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REVIEW

Semaphorin signaling in angiogenesis, lymphangiogenesis and cancer

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Angiogenesis, the formation of new blood vessels from preexisting vasculature, is essential for many physiological processes, and aberrant angiogenesis contributes to some of the most prevalent human diseases, including cancer. Angiogenesis is controlled by delicate balance between pro- and anti-angiogenic signals. While pro-angiogenic signaling has been extensively investigated, how developmentally regulated, naturally occurring anti-angiogenic molecules prevent the excessive growth of vascular and lymphatic vessels is still poorly understood. In this review, we summarize the current knowledge on how semaphorins and their receptors, plexins and neuropilins, control normal and pathological angiogenesis, with an emphasis on semaphorin-regulated anti-angiogenic signaling circuitries in vascular and lymphatic endothelial cells. This emerging body of information may afford the opportunity to develop novel anti-angiogenic therapeutic strategies.

***Keywords*:** semaphorin; signaling; angiogenesis; lymphangiogenesis; cancer

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Introduction

Angiogenesis, the formation of new blood vessels from preexisting vasculature, is essential for many physiological processes, such as wound healing, and also plays a critical role in many pathological conditions including diabetic retinopathy, age-related macular degeneration, and tumor growth. Angiogenesis is controlled by delicate balance between pro- and anti-angiogenic signals, thus elucidating the molecular mechanisms underlying normal and aberrant blood vessel growth may provide new therapeutic options for many human diseases [1]. Among the pro-angiogenic molecules, vascular endothelial growth factor (VEGF) has received a considerable amount of attention as a promising target for anti-angiogenic therapy. Anti-angiogenic drugs targeting the VEGF pathway, such as a humanized monoclonal antibody against VEGF, were developed rapidly and used in the clinical settings. However, anti-VEGF treatments often result in only modest improvement in progression-free survival, and tumors can eventually acquire resistance to anti-angiogenic therapies. To overcome these problems, a better understanding of the molecular mechanisms responsible for angiogenesis is necessary, which may afford the opportunity to develop new anti-angiogenic therapeutic strategies.

The most widely investigated angiogenesis inhibitors are the proteolytic cleavage products of extracellular matrix or serum components, such as endostatin, angiostatin, arresten, and tumstatin (reviewed in [2, 3]). Multiple cytokines can also exert anti-angiogenic properties, including interferons and certain interleukins, which often act indirectly by limiting the expression of pro-angiogenic mediators or inducing anti-angiogenic molecules (reviewed in [2, 3]). In contrast, there are very few known developmentally regulated, naturally occurring anti-angiogenic molecules, which include platelet factor 4 [4], thrombospondin-1 [5], and pigment epithelium-derived factor [6], whose precise mechanism of action is not fully understood. In this regard, emerging evidence suggests that proteins involved in transmitting axonal guidance cues, including members of the netrin, slit, eph and semaphorin families, also play a critical role in blood vessel guidance during physiological and pathological blood vessel development [7, 8]. In this review,

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we summarize the current knowledge on semaphorin family proteins in angiogenesis, with an emphasis on semaphorin-regulated anti-angiogenic signaling circuitries in endothelial cells. The possible role of semaphorin signaling in supressing lymphangiogenesis will also be discussed.

Semaphorins

Semaphorins are a family of cell surface and soluble proteins originally identified as axon guidance factors that control the development of central nervous system [9]. All semaphorins are characterized by an amino-terminal 500-amino acid Sema domain that is essential for signaling. Semaphorins are grouped into eight classes based on their structural domains, with classes 3-7 comprising the vertebrate semaphorins (Figure 1). Although initially identified as potent axon chemorepellents, several semaphorins can provide bifunctional guidance cues, functioning as repulsive or attractive molecules depending on the cell types and biological context [10, 11]. The common targets of semaphorins are the actin cytoskeleton and focal adhesions; the latter are dynamic cell-to-extracellular matrix adhesive structures that are assembled upon integrin engagement. Semaphorin signaling affects focal adhesion assembly/disassembly and induces cytoskeletal remodeling, thus consequently affecting cell shape, attachment to the extracellular matrix, cell motility, and cell migration [12, 13].

Semaphorin receptors: plexins and neuropilins (Nrps)

Semaphorins signal through two major receptor families, plexins and Nrps. In vertebrates, two Nrps (Nrp1 and Nrp2) and nine plexins have been identified [14] (Figure 1). Membrane-bound semaphorins bind directly to plexins, whereas secreted type of semaphorins (class 3 semaphorins; Sema3s) bind to a holoreceptor complex consisting of Nrps as ligand binding and plexins as signal transducing subunit. An exception to this rule is Sema3E, which binds and signals directly through plexin-D1, independently of Nrps [15].

Plexins are single-pass transmembrane receptors and subdivided into four groups, type A, B, C, and D. Similar to semaphorins, plexins have extracellular Sema domains. In addition, plexins have PSI (plexins, Sema, integrins) and IPT (Ig-like, plexins, transcription factors) domains, and share homology in their extracellular segment with the Met family tyrosine kinase receptors [14]. The intracellular domains of plexins have weak sequence similarity to GTPase activating proteins (GAPs) and display GAP activity towards the small GTPase R-Ras [16]. Type A, B, and D plexins require the association of the

