semaphorins and class V comprises the viral semaphorins. In vitro as well as genetic studies in flies and mice have shown that semaphorins primarily act as repulsive cues on a wide number of neurons (e.g., dorsal root ganglion neurons in the peripheral nervous system and hippocampal neurons in the central nervous system), by diverting axons from inappropriate regions, or driving them through a repulsive corridor [97–102]. Interestingly, semaphorins are also capable of chemoattracting certain

![Fig. 1. Attractive and repulsive cues mediating the guidance of the endothelial tip cell growth cone. VEGF and Slit-2 act as attractant cues, via Flk1 and Robo-1, respectively, while Sema3A and Slit-2 act as repulsive cues, via Npn1/PlexinD1 and Robo-4, respectively. It should be mentioned that the involvement of Slit-2/Robo-4 axis in repulsion is based on in vitro experiments.](image-url)
axonal populations, such as mitral cell axons of the olfactory bulb during development [103–106]. Cytosolic levels of cGMP seem to play a critical role in converting the response of neurons to semaphorins from repulsion to attraction [22]. Overall, these data indicate that semaphorins may be bifunctional guidance cues, endowed with attractive or repulsive activities, similar to the netrin family members. This dual activity depends on the restricted distributions of these proteins, on the receptor complexes to which semaphorins bind (see below) and on the cross-talk between semaphorin receptors and other pathways [107]. Two major family receptors have been implicated in mediating the action of semaphorins: plexins and neuropilins. Specifically, invertebrate semaphorins, membrane-associated semaphorins in vertebrates and viral semaphorins directly interact with plexins [108–110]. In contrast, vertebrate class 3 secreted semaphorins utilize receptor complexes consisting of neuropilin, serving as the ligand-binding component, and plexin, functioning as the signal-transducing component [111–114]. To date, two neuropilins, Npn-1 and Npn-2, and nine plexins (Plex-A1, -2, -3, -4; Plex-B1, -2, -3; Plex-C1, -D1) have been identified in mammals (reviewed in Ref. [115]). Neuropilins are also receptors for particular isoforms of the vascular endothelial growth factor (VEGF; see below). Additional transmembrane proteins, including the Drosophila off-track (Otk) and the hepatocyte growth factor (HGF)-receptor Met have been recently shown to be either semaphorins receptors or components of receptor complexes [96,116,117]. Thus, the wide range of possible combinations of the different members of the semaphorin receptor complex may be in part responsible of the specificity of responses of different neurons to semaphorins.

Like netrins and Slits, semaphorins are also expressed outside the central nervous system and their role is not only restricted to neural development. Indeed, they are also involved in the migration and/or morphogenesis of epithelial cells [116,118], as well as in the migration of both leukocytes [119] and tumor cells [120]. Interestingly, emerging evidence also suggests a role of Semaphorin 4D (Sema4D) and Semaphorin 3A (Sema3A) in vascular development. Indeed, Sema4D induces endothelial cell migration and tubulogenesis in vitro and stimulates blood vessel formation in vivo [121]. Zebrafish Sema3A1 regulates migration of the angioblasts, which give rise to the dorsal aorta [122], whereas endothelial-derived class 3 semaphorins control endothelial cell migration in chick embryos, via autocrine inhibition of integrin-mediated adhesion of endothelial cells to the extracellular matrix [123]. In addition, Sema3A seems to mediate the topographical congruence of nerves and blood vessels during the quail forelimb development [124]. Recent studies in mouse and zebrafish highlighted a critical role of Sema3 proteins in vessel guidance [125,126] (Table 1). In these studies, disruption of the signaling, triggered by the interaction of Sema3 proteins with endothelial PlexinD1, likely in complex with Npns, caused aberrant patterning of intersomitic vessels in zebrafish and mice, thus suggesting that semaphorins line repulsive corridors, through which the intersomitic blood vessels traverse (Fig. 1). Although genetic loss of Npn-1 and/or Npn-2 causes significant vascular defects in mice and fish [127–130], it remains unclear to what extent these defects are caused by abnormal signaling of VEGF and/or semaphorins. Indeed, vascular development, but not axonal pathfinding, was normal in mice expressing a Npn-1 variant, unable to bind to Sema3A [131]. Future work will be required to resolve the complex molecular mechanism of semaphorins and neuropilins in vascular development.

