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Understanding netrins and semaphorins in mature endothelial cell biology

Huayu Zhang[a], Dianne Vreeken[a], Caroline S. Bruikman[b], Anton Jan van Zonneveld[a], Janine M. van Gils[a,*]

[a] Department of Internal Medicine, Einthoven Laboratory for Vascular and Regenerative Medicine, Leiden University Medical Center, Albinusdreef 2, 2333 ZA, Leiden, the Netherlands
[b] Amsterdam UMC, University of Amsterdam, Department of Vascular Medicine, Amsterdam Cardiovascular Sciences, Meibergdreef 9, Amsterdam, the Netherlands

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ABSTRACT

Netrins and semaphorins are known as neuronal guidance molecules that are important to the facilitate patterning of the nervous system in embryonic development. In recent years, their function has been broadened to guide development in other systems, including the vascular system, where netrins and semaphorins critically contribute to the development of the vascular system. Evidence is accumulating that these guidance cues are also of critical importance in the biology of the mature endothelium by regulating the maintenance of endothelial quiescence. Here we review our current insights into the roles of netrins and semaphorins in endothelial cell survival, self-renewing, barrier function, response to wall shear stress, and control of the vascular tone. We also provide suggestions for future research into the functions of netrins and semaphorins in mature endothelial cell biology.

1. Introduction

The vascular system involves an extensive network of arteries, capillaries and veins, lined by endothelial cells. While the endothelial monolayer was first thought to be an inert layer between blood and tissue, a range of discoveries has led to better understanding of the complex active homeostatic functions of the endothelium including the controlling vascular tone, blood fluidity, and vascular inflammation [1]. The observation that the anatomy and gene expression patterns of the vascular system of vertebrates often overlap with that of the nervous system has led to the growing awareness that the coordinated patterning of nerves and vessels is achieved by each system separately using the same cues and signals [2,3]. These conserved patterning factors, together called the “neuronal guidance cues” (NGCs), were first identified in neural development and involve 4 major families of conserved ligands netrins, slits, semaphorins, and ephrins. NGCs act together through a complex interplay of short and long-range signals that can either repel or attract the cells of the developing network [4]. The effects of NGCs on the developing vascular network, especially with respect to angiogenesis, have been well characterized and received some excellent reviews [5–9]. However, next to their function in development, evidence is accumulating that a selective group of the NGCs, the netrins and semaphorins, have important homeostatic functions in the mature established endothelial monolayer. Here, we review the novel insights into the regulatory roles of netrins and semaphorins in endothelial cells in the mature endothelium.

2. Role of netrins and semaphorins in the survival of endothelial cells

The integrity of the vascular endothelium depends on the continued replacement of injured or senescent cells. Conditions that “stress” endothelial cells, such as an adverse hemodynamic or metabolic environment, or local and systemic inflammation, are associated with a faster turnover of endothelial cells [10,11]. This concept of endothelial cell turnover naturally raises two questions: (1) what contributes to preservation or survival of endothelial cells, and (2) what regulates the renewal activity of endothelial cells? Recent evidence points at roles for netrins and semaphorins in both phenomena.

Besides acting as a directional signal for angiogenic sprouting, vascular endothelial growth factor (VEGF) is a survival factor that plays a major role in the shaping of the vascular networks. Hypoxic tissues in need of (neo-)vascularization express VEGF that supports the generation and survival of the endothelial cells that makeup the new angiogenic network. However, this also relives the hypoxia in the tissue and, therefore, other mechanisms have to kick in to replace the pro-survival signals previously provided by VEGF. These stabilization signals include, amongst others, angiopoietin1/TIE2 signaling, platelet-derived

* Corresponding author.
E-mail address: jm.vangils@lumc.nl (J.M. van Gils).

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growth factor (receptor signaling), and signaling generated by the interaction of endothelial cells and extracellular matrix, more specifically the endothelial basement membrane, via integrins. Integrin activity has been shown to be important in stabilization and survival of mature vascular networks [12]. These stabilization signals within a functional network rely heavily on stimulation of endothelial cells by laminar shear stress. Below we discuss recent studies that demonstrate prominent roles for the netrins and semaphorins in the regulation of endothelial cell survival.

2.1. NTN4 promotes endothelial cell survival by activating integrin α6β1

The first vertebrate netrin was discovered in 1994 as a guidance cue for the embryonic development of commissural axons in the spinal cord [13,14]. Up until today, expression of six different netrins has been described in mammals. Netrin-1, 3, 4, and 5 (NTN1, 3, 4, 5) are secreted proteins, while netrin-G1 and G2 (NTNG1 and G2) are membrane-bound proteins tethered by glycosyl-phosphatidylinostitol tails [15,16]. Netrins can bind to the classical "deleted in colorectal cancer" (DCC), neogenin (NEO1) and uncoordinated-5 (UNC-5) receptors. Beside the classical ligand receptor interactions also interactions with other receptors, such as integrins [17] and adenosine A2b receptors (ADORA2B) [18], have been described (Fig. 2). In human endothelial cells, NTN4 is the highest expressed netrin class member [19–21] (Fig. 1) and can be upregulated by laminar shear stress [22]. Interestingly, NTN4 can serve as an activator of integrins, including endothelial integrin α6β1, which can bind laminin [23–25]. In human microvascular endothelial cells, NTN4 directly interacts with the integrin α6β1 complex, leading to more activated integrin β1. Functionally, a NTN4 coating indeed increases adhesion of endothelial cells *in vitro* to culture plate [23]. Studies from multiple groups also show that the presence of NTN4 increases activity of Akt and ERK1/2, suggesting that NTN4 is able to induce survival signaling in endothelial cells [26,27]. In line with this, less caspase3 activity was detected when human umbilical vein endothelial cells (HUVECs) were treated with NTN4 [21,26]. Together, these results suggest that NTN4 may provide endothelial cells with pro-survival signaling especially in stable functional vascular networks. It is important to note that NTN4 knockout mice, under normal conditions, do not have any defects in retina vascular networks [28,29]. Therefore, to fully understand the *in vivo* function of NTN4, further investigation is needed.