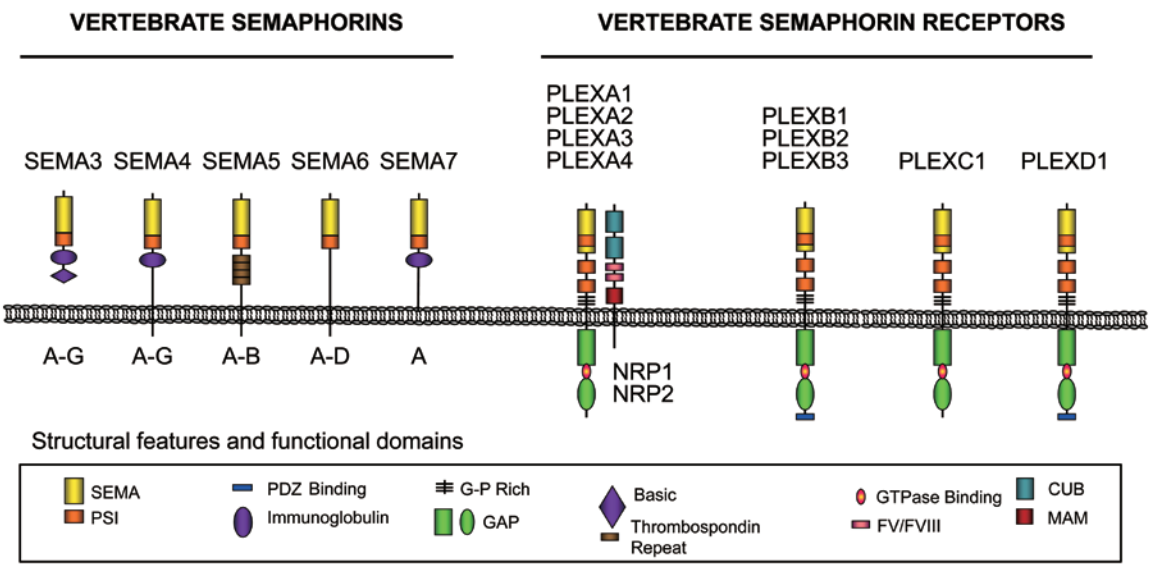

Figure 1 Human Plexins, Nrps, and Semaphorins: Vertebrates express semaphorin classes 3 through 7, plexins A, B, C, D, and Nrps 1 and 2. Semaphorins and plexins are comprised of a Sema domain and variable repeats of the PSI domain, as indicated. Members of the semaphorin 3 (Sema3, A-G) class are secreted, while the semaphorins 4-7 (Sema4-7) are membrane bound. Plexins contain cytoplasmic tails that contain both GAP and GTPase-binding domains. Additionally, plexins B1, B2, B3, and D1 contain a PDZ binding domain. Nrp1 and Nrp2 are comprised of extracellular complement binding, FV/FVIII, and MAM domains, but have only a short cytoplasmic tail, requiring their association with plexin A1-4 to facilitate signaling.

Rnd family of Rho-related GTPases to function as R-Ras GAP, while plexin-C1 displays GAP activity without Rnd [17]. Plexin-B1 also possesses GAP activity for R-Ras3/M-Ras [18].

Nrps are transmembrane proteins with a short cytoplasmic domain of about 40 amino acids, and their three C-terminal amino acids (S-E-A) constitute a PDZ-binding motif [19]. In addition to Sema3s, Nrps also bind to structurally unrelated molecules, such as VEGF family proteins, and serve as their co-receptors [20, 21]. The extracellular domains of Nrps contain two complement-binding domains (a1/a2), two coagulation factor V/VIII homology domains (b1/b2), and a MAM domain (c). Sema3s principally bind to a1/a2 domains, and VEGFs bind to b1/b2 domains [14]. Genetic studies have shown that Nrp1 is required for vascular morphogenesis. Nrp1-knockout mice are embryonically lethal due to vascular remodeling and branching defects [22, 23], whereas Nrp2-knockout mice are viable and their vasculature is grossly normal. However, Nrp2 null mice show absence of or reduced lymphatic vessel sprouting during development [24], suggesting that Nrp2 plays a key role in lymphangiogenesis (discussed below).

Anti-angiogenic semaphorins

Sema3s are the only secreted type of semaphorins in vertebrates. Seven Sema3s have been identified (designated by the letters A-G) and multiple Sema3s are reported to control physiological and pathological angiogenesis [25, 26]. Nrps and the type A/D family plexins (plexin-A1, -A2, and -A3, and plexin-D1) act as receptors for Sema3s, and each Sema3 family member shows distinct binding preference for Nrps. For example, Sema3A binds to Nrp1 [27, 28], while Sema3F binds exclusively to Nrp2 [29]. Other Sema3s, such as Sema3B, can bind to both Nrps [30, 31]. Each Sema3-Nrp complex associates with specific plexins to mediate downstream signaling. Some Sema3s, such as Sema3A and Sema3F, are expressed in endothelial cells, suggesting an endothelial-initiated autocrine regulation of angiogenesis [32].

Sema3A

Sema3A regulates endothelial cell migration and survival *in vitro* [33, 34], and tumor-induced angiogenesis *in vivo* [35]. The information emerging from the analysis of Sema3A null mice and mutant mice lacking Sema3A-Nrp1 signaling suggests that Sema3A may not be required for the early stages of developmental angiogenesis, but rather that Sema3A contributes to the reshaping of the vasculature to form a mature vascular network, such as that in the heart and brain [32, 36, 37]. The molecular mechanisms underlying the anti-angiogenic effects of Sema3A are complex, as its receptor Nrp1 also controls VEGFR2 signaling by binding VEGF165 [38, 39]. Hence, it was initially suggested that Sema3s compete for the binding to Nrp1 with VEGF, thus inhibiting VEGF-induced angiogenesis. However, recent reports have shown that Sema3s and VEGF use different domains on Nrp1 for binding [40]. Consistent with a separate function of Sema3A on Nrp1, Sema3A increases vascular permeability, inhibits endothelial cell proliferation, and induces apoptosis even in the absence of VEGF [34, 41], suggesting Sema3A activates its own signaling routes. Interestingly, Sema3A impairs endothelial cell adhesion and migration by inhibiting integrin function [32]. The molecular mechanisms by which Sema3A regulates integrins are not fully understood. From the studies in neuronal cells, it is likely that activation of plexin-A1 by Sema3A induces the intrinsic R-Ras GAP activity of Plexin-A1, thus resulting in R-Ras inhibition [16, 42]. As R-Ras is known to sustain integrin activation, the inactivation of R-Ras by plexin-A1 may lead to the inactivation of integrins, thereby inhibiting integrin-mediated cell adhesion.

Sema3B

Sema3B and Sema3F were identified as tumor suppressors that are deleted or inactivated in lung cancer [43, 44]. Consistently, overexpression of Sema3B suppresses tumorigenesis in adenocarcinoma cell lines [45], and Sema3B also decreases proliferation of lung and breast cancer cell lines [30], suggesting Sema3B exerts direct effects on cancer cells. In addition to the inhibitory effect on cancer cells, Sema3B can repel endothelial cells mainly through Nrp1, and therefore functions as an angiogenesis inhibitor [46]. Interestingly, Sema3B activity is abrogated as a result of proteolytic cleavage by furin-like pro-protein convertases, which is associated with enhanced invasion and proliferation in many cancers [46, 47]. Together, Sema3B may function as a tumor suppressor by working on both tumor cells and endothelial cells. Certainly, more work is necessary to define the precise signaling events that mediate this effect of Sema3B.

Sema3E

As described above, Sema3E binds directly to and activates plexin-D1 receptor. Plexin-D1 is highly expressed in endothelium during development [48] and also in tumor-associated blood vessels [49]. Interestingly, the expression of plexin-D1 is dynamically regulated and increased in tip cells, which extend numerous filopodia and respond to attractive and repulsive guidance cues at

