Mechanisms of vascular stabilization by PDGF-BB

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The real voyage of discovery consists not in seeking new landscapes, but in having new eyes.

Marcel Proust

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Introduction

I. Angiogenesis

Angiogenesis is the formation of new blood vessels from a pre-existing vasculature. It occurs in both health and disease, beginning during development and continuing through old age (1). The establishment of a primordial vascular network is crucial during development, as it permits the circulation of nutrients and oxygen in the tissues to sustain organism growth and viability. The first process that gives rise to blood vessels is vasculogenesis (Fig. 1), which is characterized by the differentiation, migration, and coalescence of mesoderm-derived endothelial progenitors also known as angioblasts (2). The primitive vascular plexus remodels through sprouting and pruning (angiogenesis) to form a mature circulation (3). In adult life, the formation of new capillaries occurs physiologically during wound healing, organ regeneration and the female reproductive cycle through a strict balance between stimulatory and inhibitory signals. Dysregulation in vessel growth contributes to the pathogenesis of many disorders including malignant tumors, retinopathy and psoriasis as well as obesity, asthma and infectious disease. In addition, insufficient vessel growth or regression triggers ischemia, hypertension and other pathological conditions (4).

Angiogenesis can be divided into two main phases: an activation phase characterized by the initiation of the "angiogenic cascade" and a resolution phase in which newly-induced vessels acquire proper pericyte coverage that promotes endothelial cell survival and helps to maintain vessel stability. Tissue signals involved in the activation of the endothelium are mainly hypoxic, metabolic or mechanical stimuli. Angiogenesis is stimulated in chronic hypoxic conditions through different pathways including nitric oxide or hypoxia-inducible transcription factors, or during repeated

exercises due to increases in shear stress and mechanical strain in the muscle (5). Both signals lead to the secretion of pro-angiogenic factors, i.e. vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) and angiopoietins to the target vasculature.

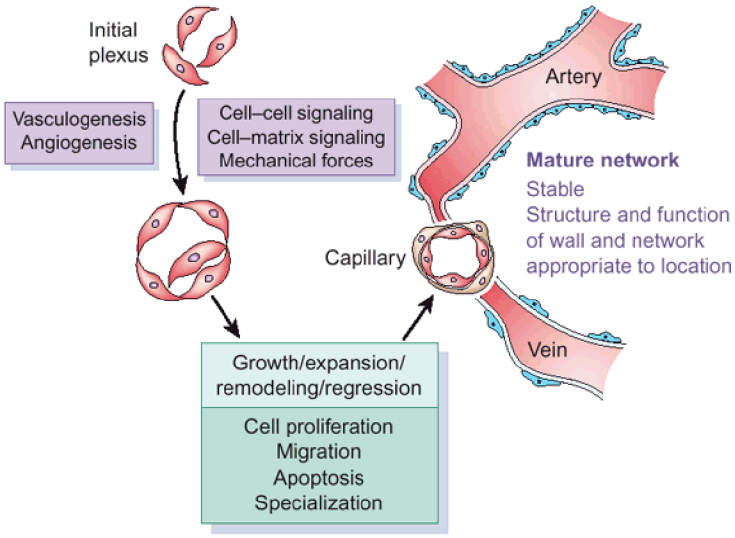

Figure 1. **Vascular development.** Endothelial progenitor (angioblasts) derived from the mesoderm gives rise to a primitive vascular plexus (vasculogenesis). The remodeling of the embryonic vascular network and the formation of new blood vessels from the pre-existing vasculature (angiogenesis) generates mature arteries and veins (Adapted from Kjain R. et al, 2003).

1.1 Molecular regulation of angiogenesis

The activation of the endothelium is a crucial step in enabling endothelial cells to exit their vessel of origin and progress along the stages of angiogenesis. In response to angiogenic signals, the vascular bed is destabilized; the basement membrane and extracellular matrix are selectively degraded to allow endothelial cell migration, proliferation and the formation of a new vascular structure (6). Among the known angiogenic factors, the vascular endothelial growth factor (VEGF) is one of the most important and specific regulators of angiogenesis. VEGF gene expression is up-regulated by many stimuli including hypoxia, the release of growth factors and cytokines or during hormonal regulation such as estrogen release. In response to low

oxygen concentration, the hydroxylation of the transcriptional factor HIF-1α (hypoxia inducible factor) is attenuated and its degradation is prevented. HIF-1α is then heterodimerize with HIF-1β and translocate to the nucleus to bind hypoxia response element (HRE) in order to transactivate target genes such as VEGF (7). The interaction between VEGF ligands and receptors is essential for starting the angiogenic process.

1.1.1 Vascular Endothelial Growth Factors

The VEGF family comprises seven subgroups of proteins: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F and placenta growth factor (PIGF). This family of secreted polypeptides has a common VEGF homology domain composed of eight conserved cysteine residues involved in intra- and intermolecular disulfide bounds (cystine knot structures) (8). VEGFs are biologically active as dimers, primarily as homodimers and sometimes as heterodimers (9).

VEGF-A is a dimeric glycoprotein of 34 to 42 kDa involved in vasculogenesis, angiogenesis and in the differentiation of hemangioblasts to hematopoietic precursor cells during embryogenesis (10). In physiological conditions, the highest levels of VEGF-A mRNA were found in adult lungs, kidneys, hearts and adrenaline glands and less in livers, spleens and gastric mucosa (6). Human VEGF-A gene is localized in the chromosome locus 6p211.3. It contains eight exons and seven introns. Alternative splicing generates at least seven VEGF-A isoforms having 121, 145, 148, 165, 183, 189 or 206 amino acids (Fig. 2). The presence or absence of the two exons influences protein solubility and receptor binding, as they encode heparin-biding domains (11). VEGF-A isoforms containing the heparin-biding motif (HPD) encoded by exon 6 (VEGF-A_{145}, VEGF-A_{189}, VEGF-A_{206}) interact highly with heparin-containing proteoglycans in the extracellular matrix (ECM). Instead, VEGF-A_{165}, which is the most abundant isoform, is

moderately diffusible, as it contains only one heparin-binding domain encoded by exon 7. The shortest isoform, VEGF-A_{121}, is a diffusible protein, as it lacks both exons necessary for encoding (HPD) (12, 13). Plasmin cleavage at the COOH terminus of higher molecular weight isoforms results in highly diffusible fragments. Both matrix binding and proteolytic release regulate the spatial distribution of VEGF in the tissue and allow the formation of extracellular VEGF gradients to stimulate endothelial growth (14). The corresponding mouse isoforms are one amino acid shorter: VEGF-A_{120}, VEGF-A_{164}, and VEGF-A_{188} (15). The importance of VEGF gradients was demonstrated *in vivo* in a mouse retinal model, where VEGF-A_{164} and VEGF-A_{188}, which bind the extracellular matrix, facilitate EC cell migration through the establishment of a gradient. VEGF-A_{120}, which represents the diffusible isoform, induced random endothelial cell migration (16).

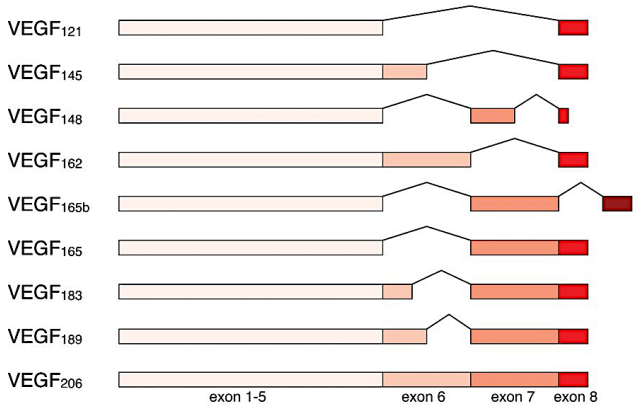

Figure 2. **VEGF-A isoforms.** Alternative splicing of VEGF-A gene gives rise to different isoforms (Adapted from Hiroyuki T. et al, 2005).

VEGF-B is a secreted homodimer abundantly expressed in mice during embryogenesis in the developing heart, skeletal muscles and spinal cord. Exon splicing of VEGF-B gene generates two isoforms: a form that binds to cell-surface heparan sulfate proteoglycans (VEGF-B_{167}) and a more diffusible form (VEGF-B_{186}). VEGF-B was first considered as an angiogenic factor; however, several studies have demonstrated a lack of angiogenic activity. As a matter of fact, VEGF-B deficiency or overexpression did not affect

angiogenesis in most organs studied. Recent studies have demonstrated that VEGF-B is crucial for blood vessel survival rather than for stimulating angiogenesis (17, 18).

VEGF-C and VEGF-D are synthesized as a preproprotein and proteolytically process to be activated. They are known as lymphangiogenic factors, implicated in tumor angiogenesis and metastasis (19). Orf virus, which is a parapoxivirus, encodes VEGF-E to induce angiogenesis in virus-infected lesions. VEGF-E has a similar propriety to that of $VEGF_{165}$ without the heparin-binding domain (6, 20). Among all VEGFs, VEGF-A and its receptors have been shown to play a major role in angiogenesis, vascular permeability, cell migration and gene expression.

1.1.2 VEGF Receptors

VEGF-A regulates angiogenesis and vascular permeability by activating two tyrosine kinases (RTKs) (Fig. 3): VEGFR-1 (Flt-1) and VEGF-R2 (KDR/Flk1 in mice) (20). Each receptor consists of seven IgG-like subdomains in the extracellular domain, a regulatory juxtamembrane domain (JMD), a single transmembrane (TM) region, a kinase insert domain (KID) and a consensus tyrosine kinase sequence which is involved in recruiting downstream signaling molecules. VEGFR-1 and VEGFR-2 are expressed predominantly on the cell surface of vascular endothelial cells (Fig. 3). They are also present on many bone marrow-derived cells such as hematopoietic cells, macrophages, and some malignant cells and on vascular smooth muscle cells (VSMCs) (21).

VEGFR-1 affinity for VEGF-A is very high with a Kd of about 15-100 pM, which is higher than VEGFR-2. However, it has a weaker tyrosine kinase activity. It is expressed not only on endothelial cells but also on the surface of monocytes where it promotes their functions. VEGFR-1 is also implicated in inflammatory diseases, cancer metastasis, and atherosclerosis via its kinase activity (22). Flt-/- null mutant mice died in the embryonic

stage at E8.5-9 due to an increased and disorganized vasculature, suggesting that VEGFR-1 plays a negative role in angiogenesis by suppressing pro-angiogenic signals in the embryo to establish a critical balance essential for physiological vessel formation (23). In fact, VEGFR-1 exists as both a full-length transmembrane form and a short soluble form (sVEGFR1) which acts as a decoy receptor for VEGF-A and therefore indirectly inhibits VEGF-A's pro-angiogenic activities.

VEGFR-2 appears to be the most important receptor in VEGF-induced endothelial cell migration and proliferation as well as in vessel permeability and dilation. It binds VEGF-A with lower affinity but has stronger receptor tyrosine auto-phosphorylation. Flk knockout caused mice death *in utero* between E8.5-9.5 with no development of a vascular system or hematopoiesis (24), thus demonstrating Flk major role in angiogenesis. VEGF-C and VEGF-D are also ligands for Flk receptor.

VEGFR-3 is a typical tyrosine kinase protein which binds only VEGF-C and VEGF-D and its expression is limited to the lymphatic epithelium (20). However, it has been reported its expression in tumor vasculature, and targeting VEGFR-3 activity is also known to retard tumor formation by inhibiting angiogenesis (25).

VEGFR signaling starts upon biding of a ligand dimer to the extracellular receptor domain. Ligand binding induces homo- or hetero-dimerization followed by phosphorylation of specific tyrosine residues located in the JMD and in the C-terminal tail of the receptor. Consequently, different signaling molecules are recruited to VEGFR dimers, giving rise to the assembly of large molecular complexes—so-called signal transduction particles (26). Src homology-2 (SH-2) and phosphotyrosine-binding (PTB) domains mainly mediate the interaction between VEGFRs and downstream signaling effectors. Specifically, the biding between VEGF-A and VEGFR-2 stimulates the activation of several signaling proteins, including mitogen-activated protein kinase (MAPKs),

phosphoinositide 3-kinase (PI3K), phospholipase C-γ (PLC- γ), small GTPase and AKT (27, 28). Moreover, mechanical forces (shear stress) and non-VEGF ligands can activate VEGFR-2 (non-canonical VEGFR-2 phosphorylation). Blood flow might activate VEGFRs through the formation of mechanosensory complexes that consist of VEGFR-2, platelet-endothelial-cell adhesion molecule-1 (PECAM1, also known as CD31) and VE-cadherin (Fig. 3). Increases in shear stress activate SRC and AKT, resulting in endothelial nitric oxide activation (eNOS) and phosphorylation of CD31 and VEGFR-2 complex (29).

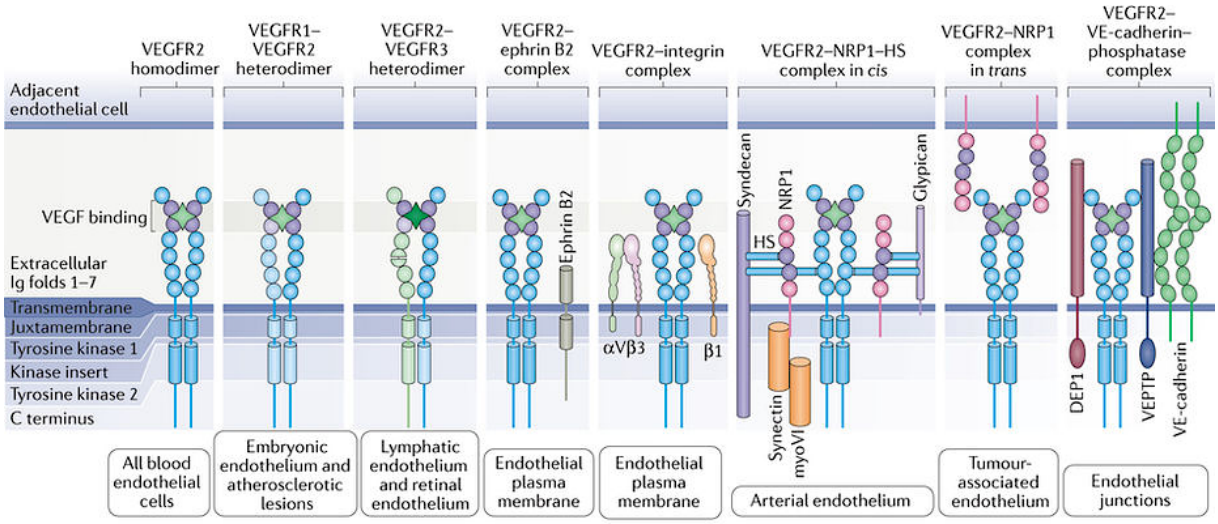

Figure 3. **VEGF family members and receptors.** Schematic representation of VEGF ligands (VEGFA: light green, VEGFC or D: dark green) and their receptor tyrosine kinases VEGFR-1, VEGFR-2 and VEGFR-3. VEGFR2 can homodimerize or heterodimerize with VEGFR1 or 3. Ligand binding results in receptor phosphorylation of tyrosine residues in the intracellular domain, which induces different biological outcomes. VEGFR2 signaling can be modulated through co-receptors binding. VEGFR has several co-receptors including heparan sulfate (HS) proteoglycans, neuropilins (NRPs), vascular endothelial cadherin (VE-cadherin), integrins, ephrin B2 and protein tyrosine phosphate (PTP) (Adapted from Simons M. et al, 2016).

1.1.3 Neuropilins co-receptors

In addition to the tyrosine kinase receptors, VEGF isoforms binds with high affinity to the neuropilin (NP) family members NP1 and NP2 and to heparan sulphate proteoglycans to modulate VEGFR-2 signaling (Fig. 3) (28). Neuropilins are glycoprotein receptors with a large extracellular region that is organized into five domains, termed a1, a2, b1, b2, and c, as well as a single pass transmembrane domain and a short cytoplasmic domain of 40

residues (30). In humans, the two proteins show 44% identity (31). NPs were first identified as receptors of class-3 semaphorins (Sema3), which are a family of secreted and membrane proteins with neuronal guidance functions. Several studies have also shown that NPs have a critical role in the development of the vascular system (32). During development, NP1 is highly expressed on the endothelial cells of capillaries, arteries and veins in postnatal mouse retina and in growing vessels in the mouse embryo hindbrain on day E11.5 (33). Many studies have demonstrated the importance of NP1 in angiogenesis. For instance, the overexpression of this receptor in mouse embryos leads to an excessive growth of leaky and hemorrhagic vessels (34). In contrast, NP-1 null mice died *in utero* due to reduced vessel sprouting, especially in the brain and spinal cord. They also experienced severe cardiovascular defects (35).

NP2 interacts with VEGF-A, VEGF-C and VEGF-D. Its signaling is also important for VEGF-C/VEGFR-2/R-3-mediated lymphangiogenesis (36). Conversely, NP1 binds the heparin-binding isoforms of VEGF-A, VEGF-B and PIGF. It has a relatively short cytoplasmic tail with no known catalytic activity, suggesting that NP1 needs a co-receptor to transduce signals in the vasculature (37). In endothelial cells, the candidates for NP1 signal transduction are VEGFR-1 and VEGFR-2, as both have been shown to interact with NRP1 *in vitro* (33). Moreover, immunoprecipitation studies reveal that NP1 interacted with VEGF-A_{165} and not with VEGF-A_{121}, suggesting that the binding to the receptor is mediated via the exon 7-encoded region of VEGF which is absent in VEGF-A_{121}. However, VEGF-A_{165} binds its receptors VEGFR-1 and VEGFR-2 via exon 3 and 4, respectively (38). Therefore, it was suggested that NP1 enhances VEGF biding by forming a bridge between NP1 and VEGFRs. Additionally, the interaction between VEGF-A_{165}, NP1 and VEGFR-2 can occur in *cis* when both receptors are present on the same endothelial cells or in *trans* if VEGFR-2 is expressed by endothelial cells and NP1 is present on another nearby cell (39).

The *cis* configuration allows a rapid VEGFR-2/NP1 complex formation and internalization where biding in *trans* delays complex formation, therefore inhibiting angiogenesis. NP1 therefore has an important role in regulating VEGFR trafficking affecting VEGF signaling and angiogenesis (29).

1.2 Cellular Mechanism of Angiogenesis

Angiogenesis is the growth of blood vessels from the existing vascular bed. There are two main cellular mechanisms: sprouting, and intussusceptive angiogenesis. Both occur in utero and in adults. As its name suggests, sprouting angiogenesis involves the formation of endothelial sprouts that grow towards an angiogenic gradient. In contrast, intussusceptive angiogenesis is called splitting angiogenesis because an existing vessel splits into two new ones (40).

1.2.1 Sprouting angiogenesis

Sprouting angiogenesis can be divided in several key phases: activation of endothelial cells (ECs) by pro-angiogenic stimuli, capillary basement membrane degradation, endothelial cell proliferation and migration, tube formation, vessel pruning and pericyte-mediated vessel stabilization (41). Endothelial cells and mural cells are embedded within the basement membrane; therefore, ECs must be liberated in order to migrate. Upon endothelial cell activation, cell-cell junctions and the basement membrane (BM) are degraded by matrix metalloproteases (MMPs) such as MT- MMP1 in tandem with mural cells detachment, allowing EC tip cells to migrate in response to guidance signals (42). Ang2, a proangiogenic factor stored in endothelial cells, is rapidly released to stimulate pericytes detachment.

