

Common Cues in Vascular and Axon Guidance

Guido Serini and
Federico Bussolino

Division of Molecular Angiogenesis, Institute for Cancer Research and Treatment, and Department of Oncological Sciences, University of Torino School of Medicine, 10060 Candiolo (TO), Italy
guido.serini@ircc.it
federico.bussolino@ircc.it

Blood vessels and nerves are structured in architecturally similar organ systems and show functional relationships. Indeed, vascular and neuronal cells are guided in their journey throughout the body by the same attractive and repulsive factors that respectively activate and inhibit the function of integrin-adhesive receptors.

The execution of complex functions by the many different tissues and organs of vertebrates is supported and regulated by the activity of two anatomic structures, namely blood vessels and nerves (29). Whereas blood streaming through the vascular system ensures provision of required nutrients and removal of toxic waste products, the nervous system directly controls and coordinates the execution of tissue and organ tasks by conveying biochemical and electrical signals. Albeit functionally distinct, these two organ systems are architecturally similar, being structured into ramifying, highly pervasive, and hierarchically ordered networks. Moreover, both systems are operatively bidirectional: the vascular network is organized in arterial and venous blood vessels and the peripheral nervous system (PNS) comprises effector and sensory pathways. Furthermore, in the adult body neurovascular bundles are sites of close physical interaction between blood vessels and peripheral nerves (47). Together, these morphological parallels and relationships would suggest some interdependence (e.g., need for oxygen and nutrients by nerves) and/or responsiveness to the same guidance cue(s) by the two systems during development, when vascular endothelial cells (ECs), neurons, and neuronal processes have to migrate over long distances through a complex embryonic terrain to reach their appropriate destinations.

Fifteen years ago in a pioneering work, Martin and Lewis (48) ablated the chick wing PNS by UV irradiation and showed that within the skin 1) blood vessels and peripheral nerves run closely parallel and branch at the same points; 2) vessels and nerves can follow the same route because they are likely controlled by the same mesenchymal signal(s); and 3) some nerves induce blood vessels to remodel around them. Over the past six years, an ever-increasing number of studies have revealed part of the complex and multifaceted nature of the guidance mechanisms shared by these two systems. It appears that vascular ECs and neuron growth cones (GCs) are guided in their journey throughout the body extracellular matrix (ECM) by

the same families of chemoattractant and chemorepulsive factors. Integrin heterodimers are primary ECM receptors, which can exist in different functional states with respect to their affinity or avidity for ECM proteins (11, 35), and directed cell motility on ECM is promoted by targeted localization of high-affinity integrins at the leading edge of migrating cells (40). There is now mounting evidence that both chemoattractant and chemorepulsive factors exert their control over cell migration by respectively activating and inhibiting the activity of integrin-adhesive receptors.

Ephrins and Eph Receptors

Ephrins are membrane-bound ligands of Eph receptor tyrosine kinases. In general, glycosylphosphatidylinositol (GPI)-anchored A-subclass ephrins (ephrinA1–ephrinA5) promiscuously bind A-subclass Eph (EphA1–EphA8) and transmembrane B-subclass ephrins (ephrinB1–ephrinB3) bind B-subclass Eph (EphB1–EphB4, EphB6). Eph/ephrin interaction at the cell surface triggers bidirectional signals both in receptor- and ligand-expressing cells, now referred to, respectively, as forward and reverse signaling (43, 56).

In the developing nervous system, ephrins and Eph receptors have been shown to regulate topographic mapping among neuronal populations, axon guidance at the midline, and migration of cerebellar granule neurons; in the adult organism they control synaptic plasticity and nerve regeneration (56). Eph/ephrin activation generally causes repulsion of neighboring cells or neuronal GCs, and in some cases it results in attraction. Paradigmatic examples are represented by topographic map formation in visual (55) and vomeronasal (41, 42) systems; expression of either A-subclass ligands or receptors on the surface of navigating GCs seems to regulate such an opposite migratory behavior. Although in the visual system EphA-bearing retinal axons are repelled, in the vomeronasal system ephrinA5-expressing axons are attracted. Notably, integrins are primary effector targets of the Eph/ephrin system (FIGURE 1). Indeed, stimulation of EphA2 by ephrinA1 has been