the leading edge of the new branching blood vessels [50]. Sema3E controls vascular patterning during development by inhibiting the expansion of intersomitic vessels into the somites [15, 51], and causes endothelial-tip cell filopodial retraction in growing blood vessels [52]. These data suggest that Sema3E is a potent chemorepellent for plexin-D1-expressing endothelial cells. Indeed, Sema3E displays anti-angiogenic properties in several different *in vivo* angiogenesis models [52, 53]. Furthermore, Sema3E is highly expressed in metastatic tumors, where a cleaved form of Sema3E may enhance tumor cell motility. Conversely, when wild-type Sema3E is overexpressed in cancer cell lines, Sema3E decreases tumor vessel density and tumor growth [54]. The interplay between cleaved products of Sema3E and potential receptors in cancer cells is an area of active investigation. More progress has been recently made on the study of how Sema3E prevents angiogenesis in endothelial cells, given the obligatory role of plexin-D1 but not of Nrps in this biological response. Sema3E may counteract the pro-angiogenic effects of VEGF by promoting the expression and release of a soluble VEGF receptor, thus inhibiting VEGF function in developing vessels [55]. In a more direct fashion in endothelial cells, upon stimulation with Sema3E, plexin-D1 initiates a two-pronged mechanism involving R-Ras inactivation and Arf6 activation, thereby affecting the activation status of β1 integrin and its intracellular trafficking, respectively [52] (Figure 2). At the molecular level, Sema3E binding to plexin-D1 induces the activation of type I phosphatidylinositol-4-phosphate-5-kinase (PIP5K) β, which may stimulate the production of phosphatidylinositol 4,5-biphosphate ($PI(4,5)P_2$) locally [56]. $PI(4,5)P_2$ then binds to the PH domain of a guanine nucleotide exchange factor (GEF) for Arf6, guanine nucleotide exchange protein 100, thus increasing its GEF activity and activating Arf6. Sema3E-induced Arf6 activation induces rapid focal adhesion disassembly and endothelial cell collapse, thereby inhibiting angiogenesis [56]. In addition, recent reports revealed that $PI(4,5)P_2$ acts as a second messenger and controls focal adhesion dynamics and actin cytoskeleton [57], both of which are critical for cell migration. In neuronal cells, inhibition of another isoform of PIP5K, PIPKIγ661, is required for Sema3A-induced axonal repulsion and growth cone collapse [42]. These data suggest that semaphorins may commonly utilize phospholipid-regulated signaling

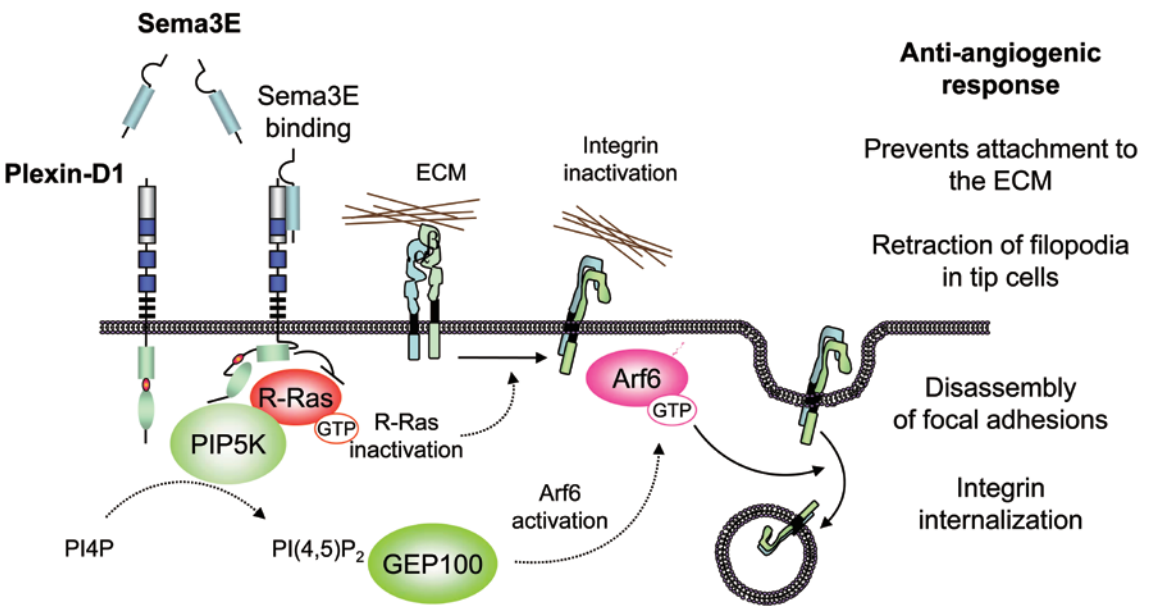

Figure 2 Anti-angiogenic signaling by Sema3E-plexin D1 in endothelial cells. The activation of plexin D1 by Sema3E induces the association of the Ras GAP domain of plexin D1 with R-Ras. This inactivates integrins and enables their subsequent internalization by the plexin D1-mediated activation of Arf6, thus inhibiting endothelial cell adhesion to the extracellular matrix (ECM) by disrupting integrin-mediated adhesive structures, and causing filopodial retraction in endothelial tip cells. The pathway by which Sema3E stimulation of plexin D1 leads to Arf6 activation involves phosphatidylinositol-4-phosphate-5-kinase β activation, which generates $PI(4,5)P_2$ locally. $PI(4,5)P_2$ then binds to the PH domain of an Arf6 GEF, guanine nucleotide exchange protein 100, resulting in Arf6 activation. Sema3E-induced Arf6 activation induces rapid focal adhesion (FA) disassembly, integrin internalization, and endothelial cell collapse, thereby inhibiting angiogenesis.

pathways to regulate cell adhesion and migration, by regulating lipid kinases such as PIP5K.

Sema3F

Sema3F decreases tumor growth in a number of *in vivo* tumor models, and although Sema3F is capable of interacting with Nrp1, its higher-affinity interaction with Nrp2 appears to be required for its tumor-suppressive activity in many models [58-60]. Sema3F exerts a repulsive effect on breast cancer cells [61] and reduces the growth and metastatic activity of colorectal carcinoma cells by modifying integrin αvβ3 [62], suggesting that Sema3F affects tumor cells directly by controlling cell adhesion and migration. Similar to other Sema3s, the Sema3F-mediated suppression of tumor growth was associated with an anti-angiogenic phenotype, where Sema3F expression significantly decreased the vascularity of tumors [25, 58]. Consistently, Sema3F can induce endothelial cell collapse [63], repel endothelial cells and inhibit their survival [59], and this anti-angiogenic effect is synergistically enhanced by the addition of Sema3A [34]. At the molecular level, it has been shown that Sema3F inhibits multiple signaling pathways in cancer cells [63, 64]; however, whether these pathways are also affected in endothelial cells remains unclear. While it is clear that Sema3F can function as a potent tumor suppressor *in vivo*, further studies are required to understand the molecular mechanism behind this function and the role that Sema3F expression plays in cancer development and progression.

Sema3G

Far less is known about the role of Sema3G in cancer and signaling. Sema3G has been identified as a significant prognostic marker in glioma [65]. Sema3G appears to bind to Nrp2, but not Nrp1 [66], and is able to suppress tumor growth and inhibit soft-agar colony formation only in cells expressing high levels of this receptor [25]. However, further work is necessary to determine whether Sema3G has anti-angiogenic effects *in vivo* and the mechanism by which it achieves its Nrp2-dependent tumor growth-suppressive activity.