Tip cells are endothelial cells with migratory capacity characterized by numerous filopodia extensions to sense VEGF gradient in the surrounding extracellular matrix (Fig. 4). In contrast, the neighboring ECs, called stalk cells, are more proliferative and thus act as building blocks for the nascent sprout (41, 43). VEGF and Notch signaling pathways are responsible for the specification of a tip and stalk cells in the vascular endothelium (Fig. 4). In mammals, there are four Notch receptors (Notch1-4) and five ligands, Jagged-1 and 2 and Delta-like proteins (Dll1, Dll3 and Dll4). Notch receptors are large single-pass type I transmembrane proteins and most of the Notch ligands are also themselves type I transmembrane proteins. Therefore, Notch signaling is mediated by cell-cell interaction and can happen in *cis* (in the same cell) or in *trans* (different cells) (44). The interaction between Notch receptors and ligands triggers a series of proteolytic cleavages that result in the release of the active Notch intracellular domain (NCID). Afterward, NCID translocate to the nucleus, where it activates the expression of target genes (43).

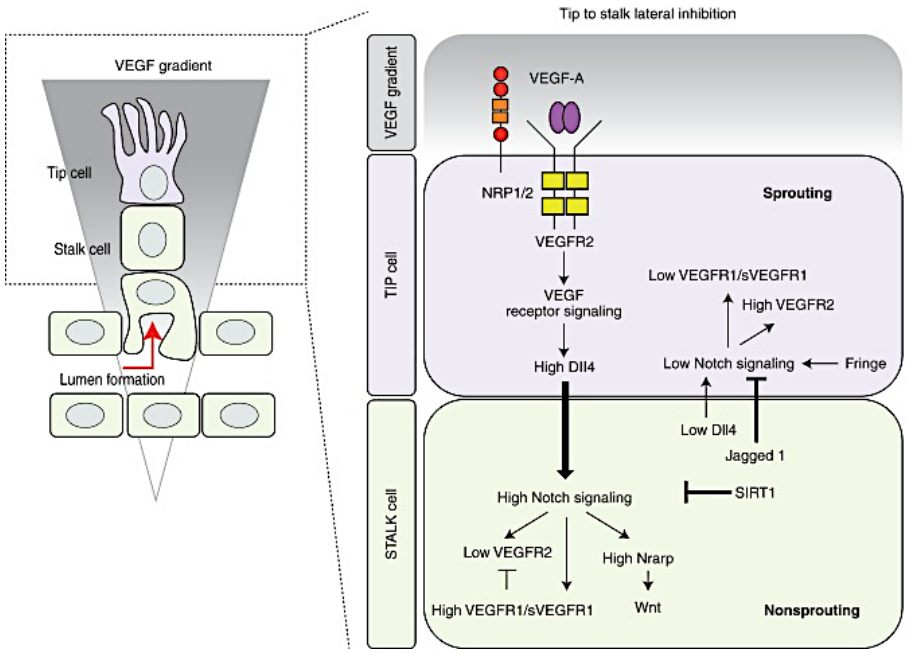

Figure 4. **Phases of tip cell selection during sprouting**. During angiogenesis, ECs extend filopodia and migrate towards an angiogenic gradient. Endothelial cells can become either migratory (tip cells) or proliferating cells (stalk cells). VEGF and Notch signaling are involved in the specification of tip/stalk cells. Activated ECs compete for the tip cell position by up-regulating Dll4, which activates notch signaling in the neighboring ECs. Dll4/Notch signaling activation down-regulates VEGFR-2 while up-regulating VEGFR-1. Jagged-1 expresses by stalk cells antagonized Dll4/Notch signaling in the migratory-leading cell. In addition, Notch is modified by Fringe. This differential Notch activity allows the specification between tip and stalking cells (Adapted from Blanco R. et al., 2013).

Dll4 signaling through Notch1 receptors regulates the formation of endothelial tip cells to control vessel sprouting. Loss of Dll4 or Notch function leads to an over-sprouting due to an excessive formation of tip cells. Conversely, the activation of Notch signaling causes a less dense vascular network as a result of a reduced number of tip cells (45). During sprouting angiogenesis, VEGF interacts with VEGFR-2 and stimulates tip cell induction and filopodia formation, and it promotes Dll4 expression in tip cells. Dll4 activates Notch1 in neighboring stalk cells, inhibiting tip cell behavior in these cells through the down-regulation of VEGFR-2 while up-regulating VEGFR1. Consequently, tip cells have a higher expression of Dll4 while stalk cells have high Notch-signaling activation and lower expression of VEGFR-2 (Fig. 4). The Notch and VEGF feedback loop allows a stable pattern of tip and stalking cells. Moreover, endothelial cells that express Dll4 at higher levels or more quickly have a reasonable advantage to become tip cells (46). In contrast to Dll4, Jagged-1 signaling antagonized Dll4-Notch activation in stalk cells to support tip cell selection and sprouting (Lateral Inhibition). The activity of the two ligands is controlled by the modification of Notch receptors by the Fringe family of glycosaminyltransferases, which has been shown to repress Jagged-1 binding and enhance Dll4-Notch signaling (47).

In developing mouse retina, VEGFR-3 expression is higher in tip cells compared with stalk cells. Additionally, the activation of VEGFR-3 by VEGF-C in tip cells increases Notch signaling, thus promoting tip to stalk conversion (48). Tip cells express guidance receptors including ROBO4, UNC5B, PLEXIN-D1, NRPs and Ephrin family members that favor the guidance of the new sprout (49).

Continued sprouting, branching, and tubulogenesis (anastomosis) are required to expand the vasculature, and lumen formation is a critical step in the development of a new vascular network. Anastomosis can occur between sprouts of two tip cells (‘head-to-

head' anastomosis) or between a tip cell sprout and a functional vessel ('Head-to-side' anastomosis) (41). Mural cells are then recruited by platelet-derived growth factor (PDGF-BB) expressed by endothelial cells. Mural cells reduce endothelial cell proliferation and migration. The establishment of a new blood flow allows vessel remodeling, which is regulated by the shear stress-responsive transcription factor KLK2. Autocrine signals, including VEGF, Angiopoietin-1 and Notch promote endothelial cell quiescence, thereby stabilizing nascent vessels (42).

1.3.2. Intussusception angiogenesis

Intussusceptive angiogenesis (IA) is a mechanism of vascular growth in which the capillary network expands "within itself." It was discovered about two decades ago in the developing lungs of neonatal rats (50). The process of intussusceptive angiogenesis can be divided in four steps: I) invagination of the capillary walls into the lumen until contact is made by transluminal endothelial protrusion; II) rearrangement of the inter-endothelial junctions and central core perforation of the endothelial pillar, this phase is characterized by the formation of a cylindrical tissue bridge wrapped by ECs; III) pillar invasion by pericytes and myofibroblast and extracellular matrix deposition; and IV) pillar enlargement and fusion resulting in the splitting of the initial capillary into to new capillaries (Fig. 5) (51).

Tissue pillar formation is the hallmark of intussusceptive angiogenesis; however, depending on the arrangement and direction of the pillars, three forms of IA can be recognized: I) intussusceptive microvascular growth (IMG); II) intussusceptive arborization (IAR); and III) intussusceptive branch remodeling (IBR). IMG was first identified in the lung vasculature of postnatal rats and subsequently in their myocardium, skeletal muscles, kidneys and retinas (52). The IMG process leads to an extension of the

existing vasculature through the continuous formation and expansion of new pillars in the vascular network. It has been suggested that IMG is driven by blood flow, resulting in a network of capillaries of similar size (53). IAR, on the other hand, contributes to the enlargement of the vascular tree through the formation of smaller vessels. During IAR, favorably-perfused capillaries can be transformed into terminal arterioles and collecting venules by changing size and position to form a new vascular entity. The third form of IA, IBR, is the mechanism that adjusts the number of vessels to efficiently supply a tissue with a proper vasculature by either remodeling the branching pattern of blood vessels or pruning the vascular network from unnecessary vessels (50, 54). Thus, IA occurs in pre-existing vascular networks formed by either vasculogenesis or sprouting. During normal cardiovascular development in an embryo, the majority of the developing vessels arise through SA and then IA takes place (53, 55). During IA, in contrast with SA, the basement membrane stays intact, and endothelial proliferation and migration is reduced as the ECs instead increase in size and flatten to proliferate (56).

Blood flow and shear stress play an important role during IA. The endothelium responds rapidly and sensitively to the mechanical conditions created by blood flow. Shear stress is defined as the tangential force of the flowing blood on the endothelial surface of the vascular wall. Laminar shear stress promotes endothelial cell survival and quiescence and alignment in the direction of flow. In contrast, turbulent or oscillatory shear stress promotes endothelial proliferation and migration—a process characteristic of SA (57). The regulation of intussusceptive angiogenesis by hemodynamics was demonstrated in the developing chick CAM vasculature (58). An artificial increase in blood flow in the cognate artery by clamping one of the branches resulted immediately in branching morphology and pillar formation. Based on *in vivo* hemodynamic parameters and 3D computational models, it was calculated that transluminal pillar development occurred

in the areas characterized by low shear and turbulent flow conditions (59). This suggests that changes in the hemodynamics of blood flow cause an immediate vascular adaptation (60). Apart from hemodynamic factor, IA can also be regulated by molecular mechanisms. However, limited data are available on the molecular mechanism governing IA due to the lack of proper experimental assays. In our group, we recently demonstrated that the over-expression of $VEGF_{164}$ in the skeletal muscles resulted in the formation of vascular enlargement followed by transluminal pillar formation and intussusceptive longitudinal remodeling (Roberto Gianni Barrera, in preparation).

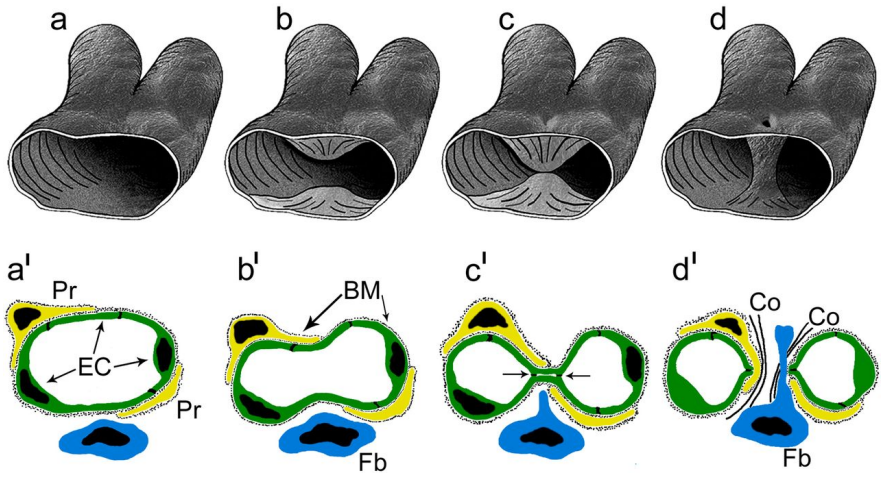

Figure 5. **Mechanism of pillar formation during IA.** Scheme illustrating the phases in the formation of transluminal pillars (a-d). During IA, endothelial cells from the opposite sides protrude into the lumen until a contact is established. After the contact has been established, the central core of the endothelial bilayer is perforated to form a transluminal pillar. 2D representation of the events described above (Adapted from Makanya A.N. et al., 2009).

II. Vascular Maturation and Stabilization

The maturation of newly-formed capillaries is a late event in the angiogenic process. This process is a crucial step in achieving a functional vascular network. It involves pericyte recruitment, deposition of the extracellular matrix, endothelium cell quiescence (endothelial phalanx phenotype) and an increased formation of cell junctions (42). Active endothelium is easily susceptible to regression on withdrawal of growth factors, whereas mature vessels are more resistant (61). An increase in blood flow and oxygen delivery promotes vessel maturation and stabilization. Vessel maturation leads to vascular stabilization—a state in which newly-induced vessels are provided with structural support, mechanical resistance and reduced endothelial cell demand for soluble survival factors, such as VEGF (62).

2.1 Pericyte-mediated vessel maturation and stabilization

Vessel maturation is characterized by a dynamic interaction between ECs, mural cells and soluble factors including PDGF-BB, transforming growth factor β (TGF-β1), angiopoietin 1 (Ang1) and the extracellular environment. During vessel maturation and stabilization, TGF-β1 promotes the differentiation of precursor cells in pericytes (Fig. 6) (63), which then migrate towards PDGF-BB expressed by endothelial cells. Ang1 it has been shown to favor pericyte recruitment on nascent vessels (64). Sphingosine-1-phosphate expressed by pericytes (S1PR) modulates EC/pericyte cell interactions by up-regulating N-cadherin between ECs and pericytes while down-regulating the destabilizing protein Ang2. Finally, basement membrane (BM) is deposited and cell-junctions are re-established to ensure ideal blood flow through the new vessels (62).

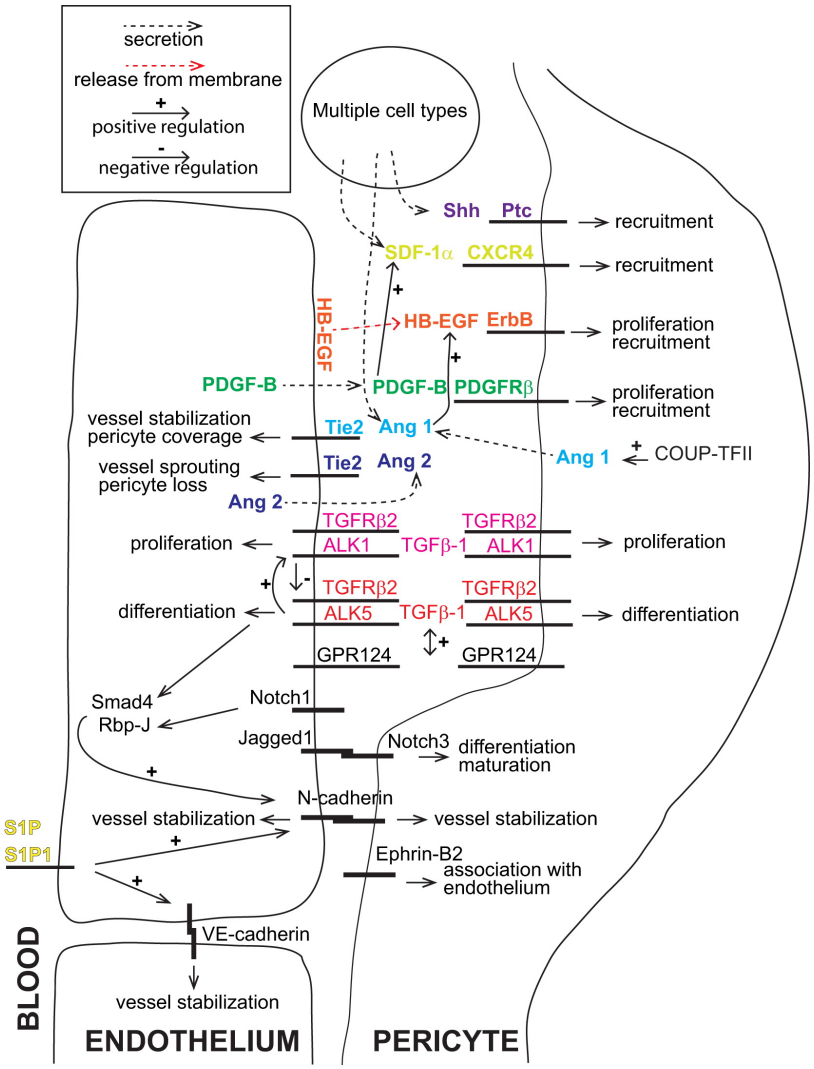

Figure 6. **Signaling pathways mediating endothelium-Pericyte cross talk.** Several factors acting in an autocrine and/or paracrine manner are involved in vascular maturation and stabilization: PDGF-B/PDGFR-β, Ang1/Tie2, TGF-β/activin receptor-like kinase 1 and 5 (ALK1 and ALK5). Notch and N-cadherin signaling requires direct contact between cells (Adapted from Armulik A. et al, 2011).

2.1. 1 Pericyte biology

Pericytes are mural cells of the microcirculation which are known to play key roles in regulating microvascular morphogenesis and stability in different tissues and organs (65). Pericytes were first described more than 100 years ago by Benjamin Rouget as perivascular contractile cells surrounding blood capillaries (66). Electron-microscopy analysis helped to elucidate their morphology. In general, mature pericytes are

considered cells with a fattened or stellate-shape with multiple long processes attached to the abluminal side of the vessels embedded within the vascular basement membrane (BM) (67). Pericytes are ubiquitously present in blood capillaries, arterioles, post-capillaries and collecting venules, but not in lymphatic capillaries (68). In contrast to small vessels, larger blood vessels are covered with single or multiple layers of smooth muscle cells (vSMCs). The abundance of pericytes varies based on tissue and vessel size. The highest density (endothelial cell-pericyte ratio 1:1) is present in neural tissues, especially in retina. In general, pericytes are more extensively present in venous capillaries and post-capillary venules (69). Pericytes typically cover from 10% to 70% of the abluminal surface of the endothelium (66).

Due to their heterogeneity, there are many molecular markers for pericytes. However, these markers cannot be used unequivocally to distinguish them from vSMC or other Mesenchymal cells (67). Commonly-used pericyte markers include neuron-glial 2 (NG2), desmin, vimentin and PDGFRβ. Pericytes on normal capillaries express desmin but not alpha smooth-muscle actin (α-SMA), whereas pericytes from venules are positive for both (70). The expression pattern of these markers is tissue specific and dependent on the development stage of the blood vessels. Different types of surface cell contact are present between endothelial cells and pericytes. Ultrastructural studies have demonstrated the presence of *peg-socket* contacts formed by pericytes' cytoplasmic fingers, which invaginate into the endothelium (71). In addition, pericytes and ECs can connect their cytoskeletons with cadherins through cytoplasmic membranes. Cadherins and gap junctions between their cytoplasms also allow the passage of metabolites and ionic currents (67, 72). Similar to vSMC, pericytes might have different origins. In fact, pericytes from the central nervous system and thymus originate from the ectoderm-derived neural crest, whereas pericytes present in coleomics have a mesothelium origin

(67). During angiogenesis, PDGF-B/PDGFRβ paracrine signals have a key role in pericyte recruitment (73).

2.1.2 PDGF-BB/PDGβR signaling

2.1.2.1 PDGF and PDGFR Family

During vessel sprouting, activated endothelial cells release PDGF-BB to chemoattract PDGF receptor-β^+ pericytes (62). Platelet-derived growth factors (PDGFs) are important mitogen for many types of cells of mesenchymal origin, including fibroblasts, smooth muscle cells and pericytes and for neuroectodermal derived cells including oligodendrocytes (74).

The PDGF family is composed of four polypeptide chains: PDGF-A, PDGF-B, PDGF-C and PDGF-D. Genes located on chromosomes 7, 22, 4, and 11 (*pdgfa*, *pdgfb*, *pdgfc* and *pdgfd*) respectively encode each chain. All PDGFs can form homodimers, whereas PDGF-A and PDGF-B can also heterodimerize (66). All PDGFs, like VEGFs, have a highly-conserved homology domain with a length of approximately 100 amino acids which is primarily responsible for recruiting receptors. The domain is a cysteine-knot motif involved in intra- and inter-disulphide bonding of the dimers (75). PDGF isoforms are produced as inactive precursors in which specific PDGF- A and PDGF-B pro-peptide sequences are cleaved intracellularly by furin-like proteases to form mature growth factors. Conversely, PDGF-C and PDGF-D are secreted in a latent form characterized by the presence of a CUB domain in the N-terminal (Fig. 7). The CUB domain is a structural motif of approximately 110 residues found in many different kinds of proteins, including numerous extracellular proteases, some components of the complement cascade and cell surface proteins (76). PDGF-C and PDGF-D are activated by limited proteolysis of the CUB domain. Thus, the principal role of the CUB domain is to interfere with ligand binding

with receptors (74). PDGF-A and PDGF-B C-terminuses are rich in positively-charged amino acids which are involved in the retention and distribution of growth factors by binding heparin and heparin sulfate proteoglycans (77). The PDGF-A tail is lost in a short isoform by alternative splicing. PDGF-C and PDGF-D lack the tail necessary for growth factor retention; however, their CUB domain may regulate the extracellular distribution of the latent from of the growth factors (75). During embryonic development, epithelial cells produce PDGF-A, whereas PDGF-B is expressed mainly in the developing vasculature by endothelial cells. PDGF-C is expressed in many cell types during embryogenesis, including mesenchymal precursors, vascular smooth muscle cells and cells of the central nervous system (74), while PDGF-D has been reported to be expressed in kidneys (78).