Intriguingly, NTN4 is itself a component of the basement membrane [30]; this is not surprising as the netrin family is structurally related to the laminin superfamily which has an important role in the structure of the extracellular matrix [31]. As a component of the basement membrane, NTN4 has been shown to regulate the assembly of laminin networks, thus altering the structure of the basement membrane and thereby affecting the survival of endothelial cells [32]. A report by Reuten et al. demonstrated that NTN4 competitively binds to the laminin γ chain, causing disassembly of the laminin network and disruption of the endothelial basement membrane [32]. Adding NTN4 to capillary networks in chick embryo chorioallantoic membranes disrupted formation of stable capillary networks [32]. It was then suggested that NTN4 causes destabilization of blood vessels. This suggestion is at first glance contradictory to the pro-survival role of NTN4. However, in order to function as a laminin network disassembling agent, a concentration of 2.1 μM NTN4 was needed, which is probably hardly reached in physiological conditions. Generally, a concentration of around 1.7 pM NTN4 can be found in endothelial cell culture [21]. Therefore, the consequences of regulation of the basement membrane structure by NTN4 in the physiological concentration range are not fully elucidated yet.

2.2. NTN1 has an anti-apoptotic effect on endothelial cells

Next to NTN4, NTN1 was found to be expressed by various types of endothelial cells. In general, NTN1 is believed to have moderate expression in endothelial cells [19,33,34]. Co-localization of NTN1 with endothelium was observed in both large blood vessels and capillaries [26,35]. There is a lack of agreement in the serum concentration NTN1. In healthy humans, several picograms to several hundred picograms per milliliter NTN1 were detected in serum using enzyme-linked immunosorbent assays, depending on the lab and reagents used [36–41]. More expression of NTN1 was found in endothelial cells cultured under shear stress and in aortic regions with higher shear stress [34]. In tumor cells and neurons, NTN1 has been shown to be a survival factor in cells expressing the netrin receptors DCC and UNC5H [42,43]. In endothelial cells, NTN1 also acts as a survival factor as NTN1 blocked the apoptotic effect of serum starvation in HUVECs and human umbilical artery endothelial cells [44]. This anti-apoptotic effect was proven to be an antagonistic effect of NTN1 binding to UNC5B, as blocking UNC5B with siRNA also reduced apoptosis while abolishing the anti-apoptotic effect of NTN1 [44]. Further evidence also demonstrated that NTN1 promotes endothelial cell survival by blocking the UNC5B-induced inhibition of death-associated protein kinase 1 (DAPK1) [44]. Similar mechanisms were described before in DCC and UNC5B expressing neurons, where binding of NTN1 to its receptors is essential to maintain survival of these neurons [42,43]. Under high glucose condition, NTN1 was shown to prevent bovine aortic endothelial from high glucose induced apoptosis by activating ERK1/2 and eNOS [45]. The effect of NTN1 on increasing nitrous oxide production is discussed in Section 6.

2.3. Ligands of PLXND1 decrease endothelial cell survival via regulation of integrin activity

The semaphorins are a large family of secreted (Class-3 semaphorins/SEMA3s) or membrane-associated proteins (Class- 4–7 semaphorins/SEMA 4–7). Almost all types of endothelial cells express high levels of SEMA3 F. SEMA3C and SEMA3G have varied expression levels among different endothelial cells. SEMA3 A has moderate expression. Class 4–7 semaphorins generally have moderate to high expression levels (Fig. 3).

The main receptors of semaphorins comprise two groups, namely neuropilins (NRP1 and NRP2) and plexins (PLXNA1-4, PLXNB1-3, PLXNC1, and PLXND1) [46–48]. In endothelial cells, high expression levels of PLXNA1, PLXNA2, PLXNB2 and PLXND1 can be found. NRP1

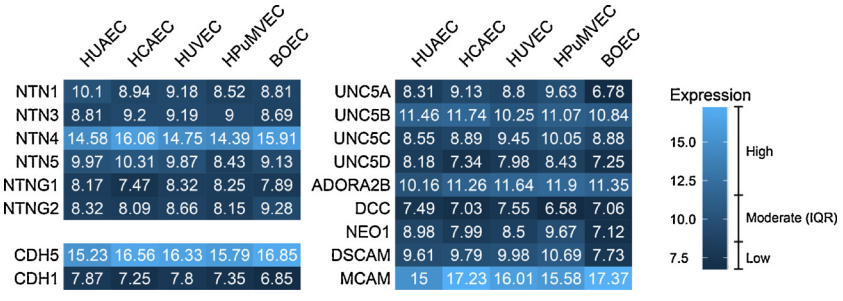

Fig. 1. Expression heatmap of netrin family proteins (left) and their receptors (right). Values are the average normalized expression signals from all published data on the Affymetrix Human Genome U133 Plus 2.0 Array (HG-U133 Plus 2.0/GPL570) platform obtained using the Genevestigator software [100]. Lighter colors indicate higher expression. Expression levels within the top quantile on GPL570 platform are recognized as "high"; levels within the inter-quantile range (IQR) are recognized as "moderate"; levels within the bottom quantile are recognized as "low". These notions are used consistently throughout this article. For comparison, we include CDH5 (VE-Cadherin) as a high-expression reference and CDH1 (E-Cadherin) as a low-expression reference (bottom left).

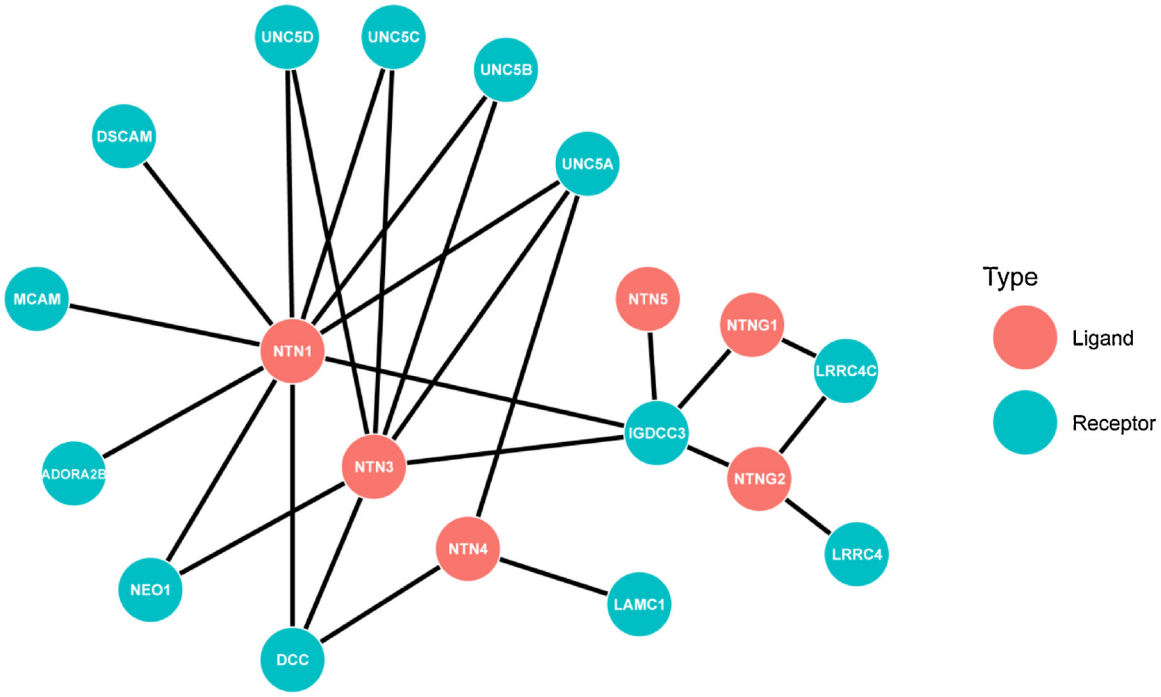

Fig. 2. Ligand-receptor binding map of netrin family proteins and their receptors. Red circles represent ligands, blue circles represent receptors (for interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