Pro-angiogenic semaphorins

Some plexins are highly expressed in endothelial cells. Among them, plexin-B1 is a receptor for Sema4D, and it has been reported that Sema4D-Plexin-B1 signaling promotes endothelial cell migration and tube formation [67, 68]. Interestingly, Sema4D expression is upregulated in head and neck squamous cell carcinoma as well as in some other solid tumors [69, 70]. Sema4D is processed and released from the membrane by membrane type 1-matrix metalloproteinase, which is frequently overexpressed in malignant tumors, and activates plexin-B1 to elicit pro-angiogenic signaling in endothelial cells [71]. Similarly, Sema4D can be released from platelets by the action of metalloprotease ADAM17 (TACE), thereby acting on endothelial cells as well as platelets that also express Sema4D receptors [72]. The pro-angiogenic effect of Sema4D is mediated by the activation of a small GTPase RhoA through Rho-specific GEFs, leukemia-associated RhoGEF, and PDZ-RhoGEF, which bind to the C-terminal PDZ-binding motif of plexin-B1 [67, 73-76]. Upon ligation of Sema4D, PDZ-RhoGEF and leukemia-associated RhoGEF are recruited to plexin-B1, thus stimulating RhoA and its downstream effector Rho kinase, and Rho kinase activation promotes angiogenic response through a chain of events involving myosin light chain phosphorylation, stress fiber contaction, non-receptor tyrosine kinase activation, and activation of the Akt and Erk pathways [77] (Figure 3). RhoA/Rho kinase signaling also activates PIP5K α and leads to the generation of the lipid second messenger $PI(4,5)P_2$, further supporting the idea that phosphoinositides may represent a common mediator for semaphorin signaling. In the case of Sema4D, $PI(4,5)P_2$ serves as a substrate for PLCγ and increases intracellular calcium level, which is required for Sema4D-induced tube formation *in vitro* [78]. In addition to the direct effect of Sema4D on plexin-B1, this semaphorin can also promote endothelial cell migration through the activation of HGF receptor Met [68], while in other cell types, plexin-B1 activates members of the EGF receptor family [79] and inhibits RhoA through p190 RhoGAP [80], resulting in changes in the actin cytoskeleton and cell migration.

Lymphangiogenesis and cancer

Like angiogenesis, lymphangiogenesis is both a physiological and pathological process required for the formation of new vasculature, and the regulation of this process is mediated by a balance of pro- and anti-lymphangiogenic factors. The lymphatic system is critical for the removal of waste products and transport of cells and proteins, and contributes to the immune response and cancer progression. During embryogenesis, expression of the transcription factor PROX1 leads to the differentiation of venous endothelial cells into lymphatic endothelial cells [81-84]. This differentiation is followed by sprouting and proliferation of the lymphatic vasculature, which is primarily attributed to signaling by VEGF-C through its receptor VEGFR3 [85-87]. VEGF-C binds to receptors VEGFR3 and Nrp2, and loss of these molecules results in a variety of lymphangiogenic defects. *Vegfc*$^{-/-}$ mice

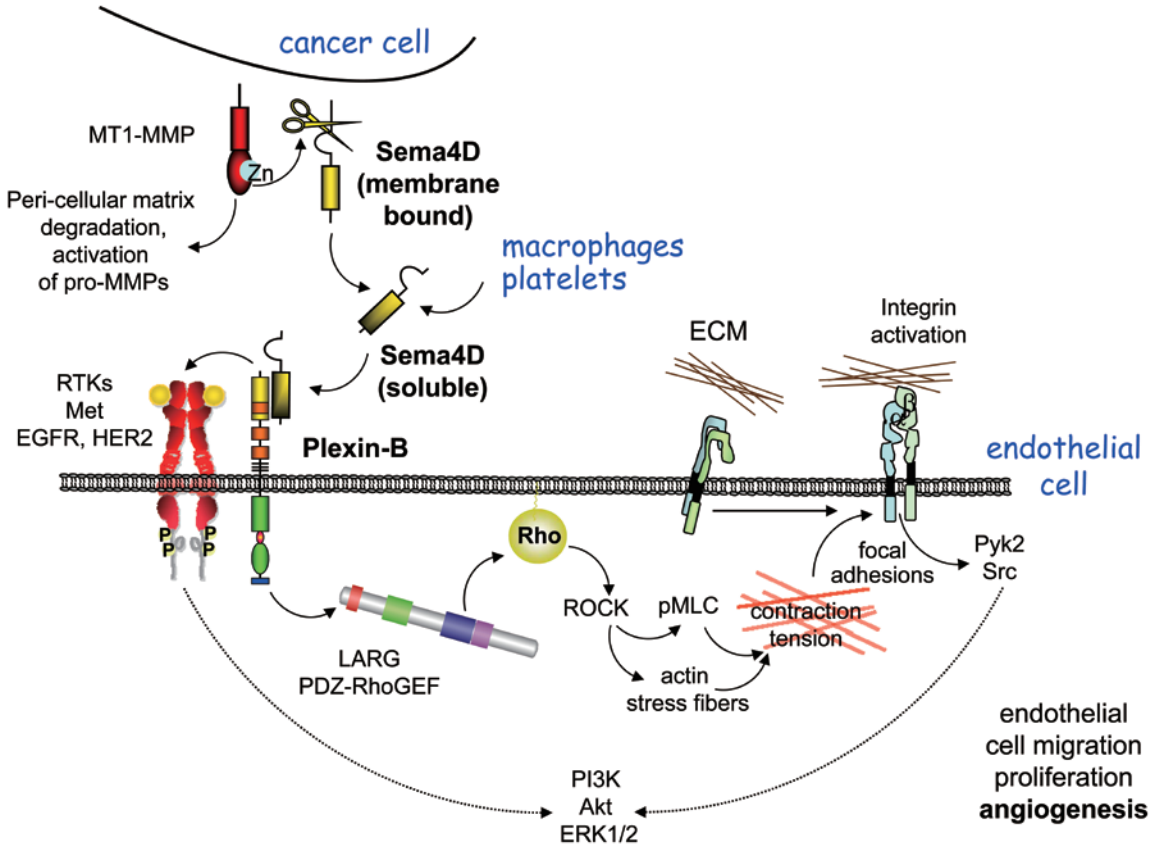

Figure 3 Pro-angiogenic signaling by semaphorins in endothelial cells. Membrane-bound Sema4D expressed by cancer cells or by tumor-associated macrophages and platelets is cleaved by matrix metalloproteinases, and soluble Sema4D can then bind and activate plexin-B members expressed on endothelial cells. After stimulation by Sema4D, plexin-B can activate certain receptor tyrosine kinases, such as Met, EGFR, and Her2, in some cellular contexts, hence activating their regulated signaling pathways indirectly. Sema4D binding to plexin-B causes the recruitment and activation of PDZ-RhoGEF and leukemia-associated RhoGEF through association mediated by their PDZ domains. These GEFs then activate Rho and its downstream effector Rho kinase (ROCK). In turn, ROCK signaling promotes the phosphorylation of myosin light chain. This induces polymerization and contraction of actin/myosin stress fibers. The tension generated by contracting stress fibers promotes the assembly of mature focal adhesion complexes at the sites of cell contact with the extracellular matrix via integrins, thereby activating non-receptor tyrosine kinases such as Pyk2 and Src. Pyk2 activation results in the phosphorylation of phosphatidylinositol 3-kinase and the activation of the Akt and Erk1/2 pathways, leading to increased endothelial cell migration and proliferation, which contribute to promoting an angiogenic response.