PDGF is a potent chemoattractant and has been involved in bone formation, erythropoiesis, wound healing and angiogenesis. It is also implicated in tumor growth and in the formation of lesions in inflammatory diseases and atherosclerosis (79). PDGF signals through two PDGFRs—PDGFRα and PDGFRβ—to stimulate cell proliferation, migration and angiogenesis. PDGF isoforms have the distinct ability to bind to PDGF receptors. PDGF-AA, PDGF-AB, PDGF-BB and PDGF-DD can bind and activate PDGFRα whereas PDGF-BB and PDGF-AB can bind to and activate PDGFRβ (Fig. 7). In addition, PDGF-AB, PDGF-BB and PDGF-CC can stimulate the heterodimer PDGFRα/β (Fig. 7) (74).

The two PDGFRs belong to the class III receptor tyrosine kinase (RTKs), a family of five members including colony-stimulating factor-1 receptor (CSF-1R), Fms-like tyrosine kinase (FLT3) and the KIT receptor tyrosine kinase. The PDGFR and VEGFR families are evolutionarily related. PDGFRs have a common domain structure characterized by five immunoglobulin (Ig)-like domains in the extracellular segment that

function in ligand binding (D1-D3) and receptor dimerization (D4). In addition, PDGFRs have a single transmembrane helix and an intracellular tyrosine kinase domain (80).

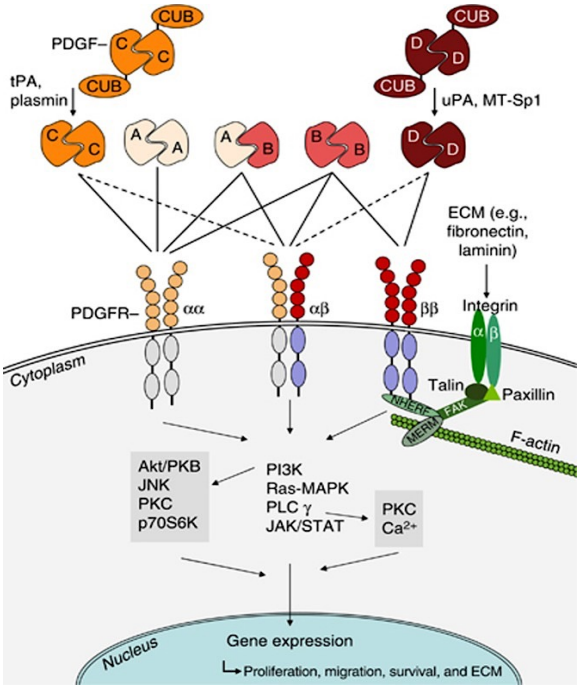

Figure 7. **PDGF/PDGFR signaling.** PDGF-A and PDGF-B are secreted as active homo- or heterodimers, while PDGF-C and PDGF -D need proteolytical cleavage of the CUB domain to bind their receptors. Plasmin can activate both PDGF-C and PDGF–D, whereas the tissue plasminogen activator (tPA) is specific for PDGF-C. Ligand binding to receptors results in receptor dimerization, autophosphorylation and activation of different pathways, including janus kinase (JAK)/ signal transducers and activators of transcription (STAT), phosphatidyl-inositol-3-kinase (PI3K), phospholipase C-γ RAS (PLC-γ) or mitogen-activated protein kinase (MAPK) pathways. These pathways are involved in cell migration, proliferation and survival (Adapted from Ostendorf T. et al, 2014).

PDGF dimer binds the receptor at Ig domains D2 and D3 and promotes receptor dimerization (homo- or heterodimerization), which is then stabilized by direct receptor-receptor interaction with the D4 and D5 domains (75). Once dimerization occurs, the intracellular domain undergoes autophosphorylation *in trans* between the receptors in the dimers. PDGFRα and PDGFRβ have 10 and 11 autophosphorylation sites, respectively. The autophosphorylation of certain tyrosine kinase has two main functions: (I) it promotes receptor activation by conformational changes of the intracellular parts, and (II) it causes subsequent recruitment of SH-domain-containing signaling proteins (81). Finally, receptor activation promotes cellular responses like proliferation and migration.

2.1.2.1 PDGF-BB/PDGFRβ signaling during blood vessel formation

During angiogenesis, PDGF-BB and PDGFRβ play an important role in pericyte recruitment to newly-induced blood vessels. PDGF-BB is release at higher levels by tip cells when compared to stalk cells. Once secreted, PDGF-BB strongly binds the extracellular matrix-forming steep gradients, allowing a proper recruitment of pericytes. As a consequence, pericytes stabilize the nascent vessels through paracrine signals (67, 82). The role of PDGF-BB/ PDGFRβ is supported by several knockout experiments. Knockout of *pdgfb* or *pdgrb* in mice is lethal at the embryonic stage due to widespread microvascular defects consisting of vessel dilatations, microaneurisms, excessive luminal folds and lack of pericyte recruitment. The failure to recruit PDGFRβ-positive pericyte progenitors leads to vascular instability and regression (70). Moreover, deletion of the PDGF-BB retention motif in mice (necessary for pericyte adhesion) results in pericyte detachment from the microvessels, suggesting that PDGF-BB retention is an absolute recruitment for pericyte recruitment and organization of nascent vessels (83).

2.1.3 Angs/Tie signaling

2.1.3.1 Ang1 and Ang2 ligands and Tie receptors

Normal vessels must have mechanisms for maintaining endothelium quiescence while simultaneously remaining able to respond to angiogenic stimuli. In this context, angiopoietins (Angs) and Tie family is a dual system which allows such a switch (62). Angiopoietins are a family of secreted multimeric proteins which collaborate with the VEGF family to regulate vascular and lymphatic vessel growth through endothelial Tie2 receptors (84).

The Angs family comprises three members: Ang1, Ang2 and, in humans, Ang4. Mouse Ang3 and human Ang4 are orthologous; both represent the third member of this family (85). Ang1 and Ang2 are the best-characterized ligand of the family and their proteins share 60% of their amino acid identity (86). Angiopoietins are characterized by the presence of a short amino-terminal domain which promotes clustering of molecules followed by a coiled-coil motif that supports multimerization. The carboxy-terminal fibrinogen domain contains the binding sites for Tie2 receptors. Angs homodimeric multimers are composed of three to six individual ligands (87). The multimerization of the ligands is crucial to activate Tie2 in the endothelium. Ang1 was the first ligand discovered for Tie2 expressed by perivascular cells. It is stored at high levels in platelet granules (87, 88). Ang2 is expressed mostly by activated endothelial cells and also by smooth muscle cells. This protein is expressed in cytoplasmic storage granules called Weibel-Palade bodies and is rapidly released after stimulation with different agonists including VEGF, angiotensin II, thrombin and leptin (89). The receptors tyrosine kinase Tie1 and Tie2 constitute a particular RTK subfamily with a unique extracellular structure consisting of two Ig domains followed by three EGF-like domains, one more Ig motif and three fibronectin type III domains. Both receptors have split tyrosine kinase domains in the intracellular portion (90).

Endothelial cells express Tie2; however this expression has also been demonstrated in subtypes of monocytes and macrophages (91). In normal conditions, Tie2 binds directly to angiopoietins and has a strong kinase activity, whereas Tie1 does not bind directly and has a weak kinase activity. The binding between angiopoietins and Tie2 receptors causes Tie2 receptor clustering. Ang1 and Tie2 interaction induces receptor phosphorylation on tyrosine kinase residues, which results in the activation of several downstream pathways like PI3-kinase/Akt and ERK. In cell-matrix or cell-cell

contacts, the complexes of Ang1/Tie2 can interact with Tie1, resulting in the activation of the receptor in order to stabilize the endothelium or stimulate endothelial migration, respectively (92).

2.1.3.1 The Ang-Tie signaling system in vessel development

The interaction between angiopoietins and Tie2 receptors plays an important role during endothelial sprouting, pericyte recruitment and vessel remodeling (Fig. 8) (70). Loss of function experiments have contributed to defining the role of angiopoietins and Tie receptors during vascular development and vascular remodeling. *Ang1* or *Tie2* knockout in mouse embryos was lethal at E10.5 and 12.5 respectively due to severe heart and vascular defects. The vessels present in *Ang1*-null embryos lack proper pericyte attachments and therefore fail to mature (93). In the Tie2-deficent mice, the primary capillary plexus fails to remodel, and it remains not well organized with few endothelial cells and breaches. Interestingly, *Tie1*-null mice died later during development because of a lack of vessel integrity (edema) without perturbation of angiogenesis. In Ang2-deficient mice, vascular development was not perturbed. This has consequently facilitated the study of the protein in adult mice. However adult Ang2-deficient mice have vascular defects (94). Transgenic overexpression of Ang2 caused a similar phenotype as that in Ang1-null mice, suggesting that Ang2 acts as a natural antagonist for Ang1 (95). The Ang/Tie system controls sprouting angiogenesis, vascular maturation and the transition between the quiescent and activated endothelium (96).

During angiogenesis, pericytes produce Ang1, which binds Tie2 receptors expressed by the stalk and phalanx to mediate vascular stabilization. The Ang1/Tie2 complex maximizes the interaction between ECs and pericytes through the establishment of adherents and tight junctions in order to reduce EC permeability (Fig. 8). Moreover,

Ang1/Tie2 signaling induces the expression of heparin-binding epidermal growth factor-like growth factor (HB-EGF), which stimulates mural migration though epidermal growth factor receptors (EGFRs) (97).

In contrast, during active angiogenesis, Ang2 released from Weibel-Palade bodies acts as a destabilizing factor in an autocrine way. Ang2 competes with Ang1 for Tie2 binding and does not induce signal transduction. This competition results in a reduction in pericyte coverage and an increase in permeability and tip cells begin to spout (Fig. 8). Recent studies have also demonstrated that pericytes express Tie2 receptors. Therefore, Ang1 expressed by pericytes acts autocrine on pericytes and contributes to vascular maturation. On the other hand, Ang2 released by endothelial cells binds Tie2 pericytes and favors vessel destabilization directly on pericytes (98).

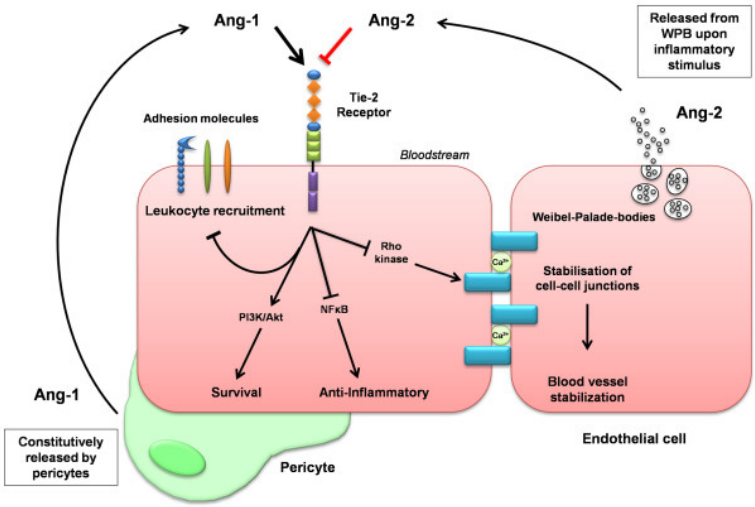

Figure 8. **Vascular effect of the Ang-Tie system.** Quiescent endothelium is covered by pericytes that secrete Ang1. Ang1/Tie2 interaction causes receptor clustering and activation of survival signal pathways (PI3K/Akt), thus promoting EC survival and stabilization. Ang2 is stored and rapidly released from WPBs by activated endothelial cells. Ang2 acts as an autocrine binding Tie2 and competing with Ang1. As a consequence, vessels are destabilized, pericytes detach and endothelial cells start to sprout (Adapted from Van Meurs M. et al., 2009).

2.1.4 S1P1/EDG1 signaling

Sphingosine-1-phosphate (S1P1) is a lipid mediator which has emerged as important molecule in the regulation of vascular formation and maturation (99). S1P1 is generated after sphingosine phosphorylation by sphingosine kinases (SphKs) (100). S1P1 is secreted by cells and transported extracellulary on lipoprotein particles. It interacts with G-protein-coupled receptors (denoted as S1P1 to 5) to trigger multiple downstream signaling processes (101). *S1P*1 (*edg1*)-null mice died *in utero* at E12.5 due to vascular abnormalities caused by the defective migration of pericytes. S1P1 regulates vessel formation positively and negatively by the activation of specific receptor subtypes. Moreover, double or triple knockout of *S1P1–3* caused more severe vascular defects and earlier lethality (Spiegel S., 2003). *In vitro*, S1P1 stimulates cytoskeletal, adhesive, and junctional changes, favoring cell migration, proliferation and survival (102). S1P is secreted by monocytes and activated platelets; S1P binding to EDG1 on endothelial cells improves ECM production. Moreover, the activation of S1P1 on pericytes enhances their migration towards endothelial cells (68). EDG1 signaling might promote vessel stabilization through N-cadherin–based endothelial–pericyte contacts (103). In contrast, S1P2 (EDG5) negatively regulates angiogenesis activity as it reduces mural cell migration through Rac inhibition (104).

2.1.5 TGF-β signaling in angiogenesis

2.1.5.1 TGF-β family and receptors

The transforming growth factor beta (TGF-β) superfamily is an evolutionarily-conserved family of secreted factors which is comprised of thirty-three members, including TGF-β isoforms, activins, anti-Müllerian hormone (AMH), bone morphogenetic proteins (BMPs)

and growth and differentiation factors (GDFs) (105). TGF-β family members play a critical role during embryogenesis and in maintaining tissue homeostasis in adult life. Dysregulation in TGF-β signaling pathway is associated with several developmental disorders and diseases comprising cancers, autoimmune diseases, fibrosis and cardiovascular diseases (106). There are TGF-β isoforms (TGF-β1, TGF-β2 and TGF-β3) and human isoforms are encoded by genes located in different chromosomes: 19q13.1, 1q41 and 14q24, respectively (107). TGF-β isoforms can act in autocrine, paracrine and endocrine ways to regulate different processes. The availability of their active forms is tightly controlled at multiple levels, including secretion and interaction with ECM components (108). In fact, polypeptides from TGF-β family are synthetized as secreted inactive latent dimeric precursors (pre-pro-TGFs) composed of a monomer with molecular weight of 55kD, N-terminal signal peptides (SP), a pro-region named latency-associated peptide (LAP), and C-terminal, which corresponds to the functionally-active cytokine (109). The association of the monomer and LAP form the small latent complex (SLC). SLC subsequently interacts covalently with the large latent TGF-β binding protein (LTBP) to form a larger complex called larger latent complex (LLC). After secretion, the LLC anchors the ECM and it is maintained in an inactive form (110). TGF-β is activated by different mechanisms, including proteolytic cleavage of LAP and LTBP by thrombospondin-1, plasmin, pH alteration, matrix metalloproteinase (MMP) and by integrin interaction. The last mechanism is unclear; however, the interaction between integrin and the RGD domains in LAP might induce conformational changes that result in the liberation of the mature protein (111).

Once the active form of the TGF-β member is released from the extracellular matrix, it can signal trough a complex of type I (TβRI, also known as Activin receptor ALK5) and type II (TβRII) serine/tyrosine kinase receptors. TGF-β receptors are

transmembrane glycoproteins, which are expressed on the surface of endothelial cells, mural cells and several other cell-type. Ligand binding induces heterotetrameric assembly of type I and type II receptors (112). In humans, there are seven type I receptors: the Alk5 group that includes Alk5, Alk4 and the Nodal receptor Alk7; the Alk3 group composed of the BMP type I receptors Alk3 and Alk6; and the Alk1 group (Alk1 and Alk2). The five type II receptors are TβRII, Activin and BMP/GDF type II receptors, BMP/GDF type II receptors (BMP RII) and Mullerian inhibitory substance (MIS) type II receptors (113). It is possible to distinguish three main receptor domains, including a small cysteine-rich extracellular domain, a transmembrane domain and a C-terminal intracellular portion containing the binding site for serine/threonine protein kinase (112, 114). Upon type I receptor activation, intracellular signals are propagated through 'canonical' effector proteins of the Smad Family. Receptor-regulated Smads (R-Smad) are then phosphorylated at two serine residues at the C-terminus allowing the formation of a complex with the common mediator Smad4. Subsequently, the complexes translocate into the nucleolus to regulate the expression of specific genes such as *SERPINE1* (also known as PAI-I, plasminogen activator inhibitor) and *ID1* (inhibitor of DNA binding-1) in cooperation with other transcriptional factors (109, 115). ALK4, 5 and 7 mediate the phosphorylation of R-Smad 2 and 3, whereas ALK 1, 2, 3 and 6 induce phosphorylation of R-Smad 1, 5 and 8 (106). TGF-β can also signal through a non-canonical pathway (Smad-independent pathway) via other intracellular signals, which may include mitogen-activated proteins (MAPK), extracellular-signal-regulated kinases 1/2 (ERK1/2) and PI3K, among others (114).

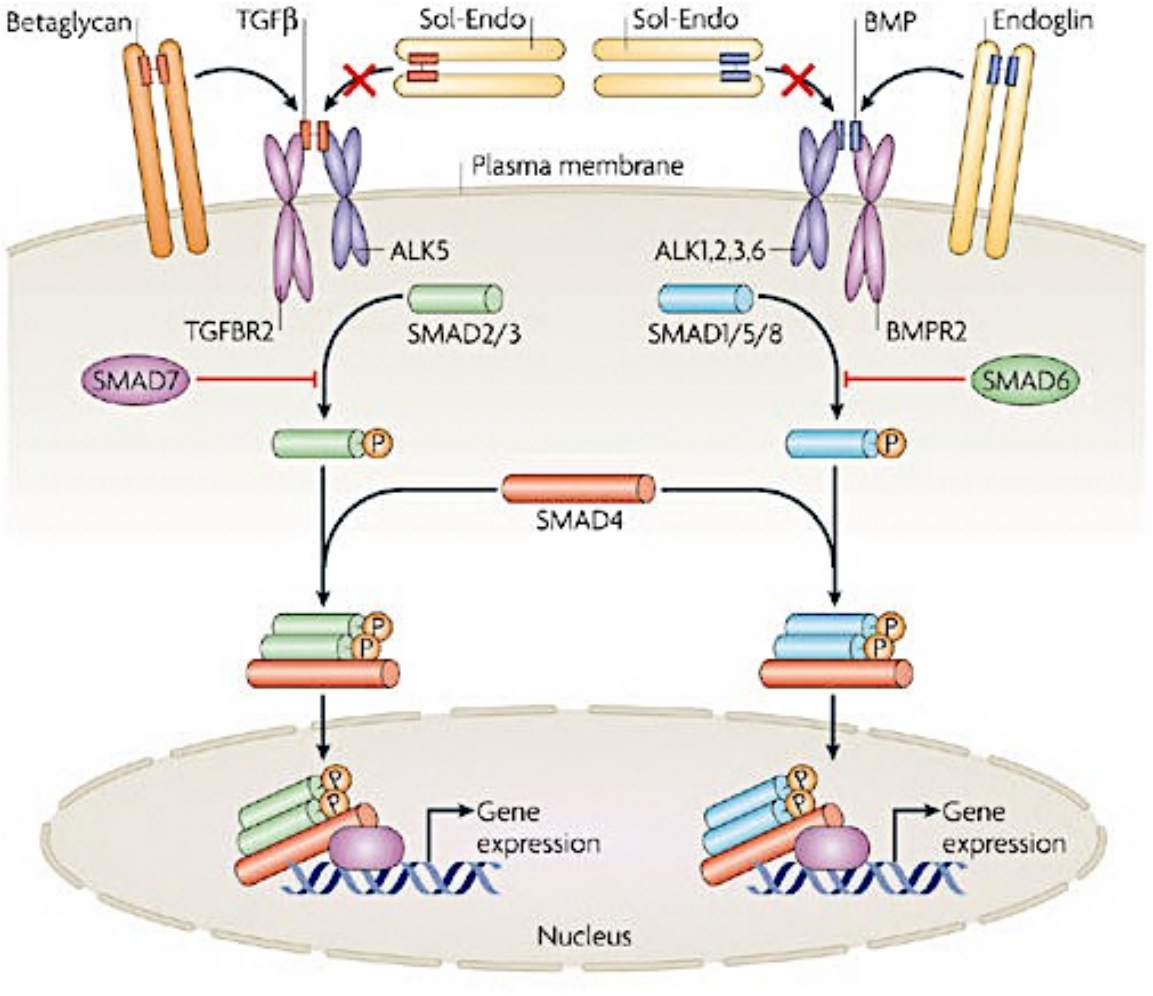

Figure 9. **TGF-β canonical pathways.** Canonical signal transduction by TGF-β family members can be divided into two main pathways based on the R-Smad effector involved. TGF-β ligands can bind to type I or type II receptors, specifically TGF-β signals via TGFBR2 and ALK5 and BMPs signals via the BMP type II receptor (BMPR2) and ALK1, -2, -3 and -6. Accessory receptors like betaglycan and endoglin can modulate receptor signaling. Receptor activation induces the phosphorylation of the intracellular effector R-Smads. Normally, TGFβ induces Smad2/3 phosphorylation and BMPs induce Smad1/5/8 phosphorylation. Phosphorylated Smads form complexes and translocate into the nucleus where, in cooperation with other transcription factors, they regulate gene expression responses. Inhibitory Smads (I-Smads) antagonize TGF-β signaling by inhibiting the activation of R-Smads (Adapted from Dijke P. et al., 2007).