and NRP2 in general also have high expression levels. PLXNA3, PLXNA4, PLXNB1 and PLXNB3 have moderate expression levels (Fig. 3). Class-4–7 semaphorins can directly bind to plexins. Class-3 semaphorins, except SEMA3E, require neuropilins as obligate co-receptors to interact with plexins, while SEMA3E can independently bind to PLXND1 (Fig. 4). Next to SEMA3E, SEMA3C and SEMA4A are also ligands for PLXND1 [49], and similar functional consequences have been found when endothelial cells are stimulated with these

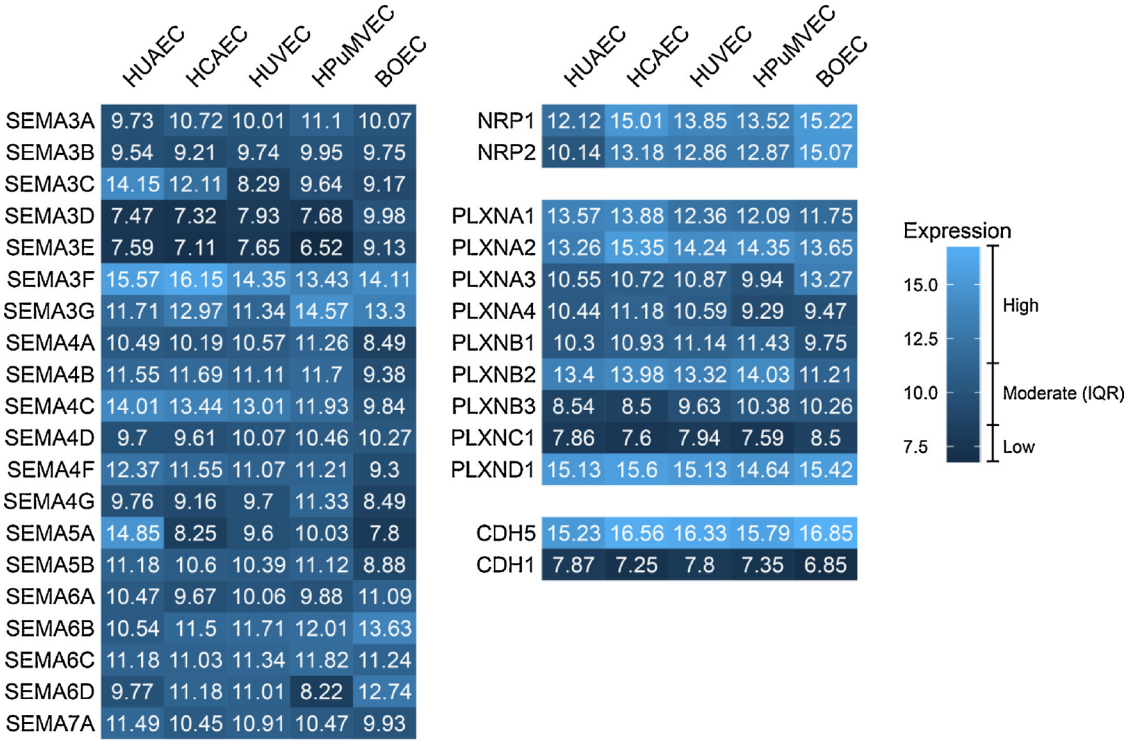

Fig. 3. Expression heatmap of semaphorin family proteins (left) and their receptors (right). Values are the average normalized expression signal from all published data on the Affymetrix Human Genome U133 Plus 2.0 Array (HG-U133 Plus 2.0/GPL570) platform obtained using the Genevestigator software [100]. Lighter colors indicate higher expression. Expression levels within the top quantile on GPL570 platform are recognized as “high”; levels within the inter-quantile range (IQR) are recognized as “moderate”; levels within the bottom quantile are recognized as “low”. These notions are used consistently throughout this article. For comparison, we include CDH5 (VE-Cadherin) as a high-expression reference, and CDH1 (E-Cadherin) as a low-expression reference (bottom right).