shown to inhibit integrin-based adhesion to the ECM by inducing Src homology 2 domain-containing tyrosine phosphatase (SHP-2)-dependent dephosphorylation of focal adhesion kinase (FAK) (50), and reverse signaling from receptor-engaged ephrinA5 enhances integrin-mediated adhesion to the ECM by activating the Src kinase family member Fyn (16). Furthermore, ephrinB1-stimulated EphB2 suppresses integrin-adhesive function by phosphorylating and inhibiting the effector region of the R-Ras small GTPase, a well-known integrin activator (79) that localizes at focal adhesions (23). Along the same line, stimulation of ephrinB ligands inhibits migration of cerebellar granule neurons elicited by the binding of stromal-derived factor 1 to the G protein-coupled receptor CXCR4 (46), which in turn triggers integrin-mediated adhesion via the small GTPase Rap1 (66). However, the observations that EphA (30) and EphB (33) receptors as well as ephrinB ligands (61) can stimulate integrin-dependent adhesion to the ECM and activate Rap1 would suggest that regulation of integrin function by the Eph/ephrin system may be cell type specific. In addition, mechanical tethering through Eph-ephrin interactions can also support

integrin-independent adhesion and spreading by activating critical components of integrin signaling such as FAK and the multiadaptor p130^{cas} (12).

In the vascular system, arterial and venous ECs (75) express ephrinB2 and its receptor EphB4, respectively. During the early phases of vascular development, i.e., vasculogenesis, EC precursors (angioblasts) aggregate in a primitive network of homogeneously sized blood vessels known as the primary plexus, which is then remodeled by angiogenesis into a hierarchically organized mature vascular tree (13). In *ephrinB2* homozygous null mice, vascular development is halted at the primary plexus stage, causing embryonic lethality at embryonic day 11 (75). The fact that targeted deletion of either *EphB4* (25) or the cytoplasmic domain of *ephrinB2* (2) phenocopies the loss of the entire *ephrinB2* (75) indicates that this ligand/receptor pair and ephrinB2 reverse signaling control angiogenic remodeling in the mouse embryo. Indeed, arteriovenous positioning within the vascular network would result from EC segregation caused by the concerted activity of EphB4-repulsive forward signaling and ephrinB2-attractive reverse signaling (22, 32). The same molecular mechanism seems to