are able to commit to the lymphatic lineage, but cannot undergo lymphangiogenesis and die *in utero* [88]. VEGFR3-knockout mice display a more severe phenotype, where they lack lymphatic vasculature entirely and fail to undergo lymphangiogenesis [89]. In contrast, Nrp2 is not required for lymphangiogenesis but appears to be a key regulator of the process. Mice deficient in Nrp2 show a variety of lymphatic defects, including reduction of small lymphatic vessels and capillaries [24]. Further, Nrp2 appears to be selectively expressed in tumor-associated lymphatic vessels, and inhibition of Nrp2 function blocked VEGF-C/VEGFR3-mediated lymphangiogenesis and reduced metastasis *in vivo* [90, 91]. By extension, this suggests that anti-angiogenic semaphorins like Sema3F can function as anti-lymphangiogenic signaling molecules *in vivo* through their interaction with Nrp2. Sema3F overexpression has been shown to have a direct chemorepulsive effect on lymphatic endothelial cells [58]. Sema3F appears to compete for Nrp2 binding with VEGF-C and decreases endothelial cell survival and migration [92]. Nevertheless, direct evidence for an anti-lymphangiogenic role of Sema3F is limited.

Indirectly, tumor-induced lymphangiogenesis is correlated with metastasis in a number of cancer models, including colorectal, breast, prostate, head and neck cancer, and melanoma [93-99], and expression of Semaphorin

molecules decreases the incidence of metastasis [58, 62, 100]. Inhibition of tumoral lymphangiogenesis through neutralization of VEGFR3 or knockdown of VEGF-C expression blocks metastasis *in vivo* [101-103], indicating that this process is essential for metastasis in a variety of cancer models. Interestingly, this inhibition did not affect the normal lymphatic vasculature in adult mice, suggesting that alternative signaling mechanisms are employed after development, and targeting the VEGF-C/VEGFR3 signaling axis in adults may represent a potent means to target the tumor-associated lymphatics [104]. The precise molecular events that signal for lymphangiogenesis are poorly understood. Inhibition of the mTOR pathway decreases lymphangiogenesis and metastasis, and prolongs survival in a head and neck cancer animal model [99]. In support of this, a separate study found that inhibition of phosphatidylinositol 3-kinase/Akt similarly blocked lymphangiogenesis [105]. However, the specific signaling events required to initiate and sustain lymphangiogenesis in the context of tumor progression and metastasis are poorly defined. Given that tumoral lymphangiogenesis is likely a critical event in the progression of metastatic disease, the potential role of semaphorin-mediated regulation in this process warrants further investigation.

Concluding remarks

Recent studies have revealed the importance of semaphorins, plexins, and Nrps in tumor progression. These molecules directly control the behavior of tumor cells and also affect the other cell types which constitute the tumor microenviroment. In this review, we focused on vascular and lymphatic endothelial cells, both of which contribute to providing a suitable environment for tumor cells to grow and metastasize by inducing angiogenesis and lymphangiogenesis. A large fraction of the current information regarding semaphorin signaling was initially obtained from extensive studies in neuronal and cancer cells. It will be important to analyze whether the pathways deployed by the semaphorin-plexin signaling systems are shared by all cell types, with emphasis on those utilized in vascular and lymphatic endothelial cells to inhibit or promote angiogenesis and lymphangiogenesis. From the studies on Sema3B and Sema3F, loss of semaphorin function may represent an early event in cancer development, thus contributing to the acquisition of the angiogenic phenotype that characterizes most solid tumors. However, a number of questions remain: Which semaphorins are cleaved by furin-like proteases, and how does the proteolytic cleavage of semaphorins affect their function? Can the differential expression of these enzymatic factors account for the discrepancies in signaling and biological effects observed among different studies? Does differential expression of semaphorin receptors on tumor and endothelial cells affect their signaling potential? How does expression of competitive factors, such as VEGF, regulate the signaling capabilities of the semaphorins? Further, can their tumor-suppressive and anti-angiogenic roles be strengthened by multi-targeted approaches that take into account both the semaphorins and the regulators of their expression and function? What are the molecular differences in the signaling pathways activated by semaphorin binding, as opposed to the withdrawal of VEGF signaling? Semaphorin expression appears to affect a number of cellular phenotypes, including proliferation, survival, anchorage dependence, angiogenesis, and migration. Which among these represent the critical processes that are central to semaphorin-mediated tumor suppression? Answering these and other questions may be central to understanding the role of semaphorins in angiogenesis and cancer.

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References

1 Carmeliet P. Angiogenesis in life, disease and medicine. *Nature* 2005; **438**:932-936.
2 Folkman J. Angiogenesis: an organizing principle for drug discovery?. *Nat Rev Drug Discov* 2007; **6**:273-286.
3 Nyberg P, Xie L, Kalluri R. Endogenous inhibitors of angiogenesis. *Cancer Res* 2005; **65**:3967-3979.
4 Maione TE, Gray GS, Petro J, *et al.* Inhibition of angiogenesis by recombinant human platelet factor-4 and related peptides. *Science* 1990; **247**:77-79.
5 Good DJ, Polverini PJ, Rastinejad F, *et al.* A tumor suppressor-dependent inhibitor of angiogenesis is immunologically and functionally indistinguishable from a fragment of thrombospondin. *Proc Natl Acad Sci USA* 1990; **87**:6624-6628.
6 Dawson DW, Volpert OV, Gillis P, *et al.* Pigment epithelium-derived factor: a potent inhibitor of angiogenesis. *Science* 1999; **285**:245-248.
7 Carmeliet P, Tessier-Lavigne M. Common mechanisms of nerve and blood vessel wiring. *Nature* 2005; **436**:193-200.
8 Adams RH, Eichmann A. Axon guidance molecules in vascular patterning. *Cold Spring Harb Perspect Biol* 2010; **2**:a001875.
9 Kruger RP, Aurandt J, Guan KL. Semaphorins command cells to move. *Nat Rev Mol Cell Biol* 2005; **6**:789-800.
10 Eichmann A, Makinen T, Alitalo K. Neural guidance molecules regulate vascular remodeling and vessel navigation. *Genes Dev* 2005; **19**:1013-1021.
11 Derijck AA, Van Erp S, Pasterkamp RJ. Semaphorin signaling: molecular switches at the midline. *Trends Cell Biol*