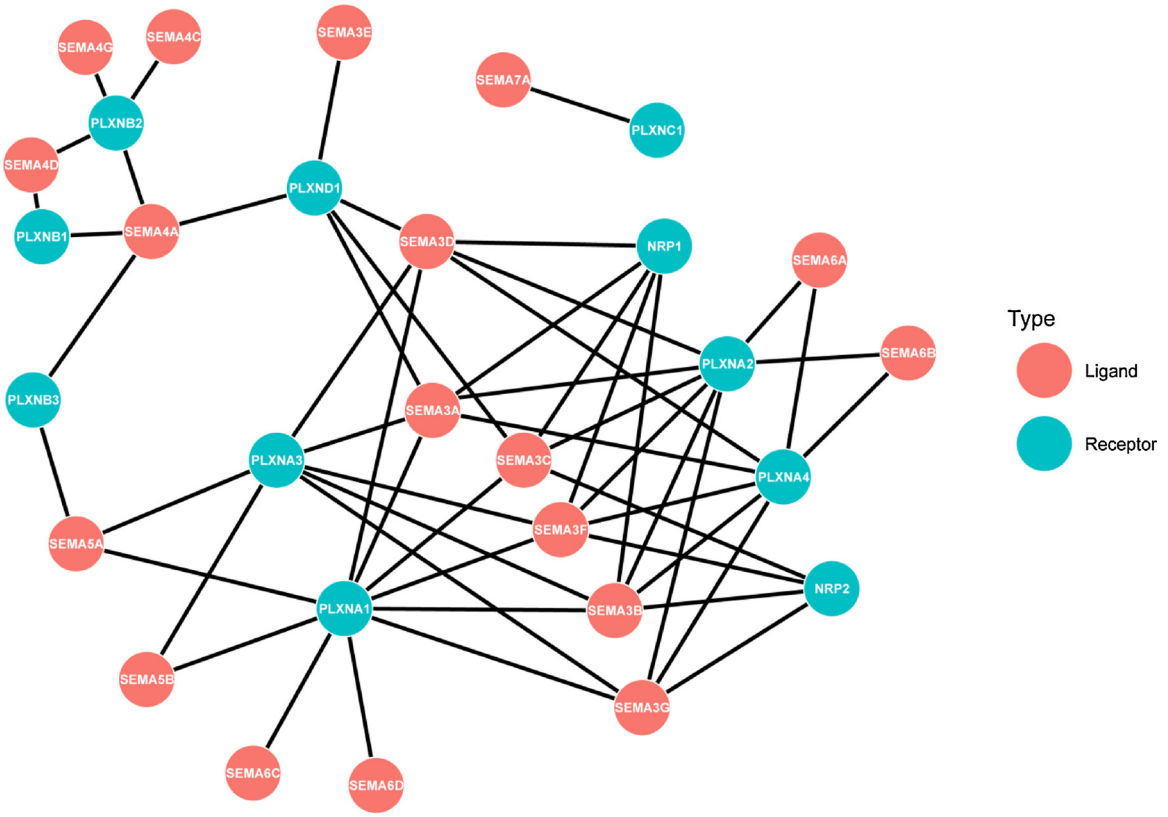

Fig. 4. The ligand-receptor binding map of semaphorin family proteins and their receptors. Red circles represent ligands, blue circles represent receptors (for interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

ligands [50,51].

Looking at a more functional level, the SEMA3E-PLXND1 axis has been shown to be able to abrogate β1 integrin catch bonds, thus negatively regulating activity of the integrin. PLXND1 itself is required for the normal cluster pattern of β1 integrin on cells [52]. Since PLXND1 is widely expressed in various types of endothelial cells [50,51,53,54], this observation is highly relevant also in endothelial cells. Sakurai et al. [55] have established that, in immortalized mouse endothelial cells, exogenous SEMA3E led to a decrease of β1 integrin activity and reduced focal adhesions, as demonstrated by a decrease of paxillin immunostaining. As a result, adhesion of these cells to collagen but not to poly-L-lysine reduced after SEMA3E treatment. Knockdown of PLXND1, on the other hand, almost completely abolished focal adhesion with or without SEMA3E, supporting a similar role of PLXND1 in endothelial cells on integrin patterning. Interestingly, they also found that SEMA3E induced internalization of β1 integrin, via PLXND1-dependent activation of Arf6 [55]. Together with the observations that integrins, as mentioned before, are important stabilization and survival signals, these mechanisms suggest that SEMA3E-PLXND1 interaction should reduce survival signals and induce apoptosis.

Similar functional consequences were observed when endothelial cells were treated with other ligands of PLXND1 [56]: conditioning a medium with SEMA3C increased caspase3/7 activity of HUVECs possibly via inhibition of Akt activity; SEMA3C also decreased focal adhesions and the activity of focal adhesion kinase. Soluble SEMA4A, which is another direct ligand of PLXND1, similarly decreased adhesion of HUVEC to fibronectin and collagen IV. The effect could be rescued by activation of β1 integrin, indicating that binding of SEMA4A to PLXND1 also affects integrin activity. As a result of SEMA4A/PLXND1 interaction, phosphorylation of Akt was also decreased, confirming the lack of integrin activity [50]. Overall, ligands of PLXND1 are able to decrease endothelial cell survival by interfering with integrin function and downstream signaling.

2.4. SEMA3C promotes endothelial cell survival via integrin α5β1 and αvβ3

Using a different mechanism, an opposite function of SEMA3C on endothelial cells has also been observed. SEMA3C is found to be expressed in mouse glomerular endothelial cells, in which SEMA3C was able to directly activate integrin β1, while activity of focal adhesion kinase was not affected [51]. With exogenous SEMA3C, adhesion of mouse glomerular endothelial cells to collagen and fibronectin could be increased, but not to gelatin [51]. Indeed, blocking antibody against α5β1 and αvβ3 integrin blocked the increase of adhesion to fibronectin induced by SEMA3C [51]. This is consistent with the fact that fibronectin is a Arg-Gly-Asp-containing ligand, to which α5β1 and αvβ3 integrins can bind [57]. The difference in the extracellular matrix coating is one possible explanation why an opposite role of SEMA3C was seen here. In a similar experiment using only gelatin coating, no pro-survival function could be detected [56].

3. Role of netrins and semaphorins in the renewal potential of endothelial cells

The second phase in endothelial cell turnover is to replace the damaged endothelium with healthy endothelial cells, which requires endothelial renewal capability. This ability is often assessed *in vitro* with proliferation assays and/or wound-healing assays, mimicking the endothelial self-renewal and repairing capability, respectively. To be noted is that the mechanisms underlying the renewal potential are sometimes similar to what we have discussed in Section 2 for endothelial survival, resulting from versatile pathways regulating both functions. In addition, interpretation of proliferative capability should be done with caution, since quiescent endothelial cells do not proliferate but make up the actual functional endothelium in stable vasculature.

3.1. NTN1 dose-dependently affects endothelial cell renewal

in vitro studies demonstrated that lower concentrations of NTN1 (~100 ng/ml) promote proliferation of endothelial cells [58–62], while an extremely high concentration of NTN1 inhibits [60,59–62] or has no effect [63] on the proliferation of endothelial cells. Considering that the typical concentration of NTN1 in human plasma is only several picograms to several hundred picograms per milliliter NTN1 [33], the physiological concentration in plasma is far from reaching the threshold for an inhibitory effect on proliferation. NTN1 has been found to play dual roles on the wound-healing capacity of endothelial cells. A low concentration of NTN1 increased the rate with which the gap in a monolayer of endothelial cells was closed in culture [64–67], while a high concentration of NTN1 decreased that [65]. Multiple studies confirmed that the canonical receptor of NTN1, UNC5B, mediated the inhibitory effect of NTN1, since siRNA knockdown of UNC5B abolished this inhibitory effect [60,62]. Interestingly, UNC5B-knockout mice displayed excessive development of blood vessels, while the same phenotype was not observed in NTN1-knockout mouse, further confirming that NTN1 in physiological concentrations does not primarily regulate the downstream signaling of UNC5B [64,68]. In 2015, Tu et al. [62] identified the melanoma cell adhesion molecule (MCAM) or CD146 as a novel NTN1 receptor that could mediate the stimulatory effect of NTN1 on proliferation. MCAM co-immunoprecipitated with NTN1 in endothelial cells and had a better binding affinity compared to UNC5B (dissociation constant Kd = 1.33 nM vs 5.10 nM), which is consistent with the finding that MCAM is responsible for NTN1 function in lower concentration ranges. Blocking MCAM expression using siRNA indeed abolished the effect of NTN1 on the proliferation of endothelial cells [62]. Therefore, the NTN1/MCAM pathway most likely mediates the stimulatory effect of (low levels of) NTN1 on proliferation, while UNC5B mediates the NTN1-induced decrease in proliferation when high levels of NTN1 are present.