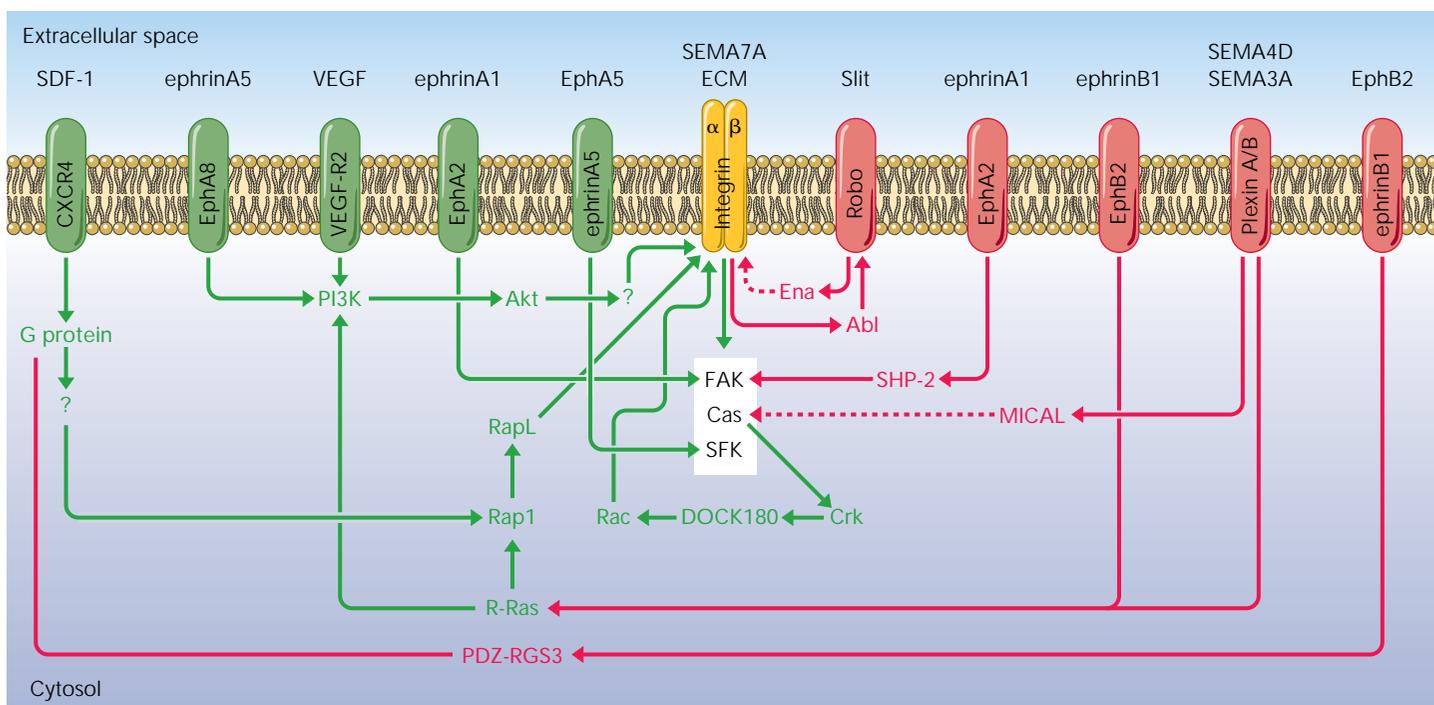


FIGURE 1. Guidance cues regulate integrin function

Chemoattractant (green) and chemorepulsive (red) factors stimulate signal transduction pathways that respectively activate or inhibit integrins (yellow). Some of them (e.g., ephrinA1) are bifunctional. EphrinA5-activated EphA8 (30) and VEGF-activated VEGF-R2 (10) switch on integrins through phosphatidylinositol 3-kinase (PI3K)/Akt, whereas stromal cell-derived factor-1 (SDF-1)-activated CXCR4 impinges on Rap1/RapL (65). EphA5 stimulates ephrinA5 reverse signaling that activates integrins via the Src-family kinase (SFK) Fyn (16) (not shown). Depending on the cellular context, ephrinA1-challenged EphA2 either activates (12) or inhibits (through SHP-2 phosphatase) (50) focal adhesion kinase (FAK). After interacting with ephrinB1, EphB2 phosphorylates and inhibits R-Ras GTPase (79), which in turn could activate integrins through either PI3K or Rap1/RapL. Although Slit-activated Robo could inhibit integrins via Ena (4, 6), activation of Abl kinase by integrin interaction with the extracellular matrix (ECM) could inhibit Robo (6, 44). EphB2 elicits ephrinB1-dependent activation of PDZ-regulator of G protein signaling 3 (RGS3), which in turn switches off G protein signaling downstream of CXCR4. ECM-engaged integrins induce FAK/Cas/Crk/ DOCK180 guanine-nucleotide exchange factor coupling, which activates Rac and provides a positive-feedback loop maintaining membrane extension and adhesion in spreading cells (65). SEMA3A/PlexinA1 and SEMA4D/PlexinB1 inhibit R-Ras GTPase (54). SEMA3A-activated PlexinA1 also interacts with molecule interacting with CasL (MICAL) (58). The proline-rich region of MICAL could compete with FAK for binding to the NH₂-terminal SH3 domain of Cas and cause FAK/Cas/Crk/DOCK180 uncoupling. SEMA7A behaves as an ECM molecule that, through its RGD motif, interacts and activates β 1-integrins (59).

be at work in the adult organism, e.g., during cancer progression when blood vessels express high levels of ephrinB2 (24, 67) and EphB4-bearing tumor cells promote angiogenesis by activating ephrinB2 reverse signaling in ECs (53). Similarly to ephrinB1 (34), ephrinB2 could elicit EC adhesion, migration, and vascularization by activating integrin function.