2010; **20**:568-576.
12 Gelfand MV, Hong S, Gu C. Guidance from above: common cues direct distinct signaling outcomes in vascular and neural patterning. *Trends Cell Biol* 2009; **19**:99-110.
13 Serini G, Napione L, Arese M, Bussolino F. Besides adhesion: new perspectives of integrin functions in angiogenesis. *Cardiovasc Res* 2008; **78**:213-222.
14 Neufeld G, Kessler O. The semaphorins: versatile regulators of tumour progression and tumour angiogenesis. *Nat Rev Cancer* 2008; **8**:632-645.
15 Gu C, Yoshida Y, Livet J, *et al.* Semaphorin 3E and plexin-D1 control vascular pattern independently of neuropilins. *Science* 2005; **307**:265-268.
16 Oinuma I, Ishikawa Y, Katoh H, Negishi M. The Semaphorin 4D receptor plexin-B1 is a GTPase activating protein for R-Ras. *Science* 2004; **305**:862-865.
17 Uesugi K, Oinuma I, Katoh H, Negishi M. Different requirement for Rnd GTPases of R-Ras GAP activity of plexin-C1 and plexin-D1. *J Biol Chem* 2009; **284**:6743-6751.
18 Saito Y, Oinuma I, Fujimoto S, Negishi M. Plexin-B1 is a GTPase activating protein for M-Ras, remodelling dendrite morphology. *EMBO Rep* 2009; **10**:614-621.
19 Schwarz Q, Ruhrberg C. Neuropilin, you gotta let me know: should I stay or should I go? *Cell Adh Migr* 2010; **4**:61-66.
20 Soker S, Takashima 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* 1998; **92**:735-745.
21 Gluzman-Poltorak Z, Cohen T, Herzog Y, Neufeld G. Neuropilin-2 is a receptor for the vascular endothelial growth factor (VEGF) forms VEGF-145 and VEGF-165. *J Biol Chem* 2000; **275**:18040-18045.
22 Kawasaki T, Kitsukawa T, Bekku Y, *et al.* A requirement for neuropilin-1 in embryonic vessel formation. *Development* 1999; **126**:4895-4902.
23 Mukouyama YS, Gerber HP, Ferrara N, Gu C, Anderson DJ. Peripheral nerve-derived VEGF promotes arterial differentiation via neuropilin 1-mediated positive feedback. *Development* 2005; **132**:941-952.
24 Yuan L, Moyon D, Pardanaud L, *et al.* Abnormal lymphatic vessel development in neuropilin 2 mutant mice. *Development* 2002; **129**:4797-4806.
25 Kigel B, Varshavsky A, Kessler O, Neufeld G. Successful inhibition of tumor development by specific class-3 semaphorins is associated with expression of appropriate semaphorin receptors by tumor cells. *PLoS ONE* 2008; **3**:e3287.
26 Serini G, Maione F, Bussolino F. Semaphorins and tumor angiogenesis. *Angiogenesis* 2009 .
27 He Z, Tessier-Lavigne M. Neuropilin is a receptor for the axonal chemorepellent Semaphorin III. *Cell* 1997; **90**:739-751.
28 Kolodkin AL, Levengood DV, Rowe EG, Tai YT, Giger RJ, Ginty DD. Neuropilin is a semaphorin III receptor. *Cell* 1997; **90**:753-762.
29 Chen H, Chedotal A, He Z, Goodman CS, Tessier-Lavigne M. Neuropilin-2, a novel member of the neuropilin family, is a high affinity receptor for the semaphorins Sema E and Sema IV but not Sema III. *Neuron* 1997; **19**:547-559.
30 Castro-Rivera E, Ran S, Thorpe P, Minna JD. Semaphorin 3B (SEMA3B) induces apoptosis in lung and breast cancer, whereas VEGF165 antagonizes this effect. *Proc Natl Acad Sci USA* 2004; **101**:11432-11437.
31 Falk J, Bechara A, Fiore R, *et al.* Dual functional activity of semaphorin 3B is required for positioning the anterior commissure. *Neuron* 2005; **48**:63-75.
32 Serini G, Valdembri D, Zanivan S, *et al.* Class 3 semaphorins control vascular morphogenesis by inhibiting integrin function. *Nature* 2003; **424**:391-397.
33 Miao HQ, Soker S, Feiner L, Alonso JL, Raper JA, Klagsbrun M. Neuropilin-1 mediates collapsin-1/semaphorin III inhibition of endothelial cell motility: functional competition of collapsin-1 and vascular endothelial growth factor-165. *J Cell Biol* 1999; **146**:233-242.
34 Guttmann-Raviv N, Shraga-Heled N, Varshavsky A, Guimaraes-Sternberg C, Kessler O, Neufeld G. Semaphorin-3A and semaphorin-3F work together to repel endothelial cells and to inhibit their survival by induction of apoptosis. *J Biol Chem* 2007; **282**:26294-26305.
35 Maione F, Molla F, Meda C, *et al.* Semaphorin 3A is an endogenous angiogenesis inhibitor that blocks tumor growth and normalizes tumor vasculature in transgenic mouse models. *J Clin Invest* 2009; **119**:3356-3372.
36 Gu C, Rodriguez ER, Reimert DV, *et al.* Neuropilin-1 conveys semaphorin and VEGF signaling during neural and cardiovascular development. *Dev Cell* 2003; **5**:45-57.
37 Arese M, Serini G, Bussolino F. Nervous vascular parallels: axon guidance and beyond. *Int J Dev Biol* 2011; **55**:439-445.
38 Soker S, Miao HQ, Nomi M, Takashima S, Klagsbrun M. VEGF165 mediates formation of complexes containing VEGFR-2 and neuropilin-1 that enhance VEGF165-receptor binding. *J Cell Biochem* 2002; **85**:357-368.
39 Whitaker GB, Limberg BJ, Rosenbaum JS. Vascular endothelial growth factor receptor-2 and neuropilin-1 form a receptor complex that is responsible for the differential signaling potency of VEGF(165) and VEGF(121). *J Biol Chem* 2001; **276**:25520-25531.
40 Appleton BA, Wu P, Maloney J, *et al.* Structural studies of neuropilin/antibody complexes provide insights into semaphorin and VEGF binding. *EMBO J* 2007; **26**:4902-4912.
41 Acevedo LM, Barillas S, Weis SM, Gothert JR, Cheresh DA. Semaphorin 3A suppresses VEGF-mediated angiogenesis yet acts as a vascular permeability factor. *Blood* 2008; **111**:2674-2680.
42 Toyofuku T, Yoshida J, Sugimoto T, *et al.* FARP2 triggers signals for Sema3A-mediated axonal repulsion. *Nat Neurosci* 2005; **8**:1712-1719.
43 Sekido Y, Bader S, Latif F, *et al.* Human semaphorins A(V) and IV reside in the 3p21.3 small cell lung cancer deletion region and demonstrate distinct expression patterns. *Proc Natl Acad Sci USA* 1996; **93**:4120-4125.
44 Tomizawa Y, Sekido Y, Kondo M, *et al.* Inhibition of lung cancer cell growth and induction of apoptosis after reexpression of 3p21.3 candidate tumor suppressor gene SEMA3B. *Proc Natl Acad Sci USA* 2001; **98**:13954-13959.
45 Tse C, Xiang RH, Bracht T, Naylor SL. Human Semaphorin 3B (SEMA3B) located at chromosome 3p21.3 suppresses tumor formation in an adenocarcinoma cell line. *Cancer Res* 2002; **62**:542-546.
46 Varshavsky A, Kessler O, Abramovitch S, *et al.* Semaphorin-

3B is an angiogenesis inhibitor that is inactivated by furin-like pro-protein convertases. *Cancer Res* 2008; **68**:6922-6931.