2.1.5.2 TGF-β/TβR signaling in EC and mural cells

TGF-β is an important cytokine expressed by endothelial cells and pericytes during vessel formation. TGF-β signaling regulates angiogenesis by different mechanisms, and is implicated in mural cell and EC differentiation and proliferation (67). *In vivo* loss of function of TGF-β signaling members resulted in an abnormal primitive capillary plexus

with impaired recruitment of mural cells. Embryos lacking one of the components die at the mid-gestation stage due to hyper-dilated and leaky vessels. Null mice of endothelial ALK5 or TβRII die at E10.5 with severe vascular defects (116, 117). It has been proposed that TGF-β regulates the activation state of the endothelium by alternating the activation of two signaling cascades with opposite effects (ALK5 and ALK1) (118). Briefly, TGF-β signaling through ALK5 induces phosphorylation of Smad2/3, expression of fibronectin and plasminogen activator inhibitor type I (PAI-1), leading to inhibition of EC migration and proliferation. In contrast, TGF-β/ALK1 signaling induces the activation of Smad1/5 and up-regulation of Id-1 to promote EC migration, proliferation and tube formation (106). ALK1 can interfere with ALK5/Smad2/3 pathway signaling. Therefore, the combined effects promote the angiogenic activation phase characterized by increased vascular permeability, basement membrane degradation and EC proliferation. In the second phase, ALK1-mediated pathways are down-regulated and ALK5 signaling regulates the resolution phase of angiogenesis, in which ECs stop to proliferate and differentiate and the basal lamina is restored (119). Moreover, it has been shown that BMPs regulate EC function as well. BMP6 promotes EC migration via Smad1/5 activation, while BMP4 induces cell proliferation and migration through the up-regulation of VEGF-A (120). TGF-β signaling also affects pericyte and SMC proliferation and differentiation. Specific knockout of TβRII vascular smooth muscle cells was lethal at E12-5 due to vascular defects in the yolk sac (117). Carvalho et al. have showed that *endoglin* knockouts and endothelium-specific knockouts of *tβrII* and *alk5* cause the loss of TGF-β signaling in the endothelium, which impairs TGF-β/ALK5 signaling in mesenchymal cells as well, consequently inhibiting their differentiation into VSMC and their association with the vessels. Furthermore, ALK1 activation stimulates pericyte recruitment to new

vasculatures while ALK 5 signaling promotes cell quiescence and vessel stabilization (68).

2.1.6 Ephrins and Eph receptors signaling

The erythropoietin-producing hepatocellular (Eph) receptor family is the largest class of tyrosine kinase receptors in mammals. Ephs have an extracellular domain consisting of a highly-conserved N-terminal ligand-binding domain, a cysteine-rich region composed of an epidermal growth factor (EGF)-like domain, and two fibronectin type III motifs (FN III) (121). The intracellular domain contains tyrosine kinases, sterile-α motifs (SAM) and PDZ-binding motif which promote oligomerization of the receptors (Fig. 10) (122). In humans, there are nine EphA receptors which are attached to the cell surface with a glycosylphosphatidynositol (GPI) and five Eph type B receptors, which are characterized by a shot cytoplasmic region. The ligands are also categorized into classes A and B and into five subclass-A ephrin ligands and five ephrin-B ligands (123). Eph type B receptors bind ephrin ligands and form the subclass B type, while Eph-A interacts with ephrin type A, with some exceptions. Eph receptors and ephrin ligands are both able to transduce a signaling cascade upon interaction. Eph-activated signaling is called forward, and ephrin-activated signaling is called reverse (Fig. 10). Another level of complexity of Eph/ephrin signaling is the fact that interactions between Ephs and ephrins can occur *in trans* (between two opposing cells) or *in cis* (within the same cell). Generally, it is assumed that *trans* interactions are activating while *cis* interactions are inhibiting (124). Eph and ephrin interactions cause sequential clustering of the receptor ligand/receptor complex to form high-affinity heteroteramer complexes (121, 125).

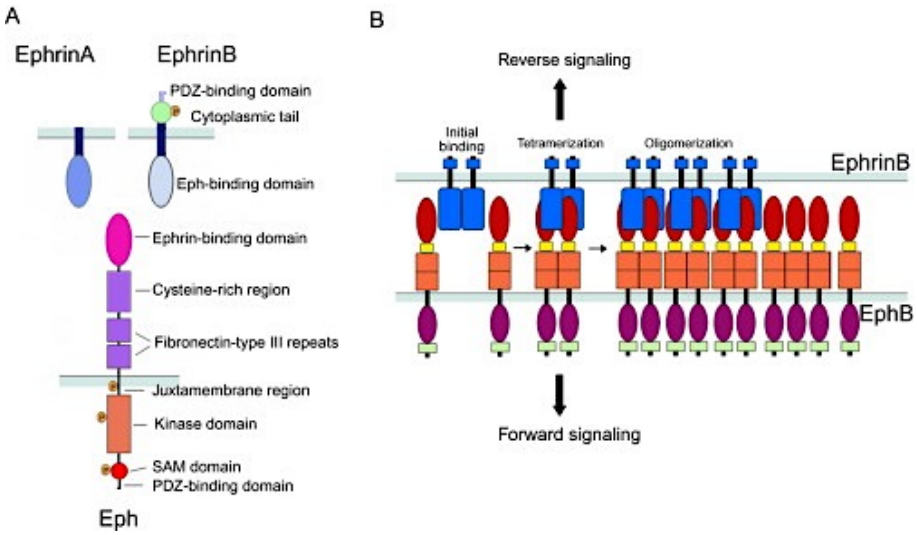

Figure 10. **Schematic representation of Eph receptor and ephrin ligand structures and bindings.** (A) Eph receptors are membrane-bound proteins consisting of a cysteine-rich region, FN type III motifs, a juxatamembrane, tyrosine kinase domain and SAM and PDZ binding sites. The ephrin-B ligands are transmembrane proteins, whereas ephrin from the subclass A are linked to the membrane via a GPI anchor. (B) Eph/ephrin pathways can signal forward through cells, expressing the receptor or reverse through ephrin-expressing cells (Adapted from Salvucci O. et al, 2012).

Eph receptors and their ephrin ligands control several cell-cell interactions, including those of vascular endothelial cells and epithelial and stem cells. They also have vital roles in bone mineral metabolism and the immune system. Veins and arteries differentially express the members of the Eph family. EphrinB2 is an arterial marker while EphB4 is expressed in veins (126). EphB4 and ephrinB2 are crucial for vascular remodeling of primitive capillary networks into distinct arteries and veins. Mutations of ephrinB2 and EphB4 are caused embryonic lethality, suggesting that both molecules are essential during the remodeling of the primary vascular plexus (127). Moreover, endothelial-specific knockout of *Efnb2* cause embryo death due to a complete arrest of angiogenesis (128). However, pericytes and vascular smooth muscle cells also express ephrinb2. Deletion of ephrinB2 in pericytes and in smooth muscle cells resulted in diffuse tissue edema, hemorrhaging and perinatal death of the mice (129). Furthermore, activation of EphB4 in mural cells reduced their ability to migrate, suggesting that EphB4 signaling in pericytes may favor their attachment to the endothelial cells, therefore stabilizing the vessel wall (130).

2.2 Role of Sema3A in Angiogenesis and Stabilization

Semaphorins are a family of secreted and transmembrane proteins, originally identified as axonal guidance modulators, which have been shown to signal through two main receptor families: plexins and Neuropilins (NPs) (131). Semaphorins are grouped into eight major classes on the basis of their sequence and overall structure characteristics. Furthermore, all the members of this family have a conserved extracellular domain of 500 amino acids known as the semaphorin domain, which is a key component through which semaphorins mediate their effects (Fig. 11) (132). Class-1 and class-2 semaphorins are found in invertebrates, while classes 3 to 7 are present only in vertebrates. Moreover, classes 1, 4, 5 and 6 are membrane-bound proteins, while classes 2 and 3 are instead secreted. Class-7 semaphorins are glycosylphosphatidylinositol (GPI)-linked proteins (133). This family of proteins is predominantly expressed during development in the nervous system; however, they are also expressed in several tissue and organ systems including the cardiovascular, endocrine, gastrointestinal, hepatic, immune, musculoskeletal, renal, reproductive and respiratory systems (132, 134). In particular, class-3 semaphorins (Sema3) have been shown to play a crucial role in cardiovascular development and vascular maturation.

2.2.1 Semaphorins class-3 Family

In mammals, class-3 semaphorins consist of 7 soluble proteins of about 100 kDa—namely Sema3A, Sema3B, Sema3C, Sema3D, Sema3E, Sema3F and Sema3G, which are secreted by several cells including endothelial cells, epithelial cells, neurons and some tumor cells (135). Sema3s comprise a conserved sema domain, a plexin-semaphorins-integrin (PSI) domain, an Ig-like motif and a C-terminal basic domain. Semaphorins are

functionally active as dimers; the sema domain, the Ig domain and the formation of disulfide bridges at the basic tail are essential for protein dimerization. Moreover, the C-terminal pro-peptide undergoes proteolytic cleavage by furine-like proteases in order to stabilize the dimers (136, 137). Apart from Sema3E, class-3 semaphorins require the formation of holoreceptor complexes (which comprise plexin and neuropilin proteins) to exert the majority of their effects (Fig. 11). Neuropilins mediate Sema3 signaling in collaboration with signal transduction receptors, like plexins and cell adhesion molecules (CAMS) (138).

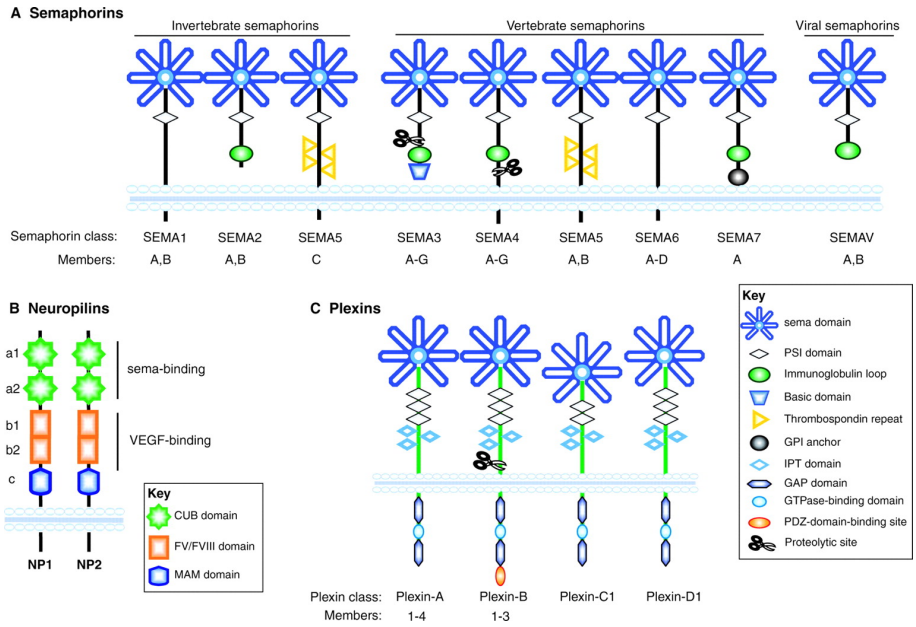

Figure 11. **Class-3 semaphorins and their receptors.** (A) Sema3s are a large family of soluble and transmembrane proteins characterized by a conserved sema domain and a PSI motif. Classes 4, 5 and 6 are membrane-bound proteins, while class 7 is anchored to the membrane via GPI. Of the vertebrate semaphorins, class-3 semaphorins are secreted proteins that have a basic C-terminal which is crucial for the binding with neuropilin receptors. Some semaphorins contain Ig-like motifs. (B) Neuropilins are transmembrane receptors which are comprised of two complement-like (CUB) domains (a1/a2 domains), two factor FV/FVIII coagulation factor-like domains (b1/b2 domains) and a MAM domain (also called the c domain). (C) Plexins are transmembrane molecules consisting of one sema domain, PSI domains and Ig-like domains. The cytoplasmic domain of plexins is composed of two GTPase-activating proteins (GAP) (Adapted from Capparuccia L. et al, 2009).

Plexins are a family of transmembrane molecules divided into four classes, A to D. The extracellular domain of plexins is a sema domain which mediates binding with Sema3 dimers (Fig. 11). The intracellular region contains a guanosine triphodphstase (GTPase)-

activating protein (GAP) homology domain. Sema3/NRP/plexin signal transduction is still poorly understood. Actually, the best-described semaphorin signal cascades are those used for axon guidance (132). There is a growing consensus that several class-3 semaphorins, such as Sema3A and Sema3E, are involved in physiological and pathological angiogenesis.

2.2.2 Role of Sema3A in angiogenesis

Semaphorin 3A was first described as an axon guidance factor which signals via the holoreceptor complex that contains neuropilin 1 (NP1) as the ligand-binding subunit, and A-Plexin that represents the signal-transducing subunit (139). Sema3A specifically binds to NP1 and not to NP2 (140). NP1 is also known as VEGF co-receptor. Therefore, it was assumed that Sema3A could interfere with NP1-mediated VEGF signaling and inhibit VEGF binding to NP1. However, it was demonstrated that Sema3A and VEGF binding domains on NP1 are completely different (141, 142). The role of Sema3A during angiogenesis appears to be controversial. Sema3A knockout mice in a CD-1 background showed a reduction in vascular remodeling when compared with wild-type animals (143). However, these results have not been reproduced in another study with Sema3A-null CD-1 mice, presumably because of mutant variation depending on mice genetic background (144). Nevertheless, Sema3A deletion in another mice strain resulted in severe renal vascular defects, supporting the importance of Sema3a in angiogenic remodeling. Sema3A treatment *in vitro* caused a significant inhibition in endothelial cell migration (145).

Maione et al. have demonstrated that Sema3A acts as an endogenous inhibitor of angiogenesis in premalignant lesions and is down-regulated during tumor progression.

Sema3A restored with an adeno-associated virus resulted in a decrease in tumor angiogenesis and tumor growth. Significantly, long-term expression of Sema3A in the tumor model strongly improved pericyte coverage of tumor-induced blood vessels, therefore normalizing tumor-induced angiogenesis. They observed that Sema3A expression is an inhibitor of tumor angiogenesis; however, it can favor vascular stabilization by increasing pericyte recruitment (146). Nevertheless, the mechanisms of Sema3A-mediated perivascular cell recruitment to tumor vessels remains to be elucidate. Recently, it has been shown that Sema3A/NP1 signaling influences arterial formation and lymphatic vessel maturation via regulating pericyte/smooth muscle cell coverage (147, 148).

2.2.3 Role of accessory cells in the formation of blood vessels

Bone marrow (BM)-derived cells have been credited with promoting vessel sprouting and stabilization of newly-induced vascular structures. During postnatal angiogenesis, BM-derived hematopoietic cells can differentiate in endothelial cells and mural cells, contributing to neovessel formation (149). Many reports have shown that hematopietic precursors are mobilized into the avascular area and maintained in a perivascular position where they promote sprouting angiogenesis by producing angiogenic signals such as VEGF and angiopoietins to chemoattract EC (150).

During adult neovascularization, VEGF signaling promotes the income of a population of bone marrow-derived CXC chemokine receptor 4 (CXCR4)-expressing cells. The stromal derived-factor-1 (SFD-1) produced by perivascular cells in response to VEGF favor $CXCR4^{+}$ cell retention within the tissues. These bone marrow-circulating cells enhance *in situ* endothelial cell proliferation during vessel formation. CXCR4 inhibition

and the resultant failure to retain $CXCR4^+$ cells leads to inhibition of angiogenesis (151, 152).

Moreover, Zacchigna et al. have demonstrated the presence of a population of bone marrow-derived cells recruited by NP1 receptors at the site of VEGF-induced angiogenesis. These infiltrating cells are $CD11b^+$ and they express the VEGF and Sema3a co-receptor NP1. They are therefore named NP1-expressing mononuclear (NEM) cells. NEM are not arteriogenic and they are not incorporated in the newly-formed vasculature. However, they favor arterial maturation through the secretion of different paracrine factors—notably Ang1, TGFβ and PBGF-BB. Their paracrine effect results in the activation and proliferation of smooth muscle cells and pericytes (147). Among myeloid cells, Tie2-expressing monocytes (TEMs), which represent 2% of circulating monocytes, play a crucial role during tumor angiogenesis. TEMs are recruited in the tumor through Ang2, where they secrete paracrine factors such as VEGF, MMP9, COX2 and Wnt5A to promote tumor angiogenesis. Depletion of TEMs impairs tumor growth and angiogenesis (153).

2.2.4 Vascular stabilization through the Sema3A/NEM Axis

Recently, we revealed the presence of an endothelial paracrine axis involving Sema3a, NP1-expressing monocytes and TGF-β1 which favors vascular maturation and stabilization (154). During VEGF-induced angiogenesis in skeletal muscles, increasing doses of VEGF have a negative impact on vessel stabilization. Vascular stabilization was defined as the portion of newly-induced vessels independent from further VEGF signaling. Withdrawal of VEGF by Aflibercept treatment at two and three weeks resulted in the regression of the vessel induced by high levels of VEGF. However, the vascular

network induced by low-VEGF doses was partially stabilized where around 50% of the vessels were independent from VEGF stimulus at three weeks. The differences in vascular stabilization did not correlate with changes in pericyte coverage. Significantly, some pro-maturation factors such as TGF-β1 and Sema3A were dose-dependently down-regulated by increasing levels of VEGF (154). VEGF and Sema3a can recruit NEM, which promotes mural cell recruitment during arteriogenesis (147). The impairment in Sema3A expression in the presence of high doses of VEGF correlated with a decrease in NEM recruitment while at low doses, the number of NEM increased when compared to control conditions matching the stabilization profile. NEM recruited by low levels of VEGF expressed paracrine factors known to favor vascular maturation such as TGF-β1. Moreover, TGF-β1 dose-dependent down-regulation prevented the activation of a Smad2/3 pathway known to mediate endothelial quiescence and vascular maturation (106).