Semaphorins

Semaphorins (SEMA) represent a large family of secreted or cell-bound repulsive cues, which affect axon steering, fasciculation, and branching in developing nervous tissue (20). Neuropilin (Nrp)-1 and -2 are transmembrane glycoproteins that regulate axon guidance and act as receptors for secreted class 3 SEMA (SEMA3) (21). Nrp do not directly transduce repulsive signals within the cell but are essential for assembling SEMA3 receptor complexes that can include several transmembrane signaling components, such as type-A plexins (PlexinA) or L1-CAM (58). Plexins are a family of transmembrane molecules with a large cytoplasmic domain containing two highly conserved stretches separated by a variable linker sequence. The conserved domains are related to GTPase-activating proteins (GAPs). The fact that mutating conserved arginine residues within GAP-like domains of PlexinA1 abrogates the response to SEMA3A suggests that plexins could exert an enzymatic GAP activity on GTPase(s) (58).

Recent evidence indicates that a SEMA3-Nrp-PlexinA system plays a key role in cardiovascular development. Indeed, SEMA3 receptor complexes are expressed on the surface of ECs (52, 65), SEMA3A (65) and SEMA3F (39, 65) inhibit EC motility, and genetic ablation of Nrp1 (37) and/or Nrp2 (72) causes vascular defects in mouse embryos. Remarkably, a series of data obtained in different experimental models provided direct evidence that SEMA3 family members are crucial for the vasculogenic aggregation of angioblasts in primitive vascular structures and in their ensuing angiogenic remodeling into an arborized network. Indeed, in zebrafish embryos, antisense knockdown of *Sema3a1*, which is expressed in early somites during vasculogenesis, interferes with the normal migration of Nrp1-bearing angioblasts, finally impairing dorsal aorta formation (68). Analyzing CD-1 mouse embryos in which the *sema3a* gene has been deleted and chick embryos transduced with dominant negative SEMA3 receptors, our group (65) further showed that during angiogenesis, ECs generate autocrine chemorepulsive signals of SEMA3 that endow the vascular system with the plasticity required for its reshaping. Lastly, Bates and colleagues (7) highlighted how *Sema3A* con-

trols the congruence of peripheral nerve and blood vessel anatomic patterns in the developing chick embryo. The fact that disruption of SEMA3/Nrp1 signaling caused vascular abnormalities in zebrafish (68), chick (7, 65), and CD-1 mice (65) but in neither 129/Sv (8) nor C57BL/6 (31) mice is likely due to genetic background effects of different mouse strains, as already shown for EphB2/EphB3 (15) and *Sema3C* (18) signaling. Moreover, it has recently been shown (27) that the EC-specific PlexinD1 associates with both Nrp1 and Nrp2 to form a novel receptor complex for SEMA3. Abrogation of PlexinD1 in zebrafish (73) and mouse (27) result in defects of blood vessel pathfinding and cardiovascular patterning. All in all, these observations indicate that SEMA3 and their receptors are prominent regulators of vascular development.

We have also shown that SEMA3 control the plasticity of the vascular system by negatively regulating integrins (65). Indeed, inhibitory autocrine loops of endothelial SEMA3 proteins would allow a tunable and fine modulation of integrin function, cell migration, and redirection during angiogenic remodeling. Notably, all of the defects we found in blood vessels of *sema3a*-null embryos (65) overlap at least in part with the vascular phenotype of *ephrin-B2* (75), *EphB4* (25), and *EphB2/EphB3* (3) mutants that act by modulating integrin function as well (see above). Therefore, guidance cues might regulate vascular morphogenesis by modulating integrin activation in general (FIGURE 1). The observation by Pasterkamp et al. (59) that SEMA7A via an RGD motif promotes axon growth by activating neuronal β_1 -integrins indicates that, independently of their attractive or repulsive activity, SEMA impinge on integrin-mediated adhesiveness and signaling to exert their functions (FIGURE 1). Accordingly, Barberis and colleagues (5) showed that SEMA4D/PlexinB1 signaling inhibits integrin-dependent adhesion and migration as well. Furthermore, the recent observation by Oinuma and colleagues (54) that inhibition of the integrin-activating GTPase R-Ras is required both for SEMA3A/PlexinA1- and SEMA4D/PlexinB1-mediated growth cone collapse further points to integrin-adhesive receptors as crucial effector targets on which SEMA/Plexin signaling converges.