3.2. The effect of NTN4 on endothelial cell renewal is ambiguous

The effect of NTN4 on the endothelial cell renewal capacity is still a matter of on-going debate. Wilson et al. [59] found that NTN4, similar to NTN1, promoted proliferation of various types of endothelial cells at lower concentrations. For very high concentrations of NTN4, Nacht et al. [20] found an inhibitory effect of NTN4 on the proliferation of HMVECs. In contrast to NTN1, Dakouane-Giudicelli et al. [69] showed that a lower concentration of NTN4 also had an inhibitory effect on both proliferation and wound-healing capacity of human placenta endothelial cells. It is very likely that different mechanisms are involved in the effect of NTN4 on endothelial cells, as both structural biology analysis and kinetic binding analysis revealed that NTN4 is not able to bind to several canonical receptors of netrin family, namely DCC, Neogenin and UNC5B [32]. Instead, NTN4 could bind to laminin γ chain to regulate laminin network formation and, potentially, stiffness of the endothelial basement membrane [32]. Since stiffness of extracellular matrix also has an influence on endothelial cell function, more investigation is needed to fully understand the mechanism of NTN4 and its effect on endothelial cell proliferation [70].

3.3. SEMA3 family members have dual roles in endothelial cell proliferation

Multiple mechanisms have been proposed to explain the involvement of SEMA3 family members in proliferation of endothelial cells. Class-3 semaphorins except SEMA3E share neuropilins as co-receptor with the VEGF165 isoform, although evidences showed that VEGF121 isoform can also bind NRP. Neuropillins serve to augment downstream signaling of VEGF165 in endothelial cells by forming a receptor complex with VEGFR2 [71,72]. Survival signaling by VEGF is especially important during development but can still be of significance in a stabilized vascular network [73]. Loss of VEGF signaling after development decreases vascular integrity, causing symptoms like brain hemorrhage and heart fibrosis in endothelial conditional VEGF-knockout mice [73]. Because of the competition for NRPs between VEGF and SEMA3s, SEMA3s were found to be endogenous inhibitors of VEGF [74–76]. SEMA3A, SEMA3B, SEMA3C and SEMA3F can all reduce endothelial proliferation in the presence of VEGF, which can be blocked by NRP antibody or silencing NRP [56,77–80]. SEMA3E, on the other hand, binds directly to PLXND1 and the binding of SEMA3E and PLXND1 could also cause upregulation of soluble VEGFR1 in endothelial cells, which serves as endogenous VEGF decoy [81]. Similar to its effect on survival of endothelial cells, SEMA3C plays dual roles on endothelial cell proliferation as well. The increase of proliferation and decrease of apoptosis was attributed to the ability of SEMA3C to induce activation of integrin β1 [51]. This effect could be blocked by NRP1 antibodies, confirming the necessity of NRP1 as a co-receptor of SEMA3C [51].

4. Roles of netrins and semaphorins in the response to vessel wall shear stress

Endothelial cells sense shear stress via mechanosensors and trigger a variety of downstream signals that are necessary for the survival of endothelial cells after initial angiogenic stimuli. Unidirectional laminar wall shear stress is a critical hemodynamic factor for endothelial cell maturation and quiescence. An unfavorable hemodynamic environment can disturb quiescent signaling and lead to endothelial cell activation and inflammation, often leading to the development of atherosclerotic lesions. Several studies have proposed roles for neuronal guidance cues as mediators of effects of shear stress.

4.1. NTN1 and SEMA3A repel leukocytes in response to laminar shear stress

Expression of both netrins and semaphorins is regulated by wall shear stress. In LDL receptor knockout mice, lower expression of NTN1 could be detected in the inner curvature of the aorta compared to the outer curvature [34]. Given that the endothelium in the inner curvature of mouse aorta experiences lower wall shear stress than in the outer curvature, NTN1 is positively regulated by shear stress. This regulation was confirmed *in vitro* using human coronary artery endothelial cells cultured under unidirectional laminar flow [34]. In addition, NTN1 was shown to have a repellent role on both migration and adhesion of leukocytes [18,34,35,82]. Neutralizing NTN1 using a blocking antibody abolished the inhibitory effect of NTN1 on leukocyte adhesion to endothelial cells both *in vitro* and in cremaster capillaries in mice [34]. UNC5B, the receptor of NTN1 involved in its inhibitory proliferative effects, is expressed in peripheral blood leukocytes [35]. Blocking UNC5B using an inhibitory antibody abolished the effect of NTN1 on monocyte migration, confirming the canonical NTN1-UNC5B pathway as the underlying mechanism for the inhibition of leukocyte adhesion and migration [34]. As we discussed above, NTN1 also has a pro-survival function for endothelial cells under physiological concentrations. These observations suggest that NTN1 is one of the mediators for the adaptation of endothelial cells to their hemodynamic environment, to promote survival of endothelial cells, and to inhibit leukocyte adhesion to quiescent endothelium under laminar shear stress.

Similar regulation of expression by wall shear stress applies to SEMA3A. Higher expression of SEMA3A was found both in the outer curvature of the aorta of LDL receptor knockout mice and in human coronary artery endothelial cells cultured under laminar flow. SEMA3A also inhibits adhesion of RAW264.7 cells (a macrophage-like cell line) and transwell migration of THP-1 cells (a monocyte-like cell line) *in vitro*. In addition, a blocking peptide of SEMA3A increases leukocyte adhesion to endothelium in cremaster capillaries [34]. These observations implicate SEMA3A as another mediator for the adaptation of endothelial cells to their hemodynamic environment. However, the

mechanism how SEMA3A has this effect needs to be further elucidated.