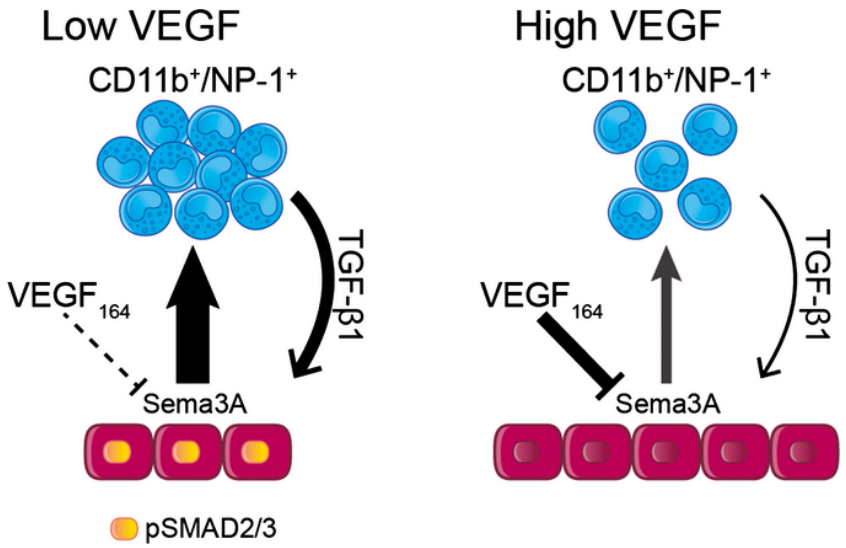

Figure 12. **Sema3A/TGF-β1/NEM axis.** Low doses of VEGF cause up-regulation of endothelial Sema3A, which recruits NEM in the area of active angiogenesis. Subsequently, NEM produces TGF-β1 which, on one hand, activates Smad2/3 pathways favoring vessel stabilization and, on the other, hand-stimulates additional Sema3A expression by endothelial cells. At a high level of VEGF, Sema3A is down-regulated in endothelial cells, causing an impairment in NEM recruitment and TGF-β1 release and resulting in a delay in vascular stabilization (Adapted from Groppa E. et al, 2015).

Additionally, we found that TGF-β1 induced Sema3a expression whereas VEGF inhibited it and TGF-β1 abrogation resulted in a down-regulation of Sema3A and reduction in NEM recruitment. Finally, Sema3A treatment favors vessel stabilization of vessels induced by high VEGF doses without inhibiting angiogenesis. These findings demonstrate the presence of a novel feedback loop where TGF-β1 secreted by Sema3A-recruited NEM up-regulates endothelial Sema3A and induces endothelial cell quiescence, promoting vascular stabilization (Fig. 12) (154).

III. Therapeutic angiogenesis

Therapeutic angiogenesis aims to stimulate the formation of new blood vessels from a preexisting vasculature to treat ischemic diseases. Delivery of angiogenic factor using genes, proteins and cells have shown a certain efficiency in animal models. However, clinical angiogenesis trials have proved challenging for demonstrating positive results in patients (155). Various reasons have been considered for the failure of clinical trials, ranging from poor gene transfer technology to short duration of transgene expression and a lack in persistency of vessels induced by therapy (156). Ischemic diseases are a group of cardiovascular diseases caused by a decrease in oxygen supply to the tissues. These diseases can occur in the heart (coronary heart disease), in the brain (cerebrovascular disease) and in the peripheral limbs (peripheral artery disease) (157). Ischemic disease represents one of the main heath care issues worldwide that continues to be the leading cause of morbidity and mortality (158).

3.1 Peripheral artery disease

Peripheral artery disease (PAD) is a common circulatory problem characterized by the narrowing of peripheral arteries due to the formation of atherosclerotic plaques and the subsequent decrease in blood supply (159). Atherosclerosis is the principal pathophysiological process leading to PAD. The pathological phases of atherosclerosis can be divided into three main stages: I) lesion initiation due to endothelial dysfunction by hypertension, hyperlipidemia, inflammation or oxidative stress; II) fatty streak formation and accumulation in the artery wall—a stage defined by the accumulation of lipoproteins and by smooth muscle cell, monocyte, macrophage and lymphocyte recruitment; and III) lesion maturation, where the lesion becomes a fibrotic plaque

composed of a growing mass of lipids (mainly cholesterol) and connective tissue. With the progression of the disease, the fibrotic plaques accumulate and grow, causing stenosis of the artery or complete occlusion. Eventually, rupture of atherosclerotic plaques can result in myocardial infarction and stroke or acute leg ischemia (160). Peripheral artery disease affects more the 13% of the population of Western countries, usually in those who are more than 50 years old. The major risk factors associated with PAD are smoking, diabetes, advancing age, hypertension and dyslipidemia. Most patients with PAD have mild or no symptoms; however, some people experience aching or burning muscles when walking (claudication). PAD can progress to a more severe form over time, leading to resting pain and even tissue loss (critical limb ischemia) (161). The current treatments for PAD include the reduction of risk factors including tobacco cessation, the adoption of a healthy diet and exercise and antiplatelet medication therapy. Moreover, patients can undergo invasive revascularization procedures such as angioplasty, artery bypass grafting and stenting. Despite advances in these treatments, however, up to 30% of patients with PAD cannot be treated due to peri-operative risk factors or patient comorbidities. In addition, some patients can develop restenosis following treatment, resulting in a diminished quality of life. Therefore, there is currently a push for novel therapeutic alternatives to improve arteriogenesis (collateral formation) and/or angiogenesis (capillary formation) in affected patients (157).

3.2 Angiogenic therapies

Therapeutic angiogenesis is a promising strategy for stimulating blood vessel growth in the ischemic tissue of patients who are not good candidates for standard revascularization procedures (162). An increase in blood supply and shear stress by

newly-induced vessels can trigger the opening of collateral arteries (arteriogenesis) to reestablish tissue function and encourage recovery (163). During arteriogenesis, pre-existing arterioles undergo processes of enlargement and remodeling into larger, functional vessels to bypass arterial occlusion (157). Neo-vascularization can be achieved by exogenous administration of several proangiogenic molecules including fibroblast growth factor (FGF), VEGF, hepatocyte growth factor (HGF), insulin growth factor (IGF) and HIF-1α. There are three main strategies for therapeutic angiogenesis: gene therapies, and protein and cell delivery. Protein therapies are accomplished by simple injection of recombinant proteins into the target site to promote vascular growth. Protein production and purification techniques are well-established and lyophilized proteins can be easily reconstituted prior to usage. However, protein therapies have shown disappointing results in trials due to the short half-life of the proteins in tissues (164). Cell therapies are based on the concept that candidate cells might differentiate in blood vessel-associated cells or mediate neovascularization by paracrine secretion of angiogenic factors. Several progenitor cells can be delivered, including bone marrow mononuclear cells (BMMNCs), mesenchymal stem cells (BMMSCs) and endothelial progenitor cells (EPCs). It has been shown that intramuscular bone marrow cell administration is safe, feasible and possibly efficient in patients with PAD (165). However, *in vivo* cell viability is quite low. In fact, most of the time, delivered cells fail to integrate into the host organism after delivery. The delivery of autologous cells is an attractive strategy for avoiding both limitations in cell sources and host immune rejection. Moreover, a recent meta-analysis of randomized, non-randomized and non-controlled studies of autologous cell therapies for peripheral artery disease showed that cell-based therapies enhance limb perfusion and functionality compared with control treatments. However, the efficacy of these therapies on all endpoints lose their significance in placebo-controlled studies and in randomized trials.

Currently, there is no significant evidence to support the efficacy of cell-based delivery treatments for patients with PAD (155, 166). Gene therapy via non-viral vector or viral vector is widely used to promote revascularization in patients with PAD (164). Non-viral gene transfer includes plasmid delivered by cationic polymers, lipids, liposomes, scaffolds or even necked plasmids. Nevertheless, this type of gene delivery presents disadvantages, including poor efficiency of vector delivery and low transgene expression. Gene transfer via viral vectors such as adeno-associated virus, lentivirus and retrovirus presents higher efficacy, as these vectors are integrated into the host genome and thus result in high protein expression. However, their use raises safety concerns with regard to immune hyper-reactivity and cytotoxicity. Moreover, viral gene integration can lead to interruption of tumor suppression, gene expression or activation of oncogene, leading to malignant cell transformation (167). Conversely, adeno-associated vectors (AAV) are safe gene therapy candidates thanks to their low immunogenicity and long-term transgene expression. Pro-angiogenic factor-based gene therapy has been administered with both intra-arterial and intramuscular delivery routes. Two plasmid trials with FGF-1 and VEGF, respectively—the TALISMAN (168) and Groningen (169) trail studies—failed to show significant functional improvements, although both trials showed a beneficial effect on the amputation rate in critical ischemia patients (170). A recent systematic review of randomized controlled trials of gene therapy in PAD showed that local administration of proangiogenic factors (VEGF, FGF, HGF, Del-1, HIF-1alpha) using gene transfer slightly improved hemodynamic measurements, ulceration recovery and residual pain in treated patients. The overall outcomes of the most recent clinical trials testing angiogenic factors have been disappointing when it comes to demonstrating a functional improvement in patients. In fact, improved exercise performance or decreases in major limb amputations and mortality were not observed (171, 172). Despite

encouraging results from preclinical studies, clinical trials using angiogenic factors did not demonstrate a successful therapeutic efficacy in patients with peripheral artery disease.

There are many factors that are likely responsible for these unsuccessful results. First, an ideal model of peripheral artery disease is not currently available. There are several differences between preclinical models and patients. Clinical manifestations in humans are heterogeneous and patients' comorbidities can influence the severity of the disease and the responsiveness to the ischemic insult. Thus far, none of the existing preclinical models have reproduced chronic ischemia, as normal reperfusion comes by two and three weeks in many mouse strains. Therefore, angiogenic therapies tested in preclinical animal models have most likely led to excessively-optimistic results which have not been observed in patients (157, 164). Moreover, over-dilated non-stable induced vessels and low and short-time transgene expression are also possible reasons for potential failure of clinical trials. Patient selection may affect the results of clinical trials. Presumably, only a subgroup of PAD patients might benefit from therapeutic vascular growth. Others with critical limb ischemia actually cannot be revascularized, as a significant number of new vessels and collateral arteries would be expected to see a proper improvement in these patients. Consequently, future clinical trials should necessarily identify patient subgroups which can respond positively to treatments and develop new biomarkers to drastically improve therapeutic angiogenic treatments (170).

3.3 Limitation in VEGF delivery: dose and duration

The most promising candidate for proangiogenic therapeutic studies is vascular endothelial growth factor A, known as the master regulator of angiogenesis. Most VEGF-

based clinical trials have been unsuccessful in show functional benefit in PAD patients. VEGF gene delivery studies have demonstrated the presence of a narrow therapeutic window *in vivo*. While low doses of VEGF are insufficient to achieve a proper angiogenic effect, high doses of these potent growth factors are unsafe, as they induce the formation of malformed vessels. Exogenous VEGF administration during embryonic neovascularization has resulted in the formation of an abnormal vascular network with alteration in vessel lumens (152, 173). Furthermore, VEGF delivery via adeno-associated viral vectors in a rabbit model of hind-limb ischemia efficiently promoted long-term angiogenesis. However, uncontrolled VEGF expression caused the formation of vascular structures and fibrosis in skeletal muscles, suggesting the need to control long-term VEGF expression to allow safe VEGF therapeutic applications (174). The over-expression of VEGF in the hearts and livers of transgenic mice resulted in an excessive angiogenic response and edema which destroyed the normal architecture of both organs. This outcome demonstrates the lack of negative feedback loops for controlling and limiting VEGF neovascularization in adult organs (175). In addition, the constitutive expression of VEGF in the skeletal muscles of mice after implantation of retrovirally-transduced myoblasts leads to the increased formation of aberrant-like structures called hemangiomas (176).

Previously, our group investigated the relationship between VEGF dosage and vessel morphology and function using a myoblast-based delivery system to constitutively express heterogeneous levels of VEGF in the skeletal muscles of mice. The results demonstrated that VEGF induces normal or aberrant angiogenesis depending on its distribution in the microenvironment and not in its total dose. In fact, decreasing the total number of VEGF-expressing myoblasts implanted, and therefore VEGF dosage, did not prevent the formation of aberrant-like structures due to the presence of VEGF hotspots

in the extracellular matrix (Fig. 13). However, the delivery of myoblasts expressing homogeneous levels of VEGF showed the presence of a threshold between normal and aberrant angiogenesis. Low and medium levels of VEGF induced the formation of normal and stable capillaries covered by pericytes, whereas high levels of VEGF induced hemangiomas (Fig. 13) (176).

The duration of VEGF expression is another crucial aspect to consider in VEGF-mediated therapeutic angiogenesis. While long-term VEGF expression is not desirable due to its toxic effect, a too-brief expression is ineffective in the induction of a functional and stable vasculature (152). The importance of VEGF duration was demonstrated in a transgenic model for conditional switching of VEGF expression in the heart and liver. Dor et al. have demonstrated that early VEGF signal termination at two weeks caused the complete regression of the vascular network induced by VEGF. However, prolonged VEGF expression for at least four weeks resulted in mature VEGF-independent vessels that persisted after VEGF withdrawal (175). Consistently, the injection of inducible VEGF-AAV or VEGF-expressing myoblasts in skeletal muscles further confirmed the need for four weeks of sustained VEGF expression in order to achieve stable and functional angiogenesis (177, 178).

Moreover, high and uncontrolled expression of VEGF by adenoviral vector delivery caused a transient VEGF expression too short to allow vessel persistency. However, local treatment with Sema3A increased the percentage of stable induced vessels at three weeks (154).

In conclusion, direct *in vivo gene* delivery of uncontrolled and heterogeneous VEGF levels is unsafe on one hand, as it can cause the formation of aberrant and unstable vessels (176). On the other hand, VEGF expression must be sustained for four weeks in order to achieve a stable vascular network (175). Therefore, there is the need to develop

new strategies for modulating the timing and dosage of the growth factor in order to increase the safety of VEGF gene delivery by enabling short-term expression.

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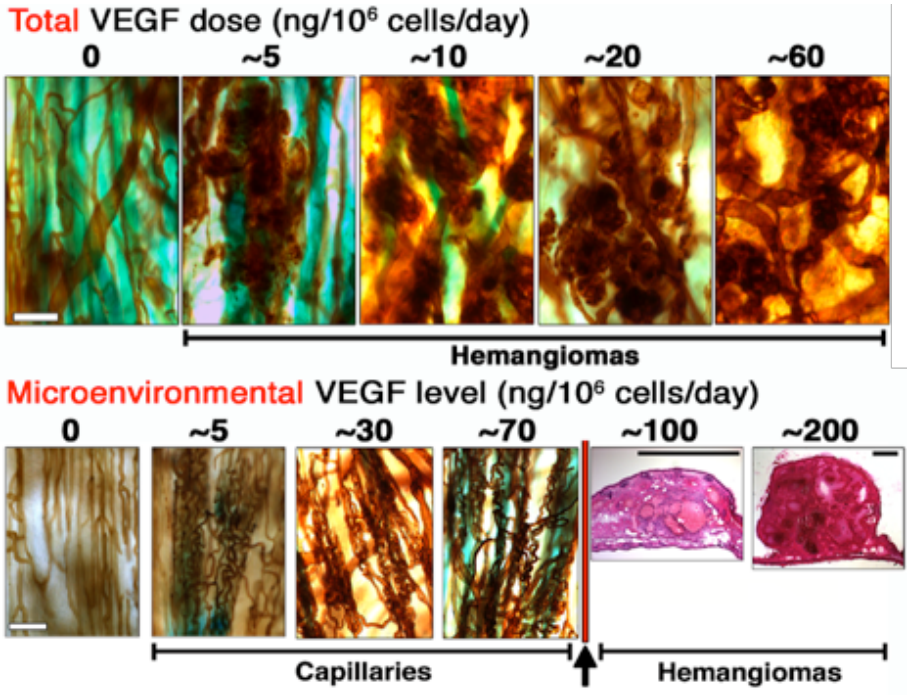

Figure 13. **VEGF induces normal or aberrant angiogenesis across a microenvironmental threshold level.** Lectin staining of the vasculature of adult mouse ears implanted with different amounts of VEGF-expressing myoblasts. Reduction in the total dose of VEGF delivered did not prevented the formation of aberrant-like structures, and bulbous vascular structures were observed even in the presence of a low percentage of myoblasts (upper image). Microenvironmental levels of VEGF produced by monoclonal myoblasts expressing homogeneous VEGF levels reveled a threshold below which normal capillaries are formed and above which hemangioma are induced (lower image) (Adapted from Ozawa CR. et al, 2004).

3.4 PDGF-B angiogenic normalization effects

Targeting the process of vascular maturation is a potential strategy for modulating or balancing VEGF-induced angiogenesis in order to overcome VEGF limitations (152). The acquisition of a proper pericyte coverage by newly-formed blood vessels is crucial for vessel survival, maturation and stabilization (66). Among the factors regulating the interplay between endothelial cells and pericytes, PDGF-BB has a primary role, as it is responsible for the recruitment of mural cells during the angiogenic process. Dual delivery of recombinant VEGF and PDGF-BB from a polymer scaffold promotes a stable and dense vasculature when compared with VEGF alone. The latter induces immature

vessels and tissue edema (179). In a rabbit model of chronic hindlimb ischemia, venous infusion of AAV vectors carrying VEGF and PDGF-B genes significantly increased microvascular maturation and collateral growth as well as perfusion and muscle function (180). Moreover, intramuscular adenoviral delivery of VEGF and PDGF-BB from two separate vectors caused the formation of normal capillaries. However, the presence of PDGF-BB could not reduce VEGF-induced edema formation after six days. Notably, VEGF and PDGF-BB co-delivery showed an impairment in pericyte recruitment on newly-induced capillaries compared with VEGF alone. Most likely, the overexpression of PDGF-BB outside the vascular wall resulted in a lack of PDGF gradients from the endothelium, leading to pericyte recruitment away from the vascular structures. Thus, the combination of the two growth factors enabled the formation of longer-lasting vessels through paracrine signals from interstitial recruited monocytes and macrophages. These results suggest the importance of establishing co-localized VEGF and PDGF-B gradients in target tissues in order to induce a proper vasculature (181).

Previously, we found that VEGF and PDGF-BB expression from a single bicistroic vector, which ensures a co-localized expression of both factors around each cell, prevented the formation of aberrant vascular structures. Instead, it caused the formation of normal capillaries covered by pericytes, which improved blood flow and collateral vessel growth in a model of hindlimb ischemia (182). Therefore, co-expression of PDGF-BB normalizes aberrant vessels induced by high and uncontrolled VEGF doses, allowing for the overcoming of the limitation of the VEGF dose and providing a safe strategy for therapeutic neovascularization.

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Aim

IV. Aim of the thesis

Peripheral artery disease (PAD) is a chronic condition characterized by narrowed arteries and decreased blood supply in the affected tissues. The disease mainly affects the lower limbs. It manifests clinically as limb claudication in the early stages and can progress to a more severe form characterized by rest pain and tissue loss (critical limb ischemia) (1). Morbidity and mortality is high amongst individuals affected by the disease. Although lifestyle changes and medical therapies have shown some efficacy, surgical and minimally-invasive revascularization are still the mainstays of treatment (2). However, current therapies for PAD are not suitable for all patients. With the advent of gene, molecular and biotechnological therapies, new strategies could be investigated in order to promote the formation of new collateral arteries (arterogenesis) and/or capillaries (angiogenesis) and to restore blood flow (3).