Slit

Slit proteins were originally identified as midline repellents of the central nervous system (CNS) that, after binding to Roundabout (Robo) receptors on the surface of commissural axons, expel and prevent them from recrossing (9). There are three Slit ligands (Slit1, Slit2, and Slit3) and four Robo receptors (Robo1, Robo2, Robo3, and Robo4) expressed in unique and complementary patterns both within the CNS and in other developing

tissues (77). Similarly to ephrins and SEMA, the Slit/Robo system deals with the integrin-adhesive machinery as well (FIGURE 1). Indeed, chemorepulsive signaling from Robo depends on Ena/vasodilator-stimulated phosphoprotein (VASP) proteins and Abl kinase functions to antagonize Robo's repulsive output (6). On the one hand, Ena/VASP proteins are part of the cytoplasmic multimolecular complex of integrin-based cell-to-ECM adhesions and VASP is already known to mediate the inhibitory effects of cyclic nucleotides on α IIb β ₃-integrin ligand binding during platelet aggregation (4). On the other hand, α ₅ β ₁-integrin-dependent cell adhesion to fibronectin leads to an increased Abl kinase activity (44), and integrin-mediated adhesion has been shown to regulate the responsiveness of *Drosophila* axons to Slit (69).

Wang et al. (74) have provided evidence that solid tumors secrete Slit2 and that associated vascular ECs express the Robo1 receptor. Expression levels of Slit2 in primary tumors correlate with microvessel density and tumor progression. Indeed, Slit2 was absent in normal and hyperplastic colon tissues, began to appear in colon adenomas, and was upregulated in colon carcinoma. In this context, Slit2 would be capable of eliciting EC migration and self-assembly in newly formed blood vessels in a Robo1- and phosphatidylinositol 3-kinase-dependent manner. The observation that, besides its repulsive activity, Slit2 could also behave as a positive regulator of EC migration is in accordance with previous findings identifying Slit2 as a potent stimulator of spinal sensory axon elongation and branching (76) and neural crest cell migration (17). It seems that neither *Slit1* nor *Slit2* plays a role in vascular development (60); therefore it would be interesting to analyze the vascular phenotype of *Slit1-Slit2-Slit3* triple-knockout mice (45).

Nogo

A major difference between adult CNS and PNS resides in the regeneration potential of damaged axons. Indeed, in the CNS of a postnatal organism, lesioned axons fail to regenerate because of the strong inhibition exerted by CNS but not PNS myelin on their growth (64). The *Nogo* gene encodes for the membrane protein Nogo-A, one of the major components of the neurite growth-inhibitory activity present in CNS myelin (63). Nogo is differentially spliced to give rise to three proteins with different NH₂ terminals. The longest isoform (1,163 amino acids), Nogo-A, contains a unique acidic NH₂-terminal sequence, known as "amino-Nogo," and is expressed mainly by CNS oligodendrocytes. Nogo-B (360 amino acids) is present in many tissues. Nogo-C (190 amino acids)

is expressed in CNS and skeletal muscles. All three Nogo isoforms share a 188-amino-acid-long COOH-terminal sequence with homology to the family of reticulon proteins that contains a 66-amino-acid loop region (known as "Nogo-66") between two very large transmembrane domains and an endoplasmic reticulum retention signal. Because the two long transmembrane stretches can span the membrane once or twice, Nogo tends to assume two possible topographies, i.e., either the NH₂ terminal or the Nogo-66 loop can face extracellularly. The neurite growth-inhibitory effect of Nogo-A depends on RhoA GTPase activation by either two NH₂-terminal stretches, the first shared with Nogo-B and the second located within amino-Nogo or Nogo-66 (63). The GPI-linked receptor NgR and the low-affinity neurotrophin receptor p75, respectively, are the ligand-binding and the signal-transduction subunits of Nogo-66 receptor downstream of Nogo-A (49).