47 Bassi DE, Fu J, Lopez de Cicco R, Klein-Szanto AJ. Proprotein convertases: "master switches" in the regulation of tumor growth and progression. *Mol Carcinog* 2005; **44**:151-161.

48 van der Zwaag B, Hellemons AJ, Leenders WP, *et al.* PLEXIN-D1, a novel plexin family member, is expressed in vascular endothelium and the central nervous system during mouse embryogenesis. *Dev Dyn* 2002; **225**:336-343.

49 Roodink I, Raats J, van der Zwaag B, *et al.* Plexin D1 expression is induced on tumor vasculature and tumor cells: a novel target for diagnosis and therapy?. *Cancer Res* 2005; **65**:8317-8323.

50 Kim J, Oh WJ, Gaiano N, Yoshida Y, Gu C. Semaphorin 3E-plexin-D1 signaling regulates VEGF function in developmental angiogenesis via a feedback mechanism. *Genes Dev* 2011; **25**:1399-1411.

51 Torres-Vazquez J, Gitler AD, Fraser SD, *et al.* Semaphorin-plexin signaling guides patterning of the developing vasculature. *Dev Cell* 2004; **7**:117-123.

52 Sakurai A, Gavard J, Annas-Linhares Y, *et al.* Semaphorin 3E initiates antiangiogenic signaling through plexin D1 by regulating Arf6 and R-Ras. *Mol Cell Biol* 2010; **30**:3086-3098.

53 Fukushima Y, Okada M, Kataoka H, *et al.* Sema3E-plexinD1 signaling selectively suppresses disoriented angiogenesis in ischemic retinopathy in mice. *J Clin Invest* 2011; **121**:1974-1985.

54 Casazza A, Finisguerra V, Capparuccia L, *et al.* Sema3E-plexin D1 signaling drives human cancer cell invasiveness and metastatic spreading in mice. *J Clin Invest* 2010; **120**:2684-2698.

55 Zygmunt T, Gay CM, Blondelle J, *et al*. Semaphorin-plexinD1 signaling limits angiogenic potential via the VEGF decoy receptor sFlt1. *Dev Cell* 2011; **21**:301-314.

56 Sakurai A, Jian X, Lee CJ, *et al*. Phosphatidylinositol-4-phosphate 5-Kinase and GEP100/Brag2 protein mediate antiangiogenic signaling by semaphorin 3E-plexin-D1 through Arf6 protein. *J Biol Chem* 2011; **286**:34335-34345.

57 van den Bout I, Divecha N. PIP5K-driven PtdIns(4,5)P2 synthesis: regulation and cellular functions. *J Cell Sci* 2009; **122**:3837-3850.

58 Bielenberg DR, Hida Y, Shimizu A, *et al.* Semaphorin 3F, a chemorepulsant for endothelial cells, induces a poorly vascularized, encapsulated, nonmetastatic tumor phenotype. *J Clin Invest* 2004; **114**:1260-1271.

59 Kessler O, Shraga-Heled N, Lange T, *et al*. Semaphorin-3F is an inhibitor of tumor angiogenesis. *Cancer Res* 2004; **64**:1008-1015.

60 Futamura M, Kamino H, Miyamoto Y, *et al.* Possible role of semaphorin 3F, a candidate tumor suppressor gene at 3p21.3, in p53-regulated tumor angiogenesis suppression. *Cancer Res* 2007; **67**:1451-1460.

61 Nasarre P, Kusy S, Constantin B, *et al.* Semaphorin SEMA3F has a repulsing activity on breast cancer cells and inhibits E-cadherin-mediated cell adhesion. *Neoplasia* 2005; **7**:180-189.

62 Wu F, Zhou Q, Yang J, *et al*. Endogenous axon guiding chemorepulsant semaphorin-3F inhibits the growth and metastasis of colorectal carcinoma. *Clin Cancer Res* 2011; **17**:2702-2711.

63 Shimizu A, Mammoto A, Italiano JE Jr, *et al.* ABL2/ARG tyrosine kinase mediates SEMA3F-induced RhoA inactivation and cytoskeleton collapse in human glioma cells. *J Biol Chem* 2008; **283**:27230-27238.

64 Potiron VA, Sharma G, Nasarre P, *et al*. Semaphorin SEMA3F affects multiple signaling pathways in lung cancer cells. *Cancer Res* 2007; **67**:8708-8715.

65 Karayan-Tapon L, Wager M, Guilhot J, *et al.* Semaphorin, neuropilin and VEGF expression in glial tumours: SEMA3G, a prognostic marker?. *Br J Cancer* 2008; **99**:1153-1160.

66 Taniguchi M, Masuda T, Fukaya M, *et al.* Identification and characterization of a novel member of murine semaphorin family. *Genes Cells* 2005; **10**:785-792.

67 Basile JR, Barac A, Zhu T, Guan KL, Gutkind JS. Class IV semaphorins promote angiogenesis by stimulating Rho-initiated pathways through plexin-B. *Cancer Res* 2004; **64**:5212-5224.

68 Conrotto P, Valdembri D, Corso S, *et al.* Sema4D induces angiogenesis through Met recruitment by plexin B1. *Blood* 2005; **105**:4321-4329.

69 Basile JR, Castilho RM, Williams VP, Gutkind JS. Semaphorin 4D provides a link between axon guidance processes and tumor-induced angiogenesis. *Proc Natl Acad Sci USA* 2006; **103**:9017-9022.

70 Ch'ng E, Tomita Y, Zhang B, *et al.* Prognostic significance of CD100 expression in soft tissue sarcoma. *Cancer* 2007; **110**:164-172.

71 Basile JR, Holmbeck K, Bugge TH, Gutkind JS. MT1-MMP controls tumor-induced angiogenesis through the release of semaphorin 4D. *J Biol Chem* 2007; **282**:6899-6905.

72 Zhu L, Bergmeier W, Wu J, *et al.* Regulated surface expression and shedding support a dual role for semaphorin 4D in platelet responses to vascular injury. *Proc Natl Acad Sci USA* 2007; **104**:1621-1626.

73 Aurandt J, Vikis HG, Gutkind JS, Ahn N, Guan KL. The semaphorin receptor plexin-B1 signals through a direct interaction with the Rho-specific nucleotide exchange factor, LARG. *Proc Natl Acad Sci USA* 2002; **99**:12085-12090.

74 Hirotani M, Ohoka Y, Yamamoto T, *et al*. Interaction of plexin-B1 with PDZ domain-containing Rho guanine nucleotide exchange factors. *Biochem Biophys Res Commun* 2002; **297**:32-37.

75 Perrot V, Vazquez-Prado J, Gutkind JS. Plexin B regulates Rho through the guanine nucleotide exchange factors leukemia-associated Rho GEF (LARG) and PDZ-RhoGEF. *J Biol Chem* 2002; **277**:43115-43120.