Vascular endothelial growth factor (VEGF) plays a crucial role in the angiogenic process. Consequently, it is widely used in clinical trials to induce therapeutic angiogenesis. VEGF delivery in ischemic tissues has been associated with improved collateral artery development, which is a key process for functional recovery (4). Nevertheless, initial positive results from clinical trials with VEGF did not demonstrate any long-term functional improvement. Additionally, the efficacy of VEGF therapy has not been confirmed in placebo-controlled phase II studies. The major obstacle of VEGF gene therapy appears to be its narrow therapeutic window. While low doses are not sufficient to induce functional improvement, levels above a specific threshold are associated with aberrant vascular structures (5). There is thus the need to precisely control the dose of VEGF delivered *in vivo*. Several studies have focused on the development of alternative

strategies for better controlling the effects of VEGF doses in order to induce normal and functional angiogenesis. Further, short-term expression of these potent growth factors is desirable for ensuring safe angiogenesis. However, it is inefficient for achieving persistent and stable vessels, as four weeks of sustained expression are required to achieve vascular stabilization (6).

Notably, we previously found that co-expression of VEGF and PDGF-BB at a fixed ratio can prevent the formation of aberrant structures by high and uncontrolled VEGF, suggesting a safe strategy for inducing therapeutic angiogenesis with direct gene therapy (7). Moreover, we recently found that increasing VEGF doses impairs vascular stabilization without affecting pericyte recruitment. Rather, it directly inhibits endothelial expression of Sema3A and leads to impaired Neuropilin-expressing monocyte recruitment and TGF-β1 signaling in the endothelium. PDGF-BB has been shown to normalize aberrant angiogenesis from excessive VEGF doses by stimulating pericyte recruitment. However, it is unknown whether PDGF-BB may also affect the stabilization kinetics of VEGF- induced angiogenesis. As the co-delivery of PDGF-BB normalizes VEGF-induced vessel growth, we will investigate whether PDGF-BB may also modulate the stabilization of VEGF-induced angiogenesis and its underlying mechanisms.

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Experimental Chapter

V. A pericyte-independent role for PDGF-BB to accelerate vascular stabilization by stimulating the Sema3A/NP1^{+} Monocyte axis

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Introduction

Therapeutic angiogenesis aims at promoting the formation of normal and functional capillaries from a preexisting vascular network in order to treat ischemic diseases (1). Neo-vascularization by delivering exogenous factors (i.e. VEGF) using genes, proteins or by progenitors cells delivery has shown promising results in animal models. However clinical trials have failed to demonstrate a functional improvement in patients (2). VEGF therapeutic potential is challenged by the need to control both the dose and duration of expression: sustained and uncontrolled levels cause the growth of angioma-like tumors (3), but transient delivery shorter than about 4 weeks is insufficient for stabilization and

persistence of induced vessels (4). The acceleration of VEGF-induced vessels stabilization would therefore help increase the safety of VEGF gene delivery by enabling short-term expression.

The maturation of new capillaries is the best understood mechanism of vascular stabilization. This process is characterized by the investment of endothelial structures with pericytes, which promote EC quiescence and make them independent from further VEGF stimulation for survival (5). A specific population of BM-derived myeloid cells, characterized by co-expression of the monocyte marker CD11b and the VEGF and Semaphorin3A (Sema3A) co-receptor Neuropilin-1 (NP1) and therefore named Neuropilin-Expressing Monocytes (NEM), have been shown to favor arteriogenesis by the secretion of paracrine factors that promote smooth muscle cell recruitment (6). Recently, we have found that NEM also play a crucial role in the stabilization of VEGF-induced angiogenesis by activating TGF-β1 signaling through SMAD2/3. Further, increasing VEGF doses impair vascular stabilization without affecting pericyte recruitment, but rather by directly inhibiting endothelial expression of Sema3A and therefore impairing the NEM/TGF-β1 axis (7).

We previously found that PDGF-BB co-expression restores normal angiogenesis despite high VEGF levels, by stimulating pericyte recruitment (8). However, it is unknown whether it may also affect the stabilization kinetics of VEGF-induced angiogenesis.

Here, we co-delivered specific doses of VEGF and PDGF-BB in skeletal muscle, taking advantage of a highly controlled cell-based gene delivery platform, to investigate whether PDGF-BB could accelerate the stabilization of VEGF dose-dependent angiogenesis and the underlying mechanisms.

Materials and methods

Cell Culture

Primary myoblasts were isolated from C57BL/6 mice and transduced to express the β-galactosidase marker gene (LacZ) from a retroviral promoter (9). Subsequently myoblasts were efficiently transduced (10) with retroviruses carrying the cDNA of murine $VEGF_{164}$ alone or either with human PDGF-BB linked through an IRES sequence (Internal-Ribosome-Entry-Site) and a truncated murine CD8a as a marker. Early passage myoblast clones were isolated and characterized as previously described (11). Briefly, myoblasts clones were isolated using a FACS Vantage SE cell sorter (Becton- Dickinson, Basel, Switzerland) and single cells isolation was confirmed visually (12). The stability of VEGF and PDGF-BB expression was assessed periodically by ELISA. All myoblast population were then cultured in 5% CO_2 on collagen-coated dishes with growth medium consisting of 40% F-10, 40%DMEM low glucose (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), 20% fetal bovine serum (hyClone, Logan, UT) supplemented with 2.5 ng/ml basic fibroblast growth factor (FGF-2), as described (13).

In vivo implantation of myoblast

Animal work was carried out in accordance to Swiss federal guidelines for animal welfare, after approval of the veterinary office of the Canton of Basel-Stadt (Basel, Switzerland; Permit 2071). CB.17 SCID mice (Charles Rivers Laboratory, Sulzfeld, Germany) of 7-15 weeks of age were used for all the experiment in order to avoid any immune response to the implanted myoblasts. Myoblast were trypsinized and resuspended in PBS with 0.5% of BSA and $1x10^6$ of cells were injected using syringe with a $29^1/_2$-gauge needle into the tibialis anterior and gastrocnemius muscle of the mice.

Aflibercept treatment

Mice were injected i.p. with Aflibercept (25mg/Kg; 100ul) in PBS or with the vehicle (100ul of PBS) 4 and 2 days before tissue harvesting. At 14 and 21 days after VEGF depletion *in vivo*, muscles were harvested and processed to examine vessels stabilization.

Anti-NRP1^{A} antibody treatment

Mice were treated systematically by i.p. injection with an anti-NRP1^{A} blocking antibody (YW64.3, Genentech Inc., South San Francisco, CA, USA) (14) in PBS with 0.5% BSA (10mg/kg) or with an IgG2A (10mg/kg) as control. Animals were treated at the time of myoblast implantation (day 0) and every 3 days till the final time point, according to the previously published treatment schedule (15).

Tissue staining

Mice were anesthetized with ketamine (100mg/kg) and Xylazine (10mg/kg) and sacrificed by intravascular perfusion with 1% paraformaldehyde in PBS pH7.4. *Tibialis anterior* and *gastrocnemius* muscles were harvested, post-fixed in 0.5% paraformaldehyde for 2 hours and then placed in 30% sucrose overnight to cryopreserve the tissues. Muscles were embedded in OCT, frozen in cold isopentane (Sigma) and then cryosectioned (10 μm thickness). The areas of effect corresponding to myoblast implantation were traced by X-gal staining (20 μm) in adjacent serial section, as previously described (11). Section of 10 μm were stained with the following primary antibodies: rat monoclonal anti-mouse CD31 (clone MEC 13.3, BD Biosciences, Basel, Switzerland) at 1:100 or hamster monoclonal anti-mouse CD31 (clone 2H8, Millipore, Merck, Germany) at 1:100; mouse monoclonal anti-mouse α-SMA (clone 1A4, MP Biomedicals, Basel, Switzerland) at 1:400; rabbit polyclonal anti-NG2 (Millipore, Merck,

Germany) at 1:200; rat monoclonal anti-CD11b (clone M1/70, Abcam, Cambridge, UK) at 1:100; rabbit polyclonal anti-p-SMAD2/3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:100; rabbit polyclonal anti-Ki67 (Abcam, Cambridge, UK) at 1:100 and rabbit polyclonal anti-Sema3A (Abcam) at 1:50. Fluorescently labeled secondary antibodies (Invitrogen, Basel, Switzerland) were used at 1:200. The Sema3A primary antibody was detected with a peroxidase-labeled anti-rabbit secondary antibody. The first chromogenic signal was developed with 3,3′-diaminobenzidinetetrahydrochloride (Sigma). To study vessel perfusion in vivo, mice were injected with 100 µl (1g/ml) of FITC-labeled *Lycopersicon esculentum* lectin (Vector Laboratories Inc., U.S.A) into the femoral vein and allowed to circulate for 4 minutes prior intravascular perfusion (7).

$VEGF_{164}$ and PDGF-BB ELISA measurements

The stability of VEGF and PDGF-BB expression by myoblasts was measured periodically during *in vitro* expansion of different cell batches. Myoblasts were cultured in 60 mm dish and subsequently incubated 4 hours with medium supplemented with 10 µg/ml heparin to prevent retention of PDGF-BB on the cell surface. One ml of medium was harvested then filtered and analyzed in duplicate. Cell culture supernatants (n = 4) were quantified for $mVEGF_{164}$ and hPDGF-BB protein using an ELISA kit (R&D Systems Europe, Abingdon, UK). ELISA results were normalized for total cell number and time of exposure to medium.

RNA in situ hybridization (ISH)

Sema3A and *CD31* mRNA were detected on frozen tissue sections with a sensitive RNA in situ hybridization (ISH) system, according to the manufacturer's instructions (QuantiGene ViewRNA, Affymetrix UK, High Wycombe, UK). Briefly, muscles were embedded in OCT as previously described followed by cryosectioning (10 μm thickness) and mounting of the tissue onto Superfrost Plus Gold glass slides (Thermo Fischer Scientific, Wohlen, Switzerland). Slides were kept at −80°C until use to avoid RNA degradation. Tissues were fixed with 4% formaldehyde overnight, washed and dehydrated in ethanol for 65 minutes. Slides were boiled for 1 min in a pretreatment solution and treated with Protease QF (Affymetrix). Tissue sections were hybridized for 2 hours at 40°C with target-specific probes for mouse Sema3A and mouse CD31 mRNAs (VB1-11132-06 and VB6-12921-01, respectively, Affymetrix). As negative control, one section for each experiment was hybridized only in diluent without any probe (Probe Set Diluent QT, Affymetrix). Subsequently each amplifier was hybridized with label probe oligonucleotides conjugated to alkaline phosphatase (LP-AP) type 1 or type 6, and the probes were detected respectively with Fast Red substrate (CD31 detection) and Fast Blue substrate (Sema3a detection). Slides were then counterstained with Meyer's hematoxylin and DAPI and mounted with aqueous mounting medium (Dako Ultramount Permanent Mounting Media S1964).

ISH image acquisition and quantification

The images were acquired with a laser scanning confocal microscope (LSM710, Carl Zeiss Microscopy, Göttingen, Germany) and Zen2 software (Carl Zeiss Microscopy). Between 5 and 10 images were acquired for each sample with a 40x objective. Red and green dots,

corresponding to the staining for *Sema3a* and *CD31* mRNA, respectively, were identified and cells positive for either or both transcripts were counted manually in each field.

Vessel analysis

Vessel length density was quantified on immunofluorescent stained cryosections by tracing the vessel length and dividing it by the area were the angiogenesis occurred (11). Vessel stabilization was calculated as the fraction of vessels that persist after Aflibercept treatment, as previously described (7).

Infiltrating $CD11b^+$ cells were quantified on cryosections stained for endothelium (CD31) and CD11b, normalizing the absolute number of $Cd11b^+$ cells by the area of effect. Vessel perfusion was quantified on section of muscles harvested after intravascular staining with fluorescent lectin, as described above. After co-staining with an anti-CD31 antibody, the total length of CD31-positive and lectin-positive vessels were quantified and vessel perfusion index was calculated as a ratio between the two values.

The immunohistochemistry staining of Sema3a was quantified as reciprocal intensity by the standard intensity function in the open source Fiji software (ImageJ) (http://fiji.sc/Fiji) as described (16). Briefly, since the maximum intensity value of an RGB image analyzed in ImageJ is 250, the intensity of a stained region of interest was subtracted from 250, thereby deriving a reciprocal intensity that is directly proportional to the amount of chromogen. For Vessel length density, vessel perfusion, $CD11b^+$ cell recruitment, Sema3a quantification, 5-10 fields (20x objective) were analyzed per muscles (n=3-4 muscle/group). All images were acquired with Olympus BX61 microscope (Olympus, Volketswil, Switzerland), and analyzed with CellSense software (Olympus, Volketswil, Switzerland).

Pericyte coverage was quantified after immunostaining for endothelium (CD31) and pericytes (NG2) from 5-10 fields per muscles (n=3). Images were taken with a 40X objective on a Carl Zeiss LSM710 3-laser scanning confocal microscope (Carl Zeiss, Feldbach, Switzerland). Briefly, the volume of CD31 and NG2 positive areas were measured with Imaris software (Bitplane AG, Zurich, Switzerland) and pericyte maturation index was calculated as a ratio between the two values obtained.

Ex-vivo cell isolation by FACS

Pools of 8 limb muscles of SCID CB17 mice (*Tibialis anterior* and *Gastrocnemius*) were harvested at 7 days after myoblast injection and processed as a single sample (*n* = 3 samples/group). Tissues were dissected and digested with Collagenase type II at 500U/ml activated with $CaCl_2$ (ThermoFisher Scientific) for 30 min at 37°C and then mashed with a syringe plunger. The digestion mix was filled with PBS and centrifuged at 850 rpm for 5 min. The supernatant was discarded then Collagenase D (Sigma) at 1.5 U/ml and Dispase at 2.4 U/ml (ThermoFisher Scientific) cocktail was added to the digest supplemented with $CaCl_2$ and incubated for 1 hour at 37°C under constant shaking. The final digest was centrifuged and cells were stained at 4°C for 30 min with the following fluorescently labeled antibodies: PE-anti-mouse CD31 (clone 390; BioLegend, San Diego, CA, USA) at 1:200; PE-Cy7-anti-mouse CD11b (clone M1/70, BioLegend) at 1:100; Alexa488-anti-mouse NG2 (Millipore, Merck, Germany). $CD31^+$, $CD11b^+$ and $NG2^+$ cells were isolated with an Influx cell sorter (BD Biosciences). RNA was extracted, and gene expression analysis was performed as described below.

Quantitative Real-Time PCR

For RNA extraction from total muscles, mice were previously injected with transgenic myoblasts and muscles were freshly harvested and then disrupted using a Qiagen Tissue Lyser (Qiagen) in 1 ml of Tri Reagent solution (Invitrogen). The RNA was extracted according to manufacturer's instructions. RNA from *in vitro* endothelial cells or FACS sorted cell was extracted with RNAeasy Mini Kit (Qiagen). The total RNA was reverse transcribed into cDNA with an Omniscript Reverse Transcription kit (Qiagen) at 37°C for 60 minutes. The quantitative Real-Time PCR was performed on an ABI 7300 Real-Time PCR system (Applied Biosystems). The commercial TaqMan gene expression assay (AppliedBiosystems) was used to determine the expression of genes of interest. The cycling parameters were: 50°C for 2 minutes, followed by 95°C for 10 minutes and 40 cycles of denaturing at 95°C for 15 seconds an at last annealing/ extension at 60°C for 1 minute. Reactions were performed two or three times for each template, averaged and normalized to expression of the GAPDH housekeeping gene.

In vitro assay with HDMEC

Primary Dermal Microvascular Endothelial Cells (HDMEC - ATCC LGS standards) were cultured according to manufacturer's protocol. Briefly, primary endothelial cells were cultured with vascular cell basal medium (ATCC LGS standards) supplemented with 0.2 % Bovine Brain Extract, 5ng/ml rhEGF, 10 mM L-glutamine, 0.75U/ml Heparin sulfate,1ug/ml Hydrocortisone, 5% Fetal Bovine Serum, 50ug/ml ascorbic acid and 100U/ml of penicillin / streptomycin. Cells were seeded (0.05 x 10^6/well) into 24 cell culture plates and cultured at 70% confluency. Cells were then starved for 2 hours and stimulated for 24 hours with different concentration of hVEGF (10ng/ml; 40ng/ml), hPDGF-BB (2.5ng/ml; 10ng/ml) or with both growth factors (R&D System).

Subsequently, cells were collected after stimulation and RNA extraction, reverse transcription and qRT-PCR was performed as explained before. To quantify VEGFR2 *in vitro* Phosphorylation, HDMEC were seeded in 12-well plate ($0.1x10^6$cells/well) and starved overnight. Cells were then stimulated with hVEGF (40ng/ml) alone or in combination with hPDGF-BB (10ng/ml) for 5 min and then lysed with Lysis Buffer (Cell Signaling Technology) supplemented with 1 mM PMSF (Sigma) for 5 min on ice. Cell lysates were assayed for VEGFR2 phosphorylation of VEGFR2 with Phospho-VEGFR-2 (Tyr1175) Sandwich ELISA (Cell Signaling Technology) as described in the manufacturer protocol.

Statistics

Data are presented as mean ±standard error. The significance differences were evaluated with the GraphPad Prism 7 software. A normality test was applied to each data set and based on data distribution we applied multiple comparisons with parametric one-way analysis of variance (ANOVA) followed by the Bonferroni test or with the nonparametric Kruskal–Wallis test followed by Dunn's post-test. Gene expression data were normalized by logarithmic transformation (ln=y) and then analyzed with one-way analysis of variance (ANOVA) followed by the Bonferroni test. A p *value* < 0.05 was considered statistically significant.

Results

PDGF-BB co-expression accelerates stabilization of vessels induced by heterogeneous VEGF levels

To assess the hypothesis that PDGF-BB co-expression can regulate vessel stabilization kinetics, we took advantage of a well-characterized myoblast-gene delivery system to achieve sustained expression of the growth factors in skeletal muscles. In order to mimic the delivery conditions of viral gene therapy, myoblasts expressing heterogeneous and uncontrolled levels of $mVEGF_{164}$, hPDGF-BB or both factors at a fixed ratio of 4:1 (VIP) were implanted in the skeletal muscles of adult SCID mice. To determine VEGF-dependence of the induced vasculature, mice were treated with Saline or Aflibercept (VEGF-Trap), a potent VEGF binder that can deplete active VEGF *in vivo*. As a control we implanted myoblast expressing only a non-functional truncated version of CD8a as a surface marker.

Delivery of PDGF-BB alone did not alter the density of the capillary network at any time point with either treatment (Fig. 1B, 1C, 1I and 1J). Heterogeneous levels of VEGF induced a mixed angiogenic response at 2 and 3 weeks consisting of both aberrant structures covered by α-SMA^+ smooth muscle cells and normal capillaries surrounded by NG2+ pericytes (Fig. 1D and 1K). As expected, all vascular structures induced by co-expression of VEGF and PDGF-BB were morphologically normal capillaries associated with $NG2^+$ pericytes, despite uncontrolled VEGF levels (Fig 1F, 1G, 1M and 1N). Vessel Length Density (VLD) was quantified after treatment with Aflibercept or saline control and vessel stabilization was expressed as the percentage of induced vessels (i.e. VLD in the V- or VIP-stimulated tissues minus pre-existing VLD measured in uninjected control muscles) that could persist independently from VEGF signaling (Resistant Fraction),

according to the mathematical equation: RF % = $((VLD_{Aflibercept} - VLD_{Ctrl})/(VLD_{saline} - VLD_{Ctrl}))*100$. In the saline-treated mice, the mean VLD of the control condition was 7.5±1.8 mm/mm^2 and values above control VLD represent newly induced vessels. Aberrant and normal vascular structures were quantified separately. Aberrant structures induced by uncontrolled doses of VEGF were completely abrogated after VEGF-Trap treatment after both 2 and 3 weeks, demonstrating their continued VEGF-dependency. Normal capillaries induced by VEGF alone caused an increase in VLD compared to control conditions to 58±7.8 mm/mm^2 and 50±7.35 mm/mm^2, respectively at 2 and 3 weeks. However, after VEGF-Trap only 25% of normal vessels induced by VEGF alone were stable after 2 weeks (Fig.1E and 1P), with a minor increase of the resistant fraction to 38% after 3 weeks. VIP-induced capillaries yielded a VLD of 50±10.2 mm/mm^2 and 47±2.88 mm/mm^2 after 2 and 3 weeks, respectively. In contrast to the VEGF alone condition, already 40% of newly induced vessels persisted after Aflibercept treatment at 2 weeks (Fig.1G, 1O and 1P), with a further increase of the resistant fraction to 76% after 3 weeks (Fig. 1R). In conclusion, the co-expression of PDGF-BB significantly accelerated vessel stabilization compared to VEGF alone, even considering only the normal fraction of induced angiogenesis.