Recently, Acevedo et al. (1) discovered that Nogo-B localizes on the cell surface of cultured ECs and smooth muscle cells (SMCs), with its NH₂ terminal exposed extracellularly. The first 200 NH₂-terminal amino acids of Nogo-B, which are homologous with Nogo-A, chemoattract ECs and counteract platelet derived growth factor (PDGF)-stimulated migration of SMCs in an NgR-independent way. Nogo-B protein is also expressed in vivo in blood vessels, where it is thought to exert a permissive effect on postnatal vascular homeostasis. Indeed, Nogo-A/B-null mice, although not displaying gross vascular abnormalities, show marked neointima formation in response to injury, likely because of either impaired reendothelialization or lack of inhibition of PDGF-driven SMC migration.

VEGF

The family of VEGF proteins controls a range of EC behaviors such as proliferation, survival, and chemotactic movement (19). During the initial phase of vascular development, VEGF, acting through VEGF receptor 2 (VEGF-R2), drives the differentiation of EC precursors from mesodermal cells and their assembly into a primary capillary plexus (14, 36). The subsequent angiogenic remodeling into a mature vascular tree requires VEGF-A/VEGF-R2 signaling in association with angiopoietin/tyrosine kinase with Ig and EGF factor homology domains (TIE) signaling (36), ephrin/Eph signaling (36, 43, 56), and SEMA3/Nrp/PlexinA-D signaling (27, 65). VEGF-A also play a permissive role in the commitment of ECs toward an arterial phenotype (62). It has been shown that VEGF-A controls EC adhesion and migration by activating integrin function (FIGURE 1) through a phosphatidylinositol 3-kinase-

dependent pathway (10).

Several pieces of experimental evidence point to VEGF-A as a key molecule employed by the nervous system to direct blood vessel patterning during development. Mukoyama and colleagues (51) provided the first molecular evidence of how peripheral sensory nerves act as a template for blood vessel branching and arterial differentiation via local secretion of VEGF-A by neurons or Schwann cells. Indeed, nerves may promote blood vessel association and arterial differentiation shortly after their arrival in the periphery to ensure access to a local vascular source of neurotrophic factors during subsequent growth. Moreover, it has also been shown that VEGF-A produced by astrocytes controls angiogenic sprouting in the early postnatal retina (26). Intriguingly, in addition to its well-known effects on ECs and angiogenesis, VEGF-A is now coming to light as a central regulator of neural cell behavior and neurogenesis, the process through which precursor cells differentiate toward a mature neuronal phenotype (38). Actually, neurons and their precursors express VEGF receptors (70) and VEGF-A can act directly on these cells to promote proliferation and survival (71) as well as directional migration (78). Together, these data support the observations of Palmer et al. (57), according to which in the adult animal neurogenesis and angiogenesis are spatiotemporally linked. In this context, the presence of a vascular niche could provide not only the necessary trophic and metabolic support but also instructive cues for neurogenesis (38). However, further work is required to understand whether the simultaneous presence of neural and endothelial precursors at the same anatomic sites is due either to the fact that these two cell types are attracted by the same guidance cue (e.g., VEGF) or because they derive from a common precursor.

Conclusions

A large amount of data from the literature points out how, during development, the spatial organization of blood vessels and nerves is controlled by the same guidance cues (e.g., ephrins, SEMA, Slit, Nogo, and VEGF-A) and at some anatomic sites the two systems are in direct physical interaction, reciprocally influencing each other. Besides the well-known control exerted by the autonomic nervous system on the vascular tone (27), the reason for blood vessels and nerves to rely on the same molecular mechanisms could be twofold. First, during their evolution these two organ systems could have been facing the same architectural issue, that is to invade the whole organism in a hierarchically and functionally ordered way (29). Second, from a metabolic point

of view the nervous system is a highly demanding tissue, which at least at some specific locations or during certain phases of development could require a direct physical interaction with blood vessels (57).

Finally, it emerges that regulation of integrin-adhesive function could represent a common critical target on which most of the vascular and nervous guidance cues are converging. Therefore, a thorough dissection and characterization of the molecular mechanisms by which guidance cues modulate integrin function could allow the identification of new pharmacological targets for different congenital and acquired neural, vascular, and tumoral diseases. ■

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