76 Swiercz JM, Kuner R, Behrens J, Offermanns S. Plexin-B1 directly interacts with PDZ-RhoGEF/LARG to regulate RhoA and growth cone morphology. *Neuron* 2002; **35**:51-63.

77 Basile JR, Gavard J, Gutkind JS. Plexin-B1 utilizes RhoA and Rho kinase to promote the integrin-dependent activation of Akt and ERK and endothelial cell motility. *J Biol Chem* 2007; **282**:34888-34895.

78 Binmadi NO, Proia P, Zhou H, Yang YH, Basile JR. Rho-mediated activation of PI(4)P5K and lipid second messengers is necessary for promotion of angiogenesis by Semaphorin 4D. *Angiogenesis* 2011; **14**:309-319.

79 Swiercz JM, Kuner R, Offermanns S. Plexin-B1/RhoGEF-

mediated RhoA activation involves the receptor tyrosine kinase ErbB-2. *J Cell Biol* 2004; **165**:869-880.
80 Barberis D, Casazza A, Sordella R, *et al.* p190 Rho-GTPase activating protein associates with plexins and it is required for semaphorin signalling. *J Cell Sci* 2005; **118**:4689-4700.
81 Wigle JT, Oliver G. Prox1 function is required for the development of the murine lymphatic system. *Cell* 1999; **98**:769-778.
82 Wigle JT, Harvey N, Detmar M, *et al.* An essential role for Prox1 in the induction of the lymphatic endothelial cell phenotype. *EMBO J* 2002; **21**:1505-1513.
83 Petrova TV, Makinen T, Makela TP, *et al.* Lymphatic endothelial reprogramming of vascular endothelial cells by the Prox-1 homeobox transcription factor. *EMBO J* 2002; **21**:4593-4599.
84 Hong YK, Harvey N, Noh YH, *et al.* Prox1 is a master control gene in the program specifying lymphatic endothelial cell fate. *Dev Dyn* 2002; **225**:351-357.
85 Karpanen T, Egeblad M, Karkkainen MJ, *et al.* Vascular endothelial growth factor C promotes tumor lymphangiogenesis and intralymphatic tumor growth. *Cancer Res* 2001; **61**:1786-1790.
86 Veikkola T, Jussila L, Makinen T, *et al.* Signalling via vascular endothelial growth factor receptor-3 is sufficient for lymphangiogenesis in transgenic mice. *EMBO J* 2001; **20**:1223-1231.
87 He Y, Kozaki K, Karpanen T, *et al.* Suppression of tumor lymphangiogenesis and lymph node metastasis by blocking vascular endothelial growth factor receptor 3 signaling. *J Natl Cancer Inst* 2002; **94**:819-825.
88 Karkkainen MJ, Haiko P, Sainio K, *et al.* Vascular endothelial growth factor C is required for sprouting of the first lymphatic vessels from embryonic veins. *Nat Immunol* 2004; **5**:74-80.
89 Haiko P, Makinen T, Keskitalo S, *et al.* Deletion of vascular endothelial growth factor C (VEGF-C) and VEGF-D is not equivalent to VEGF receptor 3 deletion in mouse embryos. *Mol Cell Biol* 2008; **28**:4843-4850.
90 Caunt M, Mak J, Liang WC, *et al.* Blocking neuropilin-2 function inhibits tumor cell metastasis. *Cancer Cell* 2008; **13**:331-342.
91 Xu Y, Yuan L, Mak J, *et al.* Neuropilin-2 mediates VEGF-C-induced lymphatic sprouting together with VEGFR3. *J Cell Biol* 2010; **188**:115-130.
92 Favier B, Alam A, Barron P, *et al.* Neuropilin-2 interacts with VEGFR-2 and VEGFR-3 and promotes human endothelial cell survival and migration. *Blood* 2006; **108**:1243-1250.
93 Nathanson SD. Insights into the mechanisms of lymph node metastasis. *Cancer* 2003; **98**:413-423.
94 Schietroma C, Cianfarani F, Lacal PM, *et al.* Vascular endothelial growth factor-C expression correlates with lymph node localization of human melanoma metastases. *Cancer* 2003; **98**:789-797.
95 Dadras SS, Lange-Asschenfeldt B, Velasco P, *et al.* Tumor lymphangiogenesis predicts melanoma metastasis to sentinel lymph nodes. *Mod Pathol* 2005; **18**:1232-1242.
96 Roma AA, Magi-Galluzzi C, Kral MA, Jin TT, Klein EA, Zhou M. Peritumoral lymphatic invasion is associated with regional lymph node metastases in prostate adenocarcinoma. *Mod Pathol* 2006; **19**:392-398.
97 He XW, Yu X, Liu T, Yu SY, Chen DJ. Vector-based RNA interference against vascular endothelial growth factor-C inhibits tumor lymphangiogenesis and growth of colorectal cancer *in vivo* in mice. *Chin Med J* 2008; **121**:439-444.
98 Das S, Ladell DS, Podgrabinska S, *et al.* Vascular endothelial growth factor-C induces lymphangitic carcinomatosis, an extremely aggressive form of lung metastases. *Cancer Res* 2010; **70**:1814-1824.
99 Patel V, Marsh CA, Dorsam RT, *et al*. Decreased lymphangiogenesis and lymph node metastasis by mTOR inhibition in head and neck cancer. *Cancer Res* 2011; **71**:7103-7112.
100 Casazza A, Fu X, Johansson I, *et al.* Systemic and targeted delivery of semaphorin 3A inhibits tumor angiogenesis and progression in mouse tumor models. *Arterioscler Thromb Vasc Biol* 2011; **31**:741-749.
101 Roberts N, Kloos B, Cassella M, *et al.* Inhibition of VEGFR-3 activation with the antagonistic antibody more potently suppresses lymph node and distant metastases than inactivation of VEGFR-2. *Cancer Res* 2006; **66**:2650-2657.
102 Khromova N, Kopnin P, Rybko V, Kopnin BP. Downregulation of VEGF-C expression in lung and colon cancer cells decelerates tumor growth and inhibits metastasis via multiple mechanisms. *Oncogene* 2011 Aug 1. doi: 10.1038/onc.2011.330
103 Kodera Y, Katanasaka Y, Kitamura Y, *et al.* Sunitinib inhibits lymphatic endothelial cell functions and lymph node metastasis in a breast cancer model through inhibition of vascular endothelial growth factor receptor 3. *Breast Cancer Res* 2011; **13**:R66.
104 Karpanen T, Wirzenius M, Makinen T, *et al.* Lymphangiogenic growth factor responsiveness is modulated by postnatal lymphatic vessel maturation. *Am J Pathol* 2006; **169**:708-718.
105 Yoo YA, Kang MH, Lee HJ, *et al.* Sonic hedgehog pathway promotes metastasis via activation of Akt, EMT, and MMP-9 in gastric cancer. *Cancer Res* 2011; **71**:7061-7070.