4.2. SEMA7A enhances atherosclerosis in response to disturbed blood flow

In contrast to NTN1 and SEMA3A, SEMA7A is upregulated in the left carotid artery of ApoE -/- mice upon disturbed flow induced by a partial carotid artery ligation procedure [83]. This positive regulation was confirmed by the elevated expression of SEMA7A in HUVECs cultured under oscillatory shear stress. The upregulation was mediated by downregulation of CREB phosphorylation, while CREB is known as a downstream effector of laminar shear stress. The disturbed flow in the left carotid artery of ApoE -/- mice with partial ligation promoted atherogenesis in this region, but much less atherosclerotic lesions could be seen in the same region in ApoE -/- SEMA7A -/- double knockout mice. Further studies found that SEMA7A overexpressing HUVECs also expressed more ICAM and VCAM, and allowed more monocyte adhesion [83]. The reason for such changes is yet to be determined, but these observations make SEMA7A a possible mediator of the effects of adverse hemodynamic environment on endothelial cells and even renders it a potential therapeutic target.

4.3. Downregulation of SEMA6A/6D by shear stress induced miR-27b promote pericyte recruitment

In the case of SEMA6A and SEMA6D, shear stress regulates their expression indirectly via upregulation of microRNA miR-27b. Downregulation of SEMA6A and SEMA6D by such mechanism in HUVECs was found to increase adhesion of human brain vascular pericytes to HUVECs. Inhibition of miR-27b in mice by intraperitoneally injection of locked nucleic acid reduce pericyte to endothelial ratio in the uterus. Since pericyte recruitment is a key step in vascular maturation, these experiments provide one of the mechanisms how semaphorins are regulated downstream of shear stress via microRNAs to allow endothelium-pericytes interaction [84].

5. Roles of netrins and semaphorins in the endothelial barrier function

Vascular endothelium provides a crucial selective barrier for molecule exchange between blood and tissue fluid and for controlled blood cell infiltration. A well-maintained endothelial barrier function relies on correct cytoskeleton arrangements and formation of endothelial cell-cell junctions. Quiescent endothelial cells have abundant cortical filamentous actin (F-actin) network and few stress fibers. Such a cytoskeletal structure limits centripetal tension in endothelial cells and keeps them in a spread morphology. The intercellular connections of endothelial cells are made possible by the formation of both tight junctions and adherens junctions. On the molecular level, tight junctions and adherens junctions 'seal' adjacent endothelial cells by a hemophilic interaction of claudin and occludin proteins, and vascular endothelial cadherin (VE-cadherin or CDH5) proteins, respectively. The cytoplasmic tails of CDH5 interact with several intracellular components resulting in actin filament binding. Factors that have influence on either cytoskeletal networks or directly on endothelial cell-cell junctions can alter the endothelial barrier function. Through these mechanisms, netrins and semaphorins are identified as regulators of the barrier function of the endothelium.

5.1. NTN1 is necessary for tight junctions in the blood-brain barrier

Intact tight junctions are critical in the blood-brain barrier to ensure more selective exchange of molecules between cerebrospinal fluid and blood. Neonatal mice with a NTN1 knockout genotype displayed impaired function of the blood-brain barrier, as the permeability for macromolecules increased [19]. The effect was due to disrupted tight junctions, as evidenced by a decrease of occludin and JAM-A (tight junction proteins) expression that was observed in the endothelium of brain blood vessels of NTN1 knockout mice [19]. It is possible that NTN1 in wild type conditions is presented to endothelial cells by astrocytes in the brain, since conditioned medium from astrocytes, similar to recombinant NTN1, upregulates several tight junction components including ZO-1, p120 and α-catenin in human brain-derived endothelial cells [19,85]. The therapeutic potential of NTN1 has been examined in experimental autoimmune encephalomyelitis and middle cerebral artery occlusion models [19,86]. In both cases, mice treated with NTN1 showed improved barrier function of the blood-brain barrier [19,86]. However, the exact mechanism on how NTN1 contributes to formation or maintenance of the tight junctions is not known yet. It is interesting to note that MCAM is part of the endothelial cell-cell junction alongside tight junctions and adherent junctions [87]. Previously, we discussed the involvement of MCAM as a receptor for NTN1 in endothelial cell survival. Whether the NTN1/MCAM signaling pathway can play a role in endothelial junction formation remains an interesting topic for future research.

5.2. SEMA3A induces endothelial permeability

Multiple studies demonstrated SEMA3A as a factor inducing permeability [88–91]. Injection of SEMA3A led to increased vessel permeability in mouse retro-orbital venous sinus [88], mouse ear vessels [89], mouse retina [90], and mouse subcutaneous capillaries [91]. This effect was confirmed *in vitro* with human brain endothelial cells [88], rat brain capillary endothelial cells [89], and HUVECs [90,91]. Increase in phosphorylation of CDH5 was observed in endothelial cells after SEMA3A treatment, resulting in internalization of CDH5 and destabilization of adherent junctions [88,90,91]. The effects were dependent on NRP1 expressed by endothelial cells [90,91]. As one of the underlying mechanisms, Le Guelte et al. [88] suggested that SEMA3A/NRP1/PLXNA2 signaling leads to phosphorylation of Src kinase, which causes dissociation of PP2A from CDH5, thereby exposing a phosphorylation site on CDH5. SEMA3A has also been found to increase association of VEGFR1 to Mical2, with Mical2 being an enhancer of F-actin depolymerization [89]. Indeed, SEMA3A was found to induce a collapse in F-actin fibers [54,80]. In addition, in SEMA3A-knockout mice ischemia-reperfusion in the brain caused less leakage from brain blood vessels compared to wild-type mice [89]. Altogether this implies that SEMA3 A increase endothelial permeability by disrupting adherens junction and collapsing cytoskeleton. However, controversy remains on the source of SEMA3A as endothelial cells express significant but low levels of SEMA3A and endothelial cell-specific knockout of SEMA3A did not cause a change in permeability of skin vessels [92].

6. Role of netrin-1 in controlling of the vascular tone

One of the important functions of endothelial cells is to control vascular tone. This function is realized by production of both vasodilators and vasoconstrictors by endothelial cells. Nitric oxide (NO), a soluble gas, is an important endogenous vasodilator. Besides vasodilation, NO also serves as an anti-inflammatory and anti-oxidative factor for endothelial cells (see the review by Tousoulis et al. [93]). Limited data is available to elucidate the role of NGCs in the regulation of vascular tone. To our knowledge, no role for semaphorins in controlling vascular tone has been described. For the netrins however, an interesting role for NTN1 in this process is described in several studies.