4. VEGF: a guidance cue for vessels and nerves?

Numerous studies established that VEGF is a key player of angiogenesis [132,133]. In this review, we will focus our attention on how VEGF regulates vessel patterning and neural development. In mammals, different isoforms are generated by alternative splicing from a single VEGF gene (reviewed in Ref. [134]). Specifically, six isoforms (VEGF121, VEGF145, VEGF165, VEGF183, VEGF189 and VEGF206) have been identified in humans, with VEGF165 being the predominant form [135]. Mouse and rat isoforms are one amino acid shorter than their human counterparts. While VEGF121 is a soluble protein and does not bind heparin, the longer isoforms show increasing binding to heparan sulfate-containing proteoglycans of the extracellular matrix, from which they can be released by proteolytic enzymes [135]. These isoforms also exhibit a distinct tissue-specific expression pattern [136] and have different biological properties (see below). All VEGF isoforms bind the tyrosine-kinase receptors VEGFR-1 [also known as fms-like tyrosine kinase (Flt1)] and VEGFR-2 [also known as fetal liver kinase (Flk1)] [134], but only VEGF165 binds both Npn-1 and -2, while VEGF145 binds Npn-2 [137,138].

VEGF stimulates endothelial cell proliferation and survival, primarily through Flk1 signaling [139–141]. Interestingly, Flk1 signaling is enhanced by Npn-1, which thus acts as a coreceptor for VEGF165 [142]. Genetic studies in mice revealed that the various VEGF isoforms have distinct roles in vessel guidance. Indeed, vascular development was normal in mice exclusively expressing VEGF164, indicating that this isoform is required and sufficient for vessel guidance [143]. In contrast, in VEGF120/120 mice, which express only the VEGF120 isoform, endothelial cells become integrated in enlarging vessels at the expense of vessel branching [143,144]. VEGF186/186 mice, expressing the matrix-associated VEGF188 isoform, exhibit opposite defects, characterized by excess branching at the expense of vessel lumen enlargement [143,144]. Overall, these results indicate that the different VEGF isoforms cooperate to form a gradient extending from the target tissue to the tip cell of the
growing vessel, thus providing long-range and short-range guidance cues for correct vessel patterning (Fig. 1).

In recent years, increasing attention has been given to the possible role of VEGF in the nervous system [145]. Both VEGF and Flk1 are expressed by neurons [146]. In vitro experiments demonstrated that VEGF/Flk1-signaling promotes axonal outgrowth [146,147], neuronal survival [146,148,149] and neuroprotection against glutamate cytotoxicity [150,151]. Moreover, VEGF, expressed by ependymal cells at sites of neurogenesis, enhances self-renewal, fate and proliferation of neuronal stem cells both in vitro and in vivo [152,153]. VEGF also acts as a chemoattractant for undifferentiated neural progenitors [154]. By competing with Sema3A for binding to Npn-1, VEGF165 prevents the collapse of dorsal root ganglia neurons [146]. Nonetheless, it remains to be established whether VEGF is a relevant axon guidance cue.

5. Concluding remarks

Several lines of evidence are starting to shed light on the molecular mechanisms underlying blood vessel pathfinding during development, while highlighting that blood vessels and nerves share common guidance cues during their navigation to reach their final target. Although additional studies are required to better elucidate this link between blood vessel and axon pathfinding mechanisms, the initial findings provide new perspectives in the design of angiogenic therapeutic strategies not only able to promote the formation of new vessels but also to guide them into a coordinated network.

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