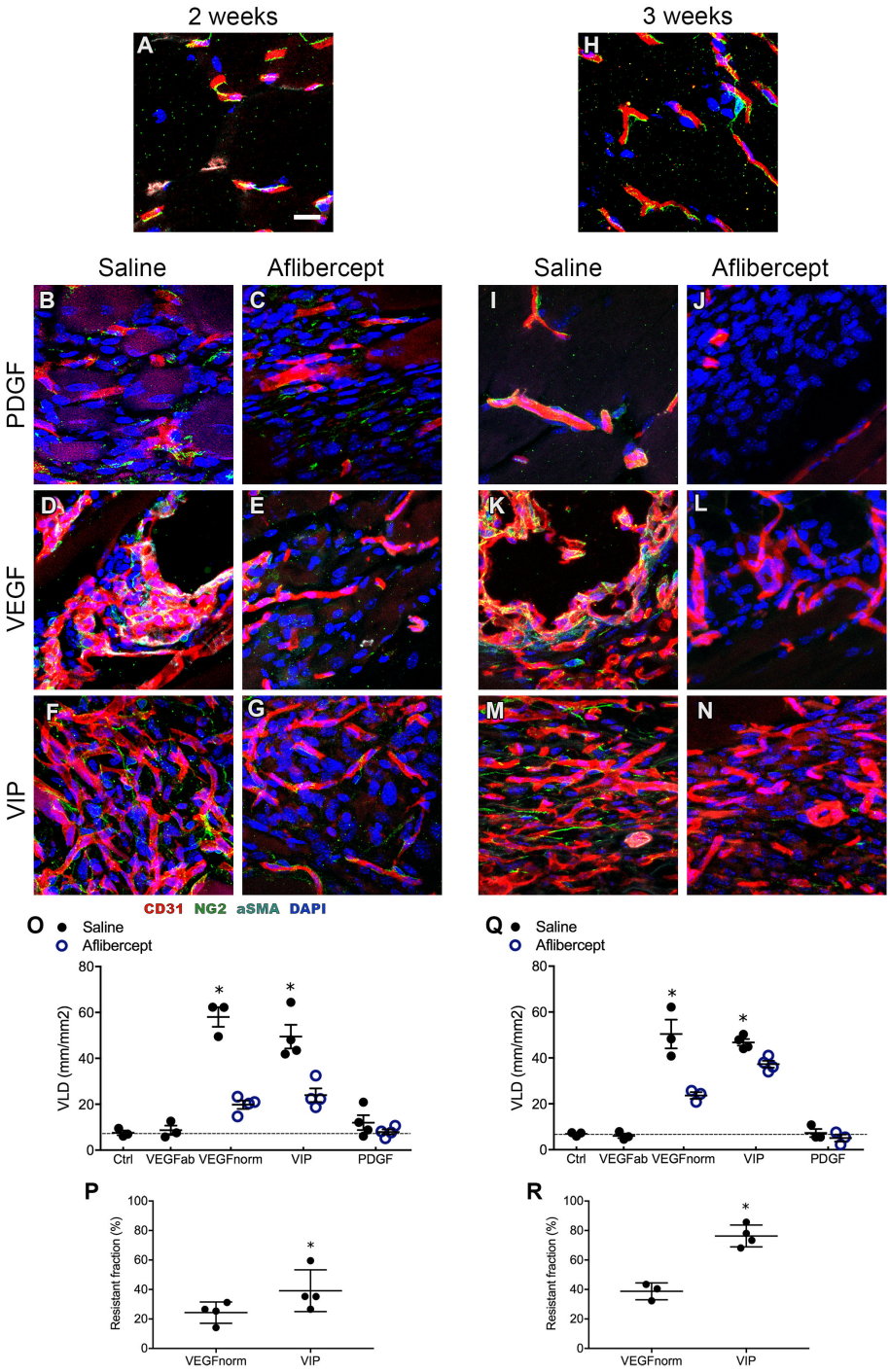

Figure 1. **PDGF-BB accelerates stabilization of VEGF induced angiogenesis at 2 and 3 weeks.** (A-N) Immunofluorescence staining of endothelium (CD31, red), pericytes (NG2, green) and smooth muscles cells (α-SMA, cyan) on frozen section of muscles injected with myoblast expressing VEGF, PDGF-BB or both growth factors (VIP). Mice were treated either with Saline of Afilbercept at 2 and 3 weeks. Normal vessels display a proper pericyte coverage, whereas aberrant-like structures induced by heterogeneous level of VEGF were covered with smooth muscle cells. Scale bar= 20µm (O) Quantification of vascular length density (VLD) and percentage of persistent vessel after Aflibercept treatment (Resistant fraction) was calculated at 2 and 3 weeks. The highest degree of vascular stabilization was observed in presence of VEGF and PDGF-BB co-expression (P-R). VEGFab=aberrant angiogenesis by VEGF alone; VEGFnorm=normal angiogenesis induced by VEGF; VIP= VEGF+PDGF; PDGF=PDGF-BB alone. Data represent the mean ± SEM of individual's muscles. **P* < 0.05 by ANOVA Kruskal-Wailles with Dunn's multiple comparisons test.

Vascular stabilization kinetics depend on PDGF-BB dose

In order to assess the role of PDGF -BB dose on the kinetics of vessels stabilization, we used a previously characterized pool of monoclonal myoblast populations, genetically modified to express specific doses of VEGF and PDGF-BB at a fixed ratio of 4:1 (VIP clones), to ensure homogeneous microenvironment levels (17). We selected 3 clonal populations based on their *in vitro* expression of the two growth factors at the fixed ratio: low (VEGF ≈10ng/10^6 cells/day – PDGFBB ≈2.5ng/10^6 cells/day), medium (VEGF ≈53ng/10^6 cells/day – PDGFBB ≈13ng/10^6 cells/day) and high (VEGF ≈150ng/10^6 cells/day – PDGFBB ≈40ng/10^6 cells/day). Myoblast clones were implanted in the skeletal muscles of adult SCID mice. Mice were treated at 2 and 3 weeks with Saline or Aflibercept, as described before.

All VIP clones induced a similar network of capillaries at 2 and 3 weeks in the Saline conditions. PDGF-BB co-expression did not significantly increase vessel stabilization at low and medium levels at 2 weeks compared to equivalent doses of VEGF alone, as only 36% and 23% of vessels persisted respectively (Fig.2C, 2E, 2O and 2P). However, in the presence of high VEGF and PDGF-BB levels already 62% of the induced vessels were stable. By 3 weeks after myoblast implantation, vessel stabilization gradually increased for both VIP-low and VIP-med (64% and 39%, respectively; Fig2J, 2L, 2Q and 2R), but PDGF-BB co-expression greatly increased vessel stabilization at high doses (95% Resistant fraction; Fig.2N, 2Q and 2R). In conclusion, the coordinated co-expression of PDGF-BB promotes the stabilization of new induced vessels, primarily at high VEGF levels, but not at low and medium VEGF levels.

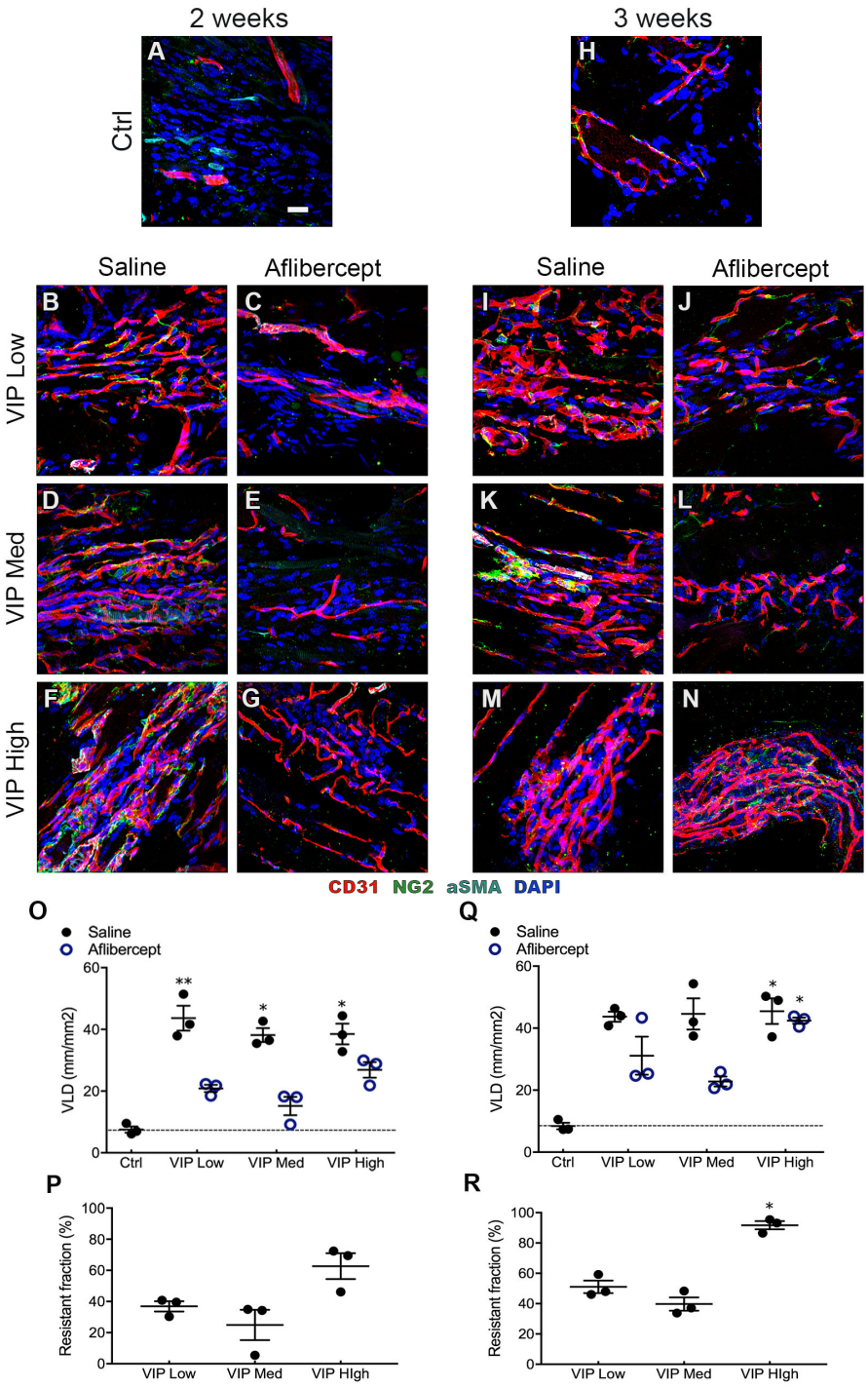

Figure 2. **PDGF-BB accelerate vessel stabilization only at high doses**. Immunofluorescence staining of endothelium (CD31, red), pericytes (NG2, green) and smooth muscles cells (α-SMA, cyan) of mice skeletal muscles injected with clonal population of myoblast expressing increasing doses of VEGF+PDGF-BB (VIP). Mice were treated with Saline or Afilbercept at 2 and 3 weeks. All VIP clones induced normal capillaries covered by pericytes. Scale bar= 20µm (A-N) Quantification of vessel length density and vessel regression (Resistant fraction) was measured at 2 and 3 weeks (VLD) (O-Q). Quantification of the resistant fraction reveled that a moderate fraction of vessels induced by low and medium doses of VIP were stable. However, the fastest stabilization rate was obtained with high VEGF and PDGF-BB levels both at 2 and 3 weeks (P-R). Data represent the mean ± SEM of individual's muscles. *$P < 0.05$ by ANOVA Kruskal-Waills with Dunn's multiple comparisons test.

Stabilization kinetics does not correlate with differential pericyte coverage or vascular perfusion

Pericytes have been shown to regulate vessel maturation and therefore vascular stabilization through their interaction with nascent endothelial tubes (18). Therefore, we sought to determine if the differences in VIP stabilization kinetics were correlating with changes in pericyte recruitment. Pericyte coverage of VIP-induced vessels was quantified two weeks after myoblast implantation in skeletal muscles. After immunofluorescence staining, the ratio between NG2-positive area and CD31-positive area was measured (maturation index). All conditions displayed similar pericyte coverage (VIP Low = 0.51±0.06, VIP Med = 0.47±0.09, VIP High = 0.53±0.02), demonstrating that differences in vascular stabilization did not correlate with differential pericyte recruitment (Fig. 3A-D).

The establishment of blood flow in newly induced vessels is a requirement for vessel remodeling and therefore vessel maturation and stabilization (19). Consequently, observed differences in stabilization kinetics could correlate with differences in vessel perfusion. To assess this hypothesis, mice were injected 4 minutes prior sacrifice with a fluorescein-labeled lectin (FITC-lectin), which binds the endothelial glycocalyx and therefore marks the lumen of vessels connected with the general circulation. The ratio between lectin-positive and CD31-positive vessel length was used to identify perfused vascular structure. As shown in Fig. 3 (E-M), vessels induced by increasing doses of VEGF and PDGF-BB were similarly perfused and therefore functional (Fig. 3N) and only a similarly small fraction of the new vascular bed was not perfused in all conditions. These results suggest that differences in vessel stabilization rates are not due to the establishment of a functional flow throughout the new vasculature.

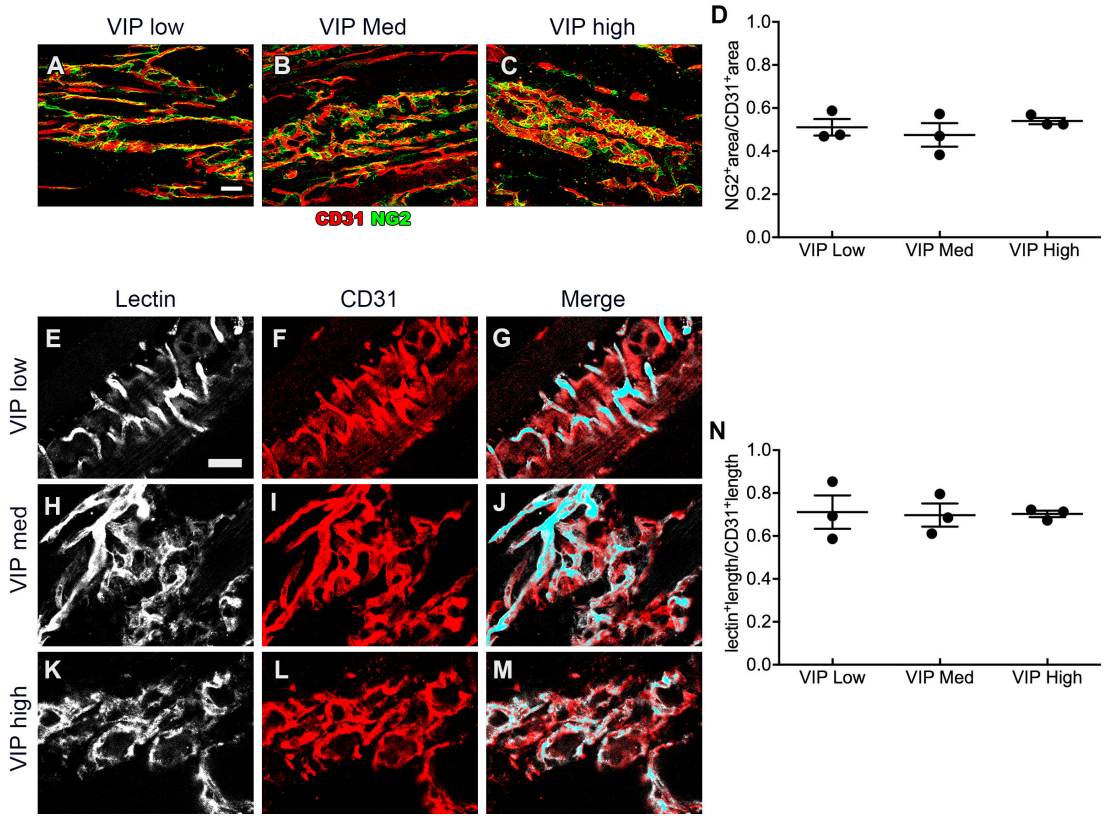

Figure 3. **Vessel stabilization is not associated with differences in pericytes recruitment and vessel perfusion.** Immunofluorescence staining of endothelium (CD31, red) and pericytes (NG2, green) of muscles implanted with myoblast expressing Low, Med and High levels of VEGF and PDGF-BB at fixed ratio at 2 weeks' time point (a-c). Size bar=20µm. Pericyte coverage was comparable among the conditions as demonstrated by quantification of the ratio between positive area for CD31 and NG2 (d). Immunofluorescence staining of endothelium (CD31, red) and lectin-positive structures (lectin, white) of mice muscles exposed to increasing doses of VEGF and PDGF-BB. Mice were injected with FITC-labeled lectin prior sacrifice in order to reveal functional and perfused vessels (e-m). Scale bar=20µm. Vessel perfusion quantification showed that most of the new vascular structures induced by different VIP levels were perfused at 2 weeks (≈ 70% perfused capillaries) (n). Data from both experiments represent the mean ± SEM of individual's muscles. Data were subjected to ANOVA Kruskal-Waills with Dunn's multiple comparisons test., No significant difference was detected.

Increasing PDGF-BB levels up-regulate Sema3a and TGF-β1 expression with increased NEM recruitment

Recently it has been shown that increasing VEGF doses impair vascular stabilization without affecting pericyte recruitment, but rather by directly inhibiting endothelial expression of Sema3A and leading to impaired recruitment of neuropilin-expressing monocytes (NEM) and TGF-β1 signaling in the endothelium (7). Specifically, low VEGF levels enable vessel stabilization by allowing expression of endothelial Sema3A, leading to robust NEM recruitment and high TGF-β1 levels, which in turn start a positive feedback loop to stimulate further Sema3A expression and maintain the stabilizing signals. Conversely, high doses of VEGF impair stabilization by inhibiting this Sema3A/NEM/TGFβ1 axis. Therefore, we sought to investigate whether the expression of maturation factors (Ang-1, TGF-β1 and Sema3a) governing endothelial-pericytes crosstalk and NEM recruitment were differentially regulated and which might correlate with the observed differences in VIP stabilization rates. Mice were injected with control cells and the different VIP-expressing myoblasts and gene expression was measured after 7 days, when pericyte-endothelium crosstalk is fully active in nascent vessels.

As shown in Fig.4A, different levels of VEGF and PDGF-BB did not regulate *Ang1* expression. *Sema3a* and *Tgfb1* were significantly up-regulated with increasing doses of VEGF and PDGF-BB (Fig.4A), in contrast to the effects of high VEGF alone, which caused instead a down-regulation of both factors (7). Specifically, VIP High increased *Sema3a* expression 5-fold and that of *Tgfb1* 9-fold compared to control levels. These data show that the expression trends of *Sema3a* and *Tgfb1* in presence of increasing doses of VEGF and PDGF-BB correlate with the observed vascular stabilization pattern and suggest that they might be regulated directly by PDGF-BB. To assess this hypothesis, myoblasts expressing low, medium and high doses of PDGF-BB alone were implanted in mouse

muscles and the expression of the maturation factors was quantified after 7 days. Interestingly, increasing doses of PDGF-BB alone caused an upregulation of *Sema3a* and *Tgfb1* expression in total muscles even in the absence of VEGF (Fig.4B), suggesting a direct regulation of *Sema3a* expression by PDGF-BB. Gene expression data of *Sema3a* expression in the VIP conditions were confirmed by immunostaining for Sema3A protein, which showed a progressive increase of Sema3A in the tissues (Fig.4C-H). All the implanted myoblast populations (VIP and P clones) expressed low levels of Sema3A *in vitro,* thereby excluding that the implanted cells could be the source of the observed changes in Sema3A expression *in vivo* (Fig.4 I-J).