6.1. NTN1 controls vascular tone by stimulating release of nitric oxide via DCC receptors

In 2006, Nguyen and Cai [67] found that NTN1 increased activity of endothelial nitric oxide synthase (eNOS) via phosphorylation of extracellular signal-regulated kinase (ERK1/2). Phosphorylation of ERK1/2 led to an increased activity of eNOS, which then catalyzed the oxidation

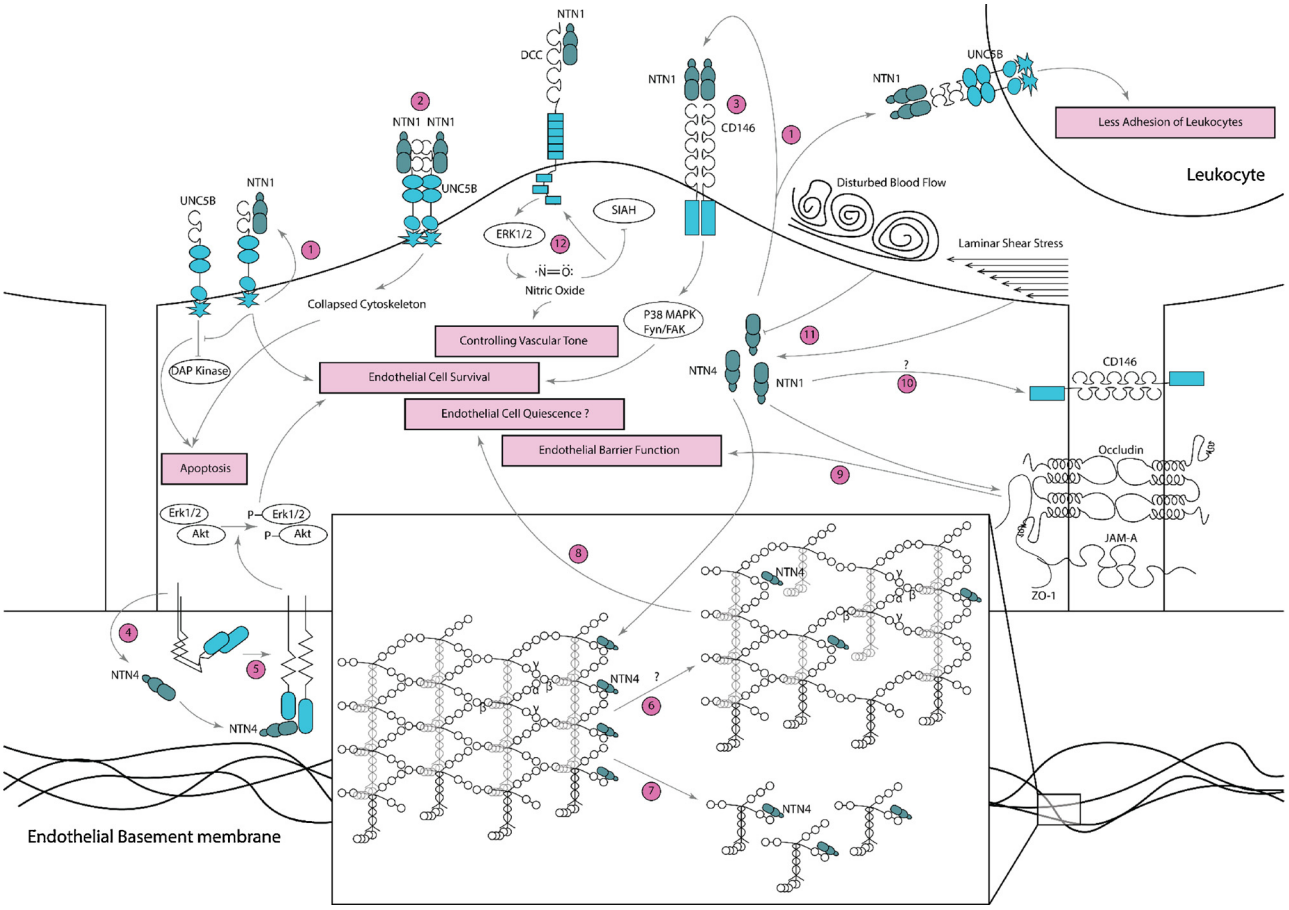

Fig. 5. Schematic representation of mature endothelial cell biology, indicating the roles of netrin family proteins.
1. Moderate secretion of NTN1.
2. Saturated concentration of NTN1.
3. High affinity binding.
4. Basolateral secretion.
5. Activation of integrin α6β1.
6. Physiological concentration of NTN4 possibly changes the stiffness of the laminin matrix.
7. Saturating concentration of NTN4 dissociates the laminin network.
8. Low stiffness of extracellular matrix promotes endothelial cell quiescence.
9. NTN1 is necessary for tight junction stability in the blood-brain barrier.
10. NTN1 binds to MCAM; this could have an influence on endothelial barrier.
11. Laminar shear stress increases the expression of NTN1, while disturbed flow decreases it.
12. NTN1 increases NO production via DCC receptors and ERK1/2. In addition, NO protects DCC from degradation through inhibition of E3 ubiquitin ligase SIAH.

of L-arginine to produce NO [67,94]. This effect could be blocked by a DCC antibody, suggesting involvement of DCC as the responsible receptor [45,67,95–97]. Despite low expression of DCC on endothelial cells, it was shown that NO itself preserves DCC from degradation in endothelial cells via inhibition of the E3 ubiquitin ligase "seven in absentia homolog" (SIAH) [98]. The ability of NTN1 to activate NO production gives it a protective function in organ ischemia reperfusion. Infusion of NTN1 following ischemia reperfusion of mouse hearts decreases infarct size and improves mitochondrial dysfunction, resulting from the increased bio-availability of NO [96,97,99]. Very recently, induction of NO production by NTN1 was confirmed *in vivo* in NTN1 transgenic mice. Compared to WT mice, NTN1 transgenic mice showed increased level of NO in aortic ring assessed by 4,5-diaminofluorescein (DAF-2) staining. Under normal condition, response of aortas to acetylcholine (relaxation), nitroprusside (relaxation) and phenylephrine (contraction) did not change by NTN1 transgene. In diabetic condition, NTN1 overexpression transgene could protect the mice from dampened response to relaxation agents and exaggerated response to contraction agent. [45]

7. Concluding remarks

In this review, we summarized relevant studies about the involvement of netrins (Fig. 5) and semaphorins (Fig. 6) in the function of mature endothelial cells. Netrins and semaphorins were originally identified to have axonal guidance function in the nervous system. Given the striking similarity in anatomy of the peripheral nervous and vascular systems, it is not surprising that the vascular system uses the same group of molecules in development. A lesson learned in recent years is that the function of mature and quiescent endothelial cells is of great importance and requires active regulation. Besides other well-known mechanisms, evidence accumulates suggesting that netrins and semaphorins continue to serve as intercellular and extracellular cues for endothelial cells to maintain their quiescent state and related functions as described in this review. These functions also give both netrins and semaphorins implications in the development of several diseases. Despite the fact that more and more attention is being paid to the regulation of endothelial cell function by NGCs, most research has been performed *in vitro*. More *in vivo* evidence is required to fully confirm the

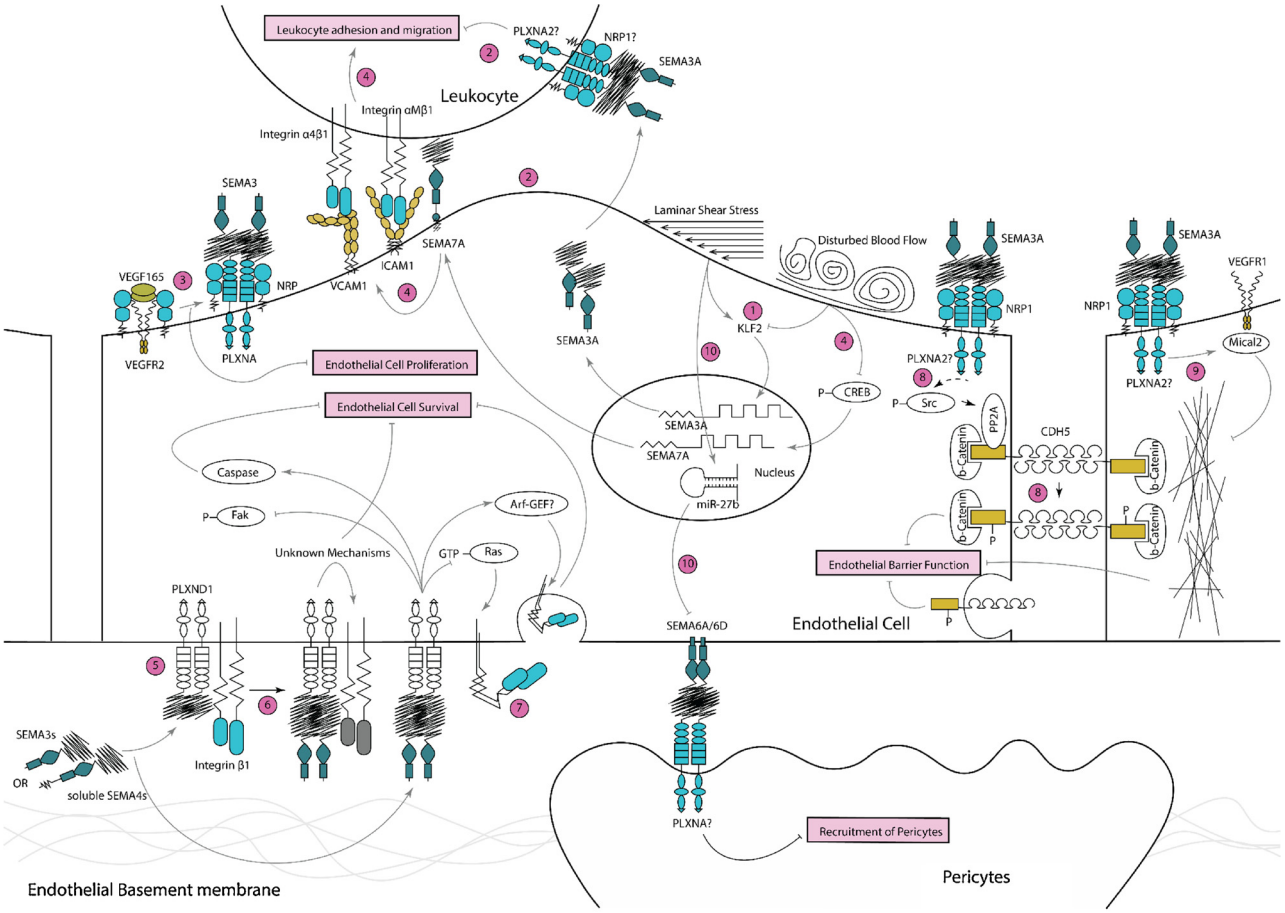

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Fig. 6. Schematic representation of mature endothelial cell biology, indicating the roles of semaphorin family proteins.
1. Laminar shear stress upregulates the flow-dependent transcription factor KLF2. Translocation of KLF2 into the nucleus increases the expression of its target gene SEMA3 A.
2. SEMA3 A secreted by endothelial cells reduce adhesion of leukocytes and repel leukocyte migration.
3. Competition on co-receptor neuropillin between Class-3 semaphorin and VEGF decreases VEGF signaling, thus decreasing endothelial cell proliferation.
4. SEMA7 A, upregulated by turbulent flow on endothelial cells, increases expression of adhesion molecules ICAM-1 and VCAM-1, thereby increasing monocyte adhesion to endothelial cells.
5. PLXND1 itself patterns integrin β1 on the endothelial cell surface.
6. Binding of ligands to PLXND1 causes loss of integrin catch bond, thus decreasing integrin activity.
7. Binding of ligands to PLXND1 inactivate integrin activity and induce integrin internalization.
8. Binding of SEMA3 A to its receptor complex increases phosphorylation of Src kinase, which causes dissociation of PP2A from CDH5, resulting in phosphorylation of CDH5 and thereby its internalization. This disturbs the adherent junction between endothelial cells.
9. Binding of SEMA3 A to its receptor complex induces association of Mical2 to VEGFR1, which leads to depolymerization of cortical F-actin. Loss of cortical actin filaments undermines the ability of endothelial cells to counteract centripetal tension, thus destabilizing the endothelial barrier.
10. Laminar shear stress increases expression of miR-27b, which represses expression of SEMA6A and SEMA6B. Repression of these 2 semaphorins increases recruitment of pericytes to endothelium.

necessity of semaphorins and netrins. As research into this aspect is ongoing, we expect new insights in the near future. For example, it has already been shown that NTN1 promotes endothelial tight junctions in the blood-brain barrier in a model of experimental autoimmune encephalomyelitis (which is a mouse model for brain inflammation caused by loss of blood-brain barrier function) and middle cerebral artery occlusion models (mouse models for stroke). Moreover, SEMA7A can elevate monocyte adhesion in regions with disturbed flow and thereby promote atherosclerosis. Taken together, NGCs are intrinsically involved in endothelial physiology and pathophysiology. Further understanding their roles can be of relevance for vascular diseases. For instance the role of NGCs in leukocyte recruitment and endothelial barrier function would be of relevance in atherosclerosis and vasculitis. While their role on endothelial-pericyte interaction are of vast importance in nephropathy. Further investigations of NGCs could reveal novel disease targets towards sustaining vascular health and integrity.

Conflict of interest

The authors declare no conflicts of interest.

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