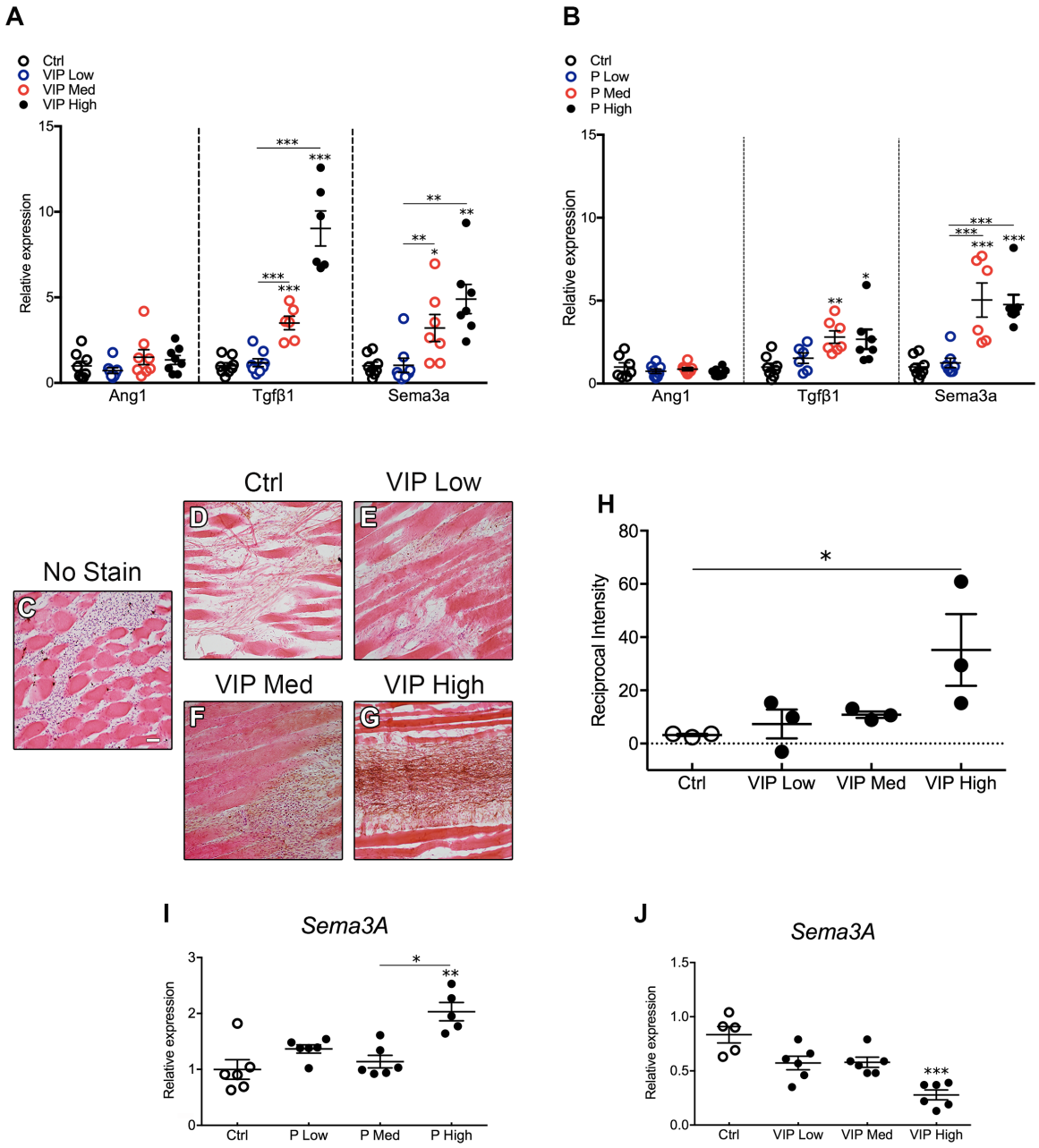

Figure 4. **TGF-β1 and Sema3A are up-regulated by increasing doses of VEGF and PDGF-BB.** Gene expression analysis at 7 days after implantation of myoblasts expressing VEGF+PDGF-BB (VIP clones), PDGF-BB alone (P clones) and control cells. Relative mRNA expression of *Ang1*, *Tgfb1* and *Sema3a* was quantified by RT-PCR and normalized to control conditions (A-B). *Tgfb1*and *Sema3a* expression trends matched with the vascular stabilization pattern in the VIP conditions, with a 7-fold and 4-fold up-regulation at high VIP levels respectively despite high VEGF levels (A). Interestingly, delivery of PDGF-BB alone caused a direct upregulation of Sema3A in absence of VEGF (B). Data represent the mean ± SEM of individual's muscles. *$P < 0.05$, **$P < 0.001$, ***$P < 0.0001$ ANOVA with Bonferroni multiple comparisons test, after data normalization by logarithmic transformation. Immunohistochemistry for Sema3A protein on frozen muscle sections. The intensity of Sema3A was quantified as reciprocal intensity on a scale out of 250. At 1 week IHC staining confirmed an up-regulation of Sema3A expression by increasing VIP doses (C-H). Scale bar= 50 μm; Data represent the mean ± SEM of individual's muscles. *$P < 0.05$ by ANOVA Kruskal-Wailles with Dunn's multiple comparisons test. *Sema3a* gene expression was performed on myoblast expressing Low, Med and High levels of VEGF+PDGF-BB or PDGF-BB alone *in vitro* (I-J). Data were represented as the mean ± SEM of individual samples. *$P < 0.05$, **$P < 0.001$ ***$P < 0.0001$ by ANOVA Kruskal-Wailles with Dunn's multiple comparisons test.

NEM recruitment by Sema3A was previously shown to promote vascular stabilization (7). Therefore, NEM recruitment was assessed one week after VIP myoblast implantation by immunostaining for CD11b and their frequency was quantified and normalized by the area of active angiogenesis ($CD11b^+$ cells/area mm^2). As shown in Fig.5 (D, H, L and M) a robust recruitment of NEM was detected in the VIP high condition that correlated with increased expression of Sema3a and faster vessel stabilization. $Cd11b^+$ NEMs were isolated *ex-vivo* by FACS in order to investigate cell-specific changes in gene expression. TGF-β1 and NP1 expression by $CD11b^+$ cells did not show any difference among the groups (Fig.5N), in agreement with previous findings (7).

Taken together, these results suggest that increasing VEGF and PDGF-BB doses might accelerate vascular stabilization by up-regulating Sema3A expression and increasing NEM recruitment, which in turn lead to an increased production of TGF-β1 in the tissue, though TGF-β1 expression levels in NEM themselves were not directly regulated by PDGF-BB co-delivery.

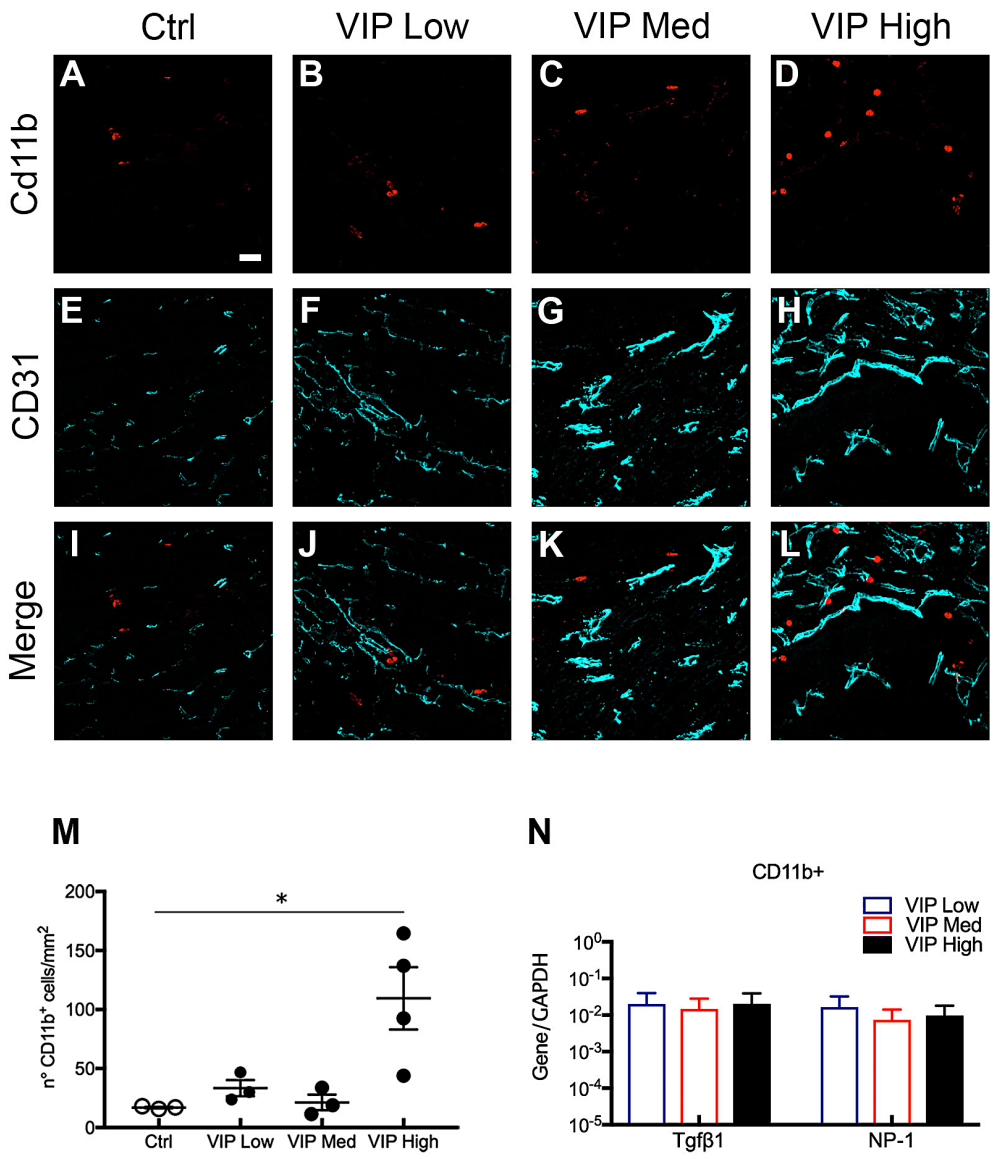

Figure 5. **Increasing VIP doses favor CD11b⁺/Nrp1⁺ monocyte recruitment.** Immunofluorescence staining of endothelial cells (CD31, in cyan) and NEM (CD11b, in red) 1 week after injection of VIP myoblast clones. Increased recruitment of NEM is observed in the VIP high condition that correlates with the increased expression of Sema3A (A-L). Quantification of NEM recruitment in the area of VIP-induced angiogenesis (CD11b⁺/mm²). Data were represented as the mean ± SEM of individual's muscles. *$P < 0.05$ by ANOVA Kruskal-Waills with Dunn's multiple comparisons test (M). CD11b⁺ cells were isolated by FACS sorting from muscles injected with VIP Low, VIP Med and VIP High. Gene expression data relative to GAPDH showed that TGF-β1 and Neuropilin-1 were similarly expressed by NEM. Data represent the mean ± SEM of individual values. Data were subjected to ANOVA with Bonferroni multiple comparisons test, after data normalization by logarithmic transformation. No significant difference was detected.

Increasing VEGF and PDGF-BB doses promote endothelium quiescence via TGF-β1 pathway

TGF-β1 is known to regulate endothelium state by differentially activating ALK1 and ALK5 receptors (20). TGFβ/ALK1 signaling induces Smad1/5 phosphorylation and stimulates EC proliferation and migration during tube formation (21). In contrast, TGF-β/ALK5 signaling stimulates Smad2/3 pathway, which has been shown to mediate vessel maturation and stability through inhibition of EC proliferation and migration (20). Therefore, we sought to assess whether TGF-β signaling on endothelial cells was differentially regulated upon stimulation with increasing doses of VEGF and PDGF-BB and increased TGF-β1 expression in tissue. As shown in Fig.6 (A-I) immunofluorescence staining of phosphorylated Smad2/3 complex was detectable in EC nuclei only in presence of high levels of VEGF and PDGF-BB, which cause the greater increase in TGF-β1 levels, but not at lower doses. Consistently, VIP doses increased dose-dependently the expression of *Serpine1* (PAI-I), which is specifically induced by the Smad2/3 pathway and not by Smad1/5. The highest *Serpine1*expression (15-fold compared to control) was cause by high levels of VEGF and PDGF-BB (Fig.6J). Instead the expression of *Id1*, which is induced specifically by activation of the Smad1/5 pathway, did not change significantly among the conditions (Fig.6J).

Furthermore, the percentage of proliferating cells ($Ki67^{+}$) was quantified in order to confirm the observed differences in the activation state of EC in VIP-induced vessels. At 7 days, vessels induced by VIP Low and VIP Med had respectively 25% and 8% of actively proliferating endothelium. Instead at high level of VEGF and PDGF-BB, the percentage of Ki-67-positive EC cells further decreased to 2%, i.e. lower than the control conditions (Fig.6 K-O). Therefore, these data show that VIP dose-dependent up-regulation of TGF-β1 in the tissues caused the specific activation of Smad2/3 signaling

and suppression of endothelial proliferation, i.e. the processes that favor vessel stabilization.

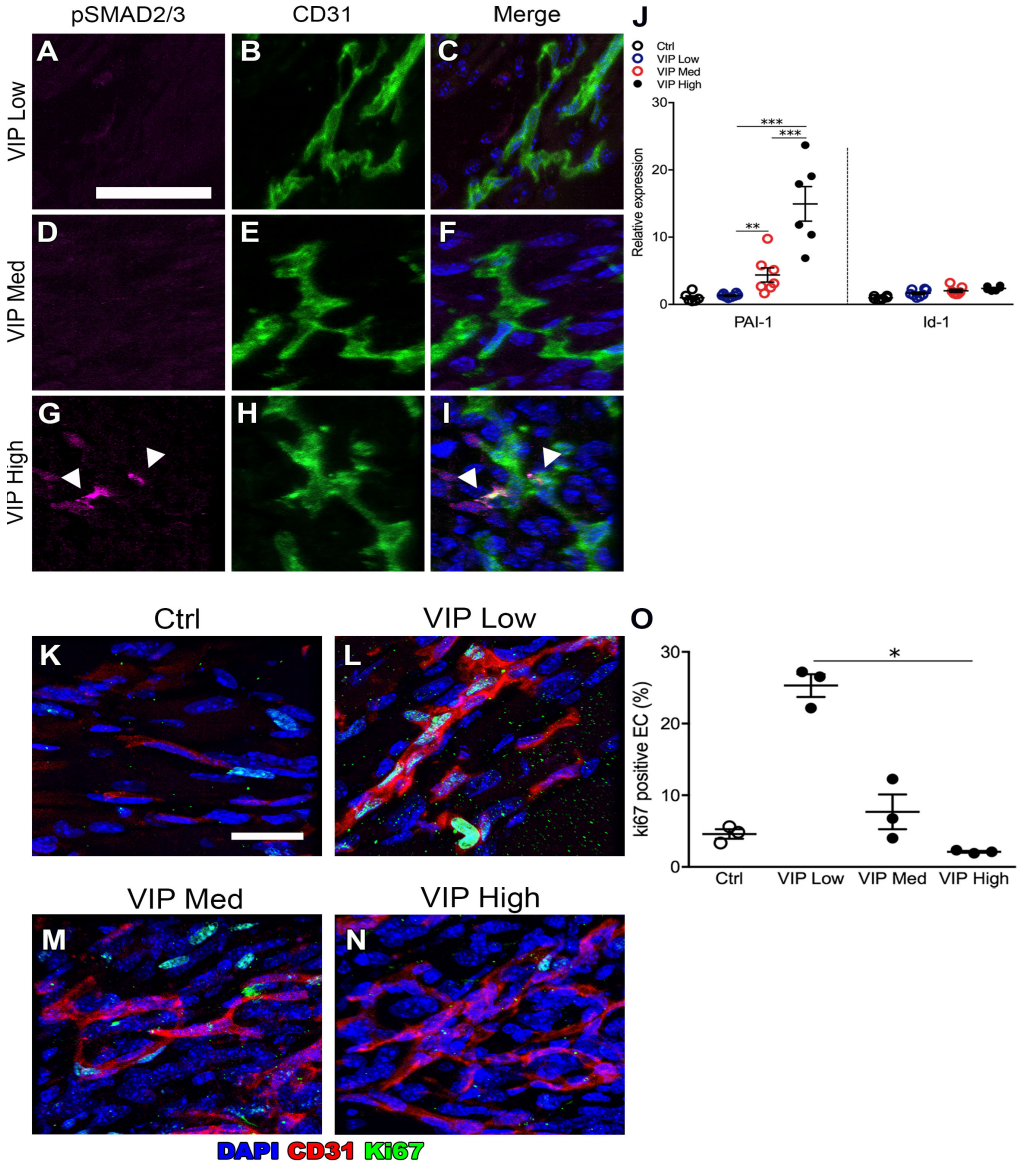

Figure 6. **Increasing doses of VEGF+PDGF-BB promote endothelial cells quiescence that mediates vessel stabilization.** Immunofluorescence staining of endothelium (CD31, green) and pSMAD2/3 (violet) 1 week after VIP myoblast injection showed that TGF-β1 pathway activity was present only at doses of VEGF and PDGF-BB (A-I). Scale bar= 50μm. Total muscles gene expression analysis revealed that *Serpine1* (PAI-I) expression, induced by the activation of Smad2/3, was upregulated by increasing doses of VEGF+PDGF-BB. Instead, the expression of *Id1* induced by activated Smad1/5 was not regulated by VIP doses (J). Data represent the mean ± SEM of individual's muscles. $^{**}P < 0.001$, $^{***}P < 0.0001$ ANOVA with Bonferroni multiple comparisons test, after data normalization by logarithmic transformation. Immunostaining for endothelium (CD31, red) and proliferating cells (Ki67, green) at 1 week (K-N). The percentage of proliferating cells was quantified in the area of VIP-induced angiogenesis (O). VEGF and PDGF-BB co-expression decreased dose-dependently the total amount of proliferating endothelial cells (K-O). Data represent the mean ± SEM of individual's muscles. $^{*}P < 0.05$ by ANOVA Kruskal-Wailles with Dunn's multiple comparisons test. Scale bar= 20μm.

Sema3A/Nrp1 binding is required for vessel stabilization by PDGF-BB

Neuropilin-1 (NP1) is a non-tyrosine kinase receptor present in different cell types including NEM, which can act as a co-receptor for Sema3a and $VEGF_{164/165}$ (22). NP1 receptor is essential for NEM recruitment as silencing of neuropilin-1 by RNAi reduced the *in vivo* migration of $CD11b^+$ cells in response to $VEGF_{165}$ and Sema3A (6). Recently, it has been shown that Sema3A signaling through Neuropilin-1 is required to maintain the Sema3A/NEM/TGF-β1 axis during vessel stabilization after VEGF delivery (7). To determine the role of the Sema3A/NEM/TGF-β1 feedback loop in the accelerated vascular stabilization at high PDGF-BB doses, we inhibited Sema3A/NP1 interaction by systemic treatment with an antibody that blocks specifically the Sema3A-binding domain of NP1 ($NP1^A$, YW64.3, Genentech), but doesn't interfere with VEGF interactions (14), while also abrogating VEGF signaling by Aflibercept treatment to determine the fraction of newly induced vessels that had successfully stabilized independently of Sema3A signaling.

Adult SCID mice were implanted with myoblast clones expressing high VEGF and PDGF-BB (VIP High), i.e. the condition with the fastest PDGF-BB induced stabilization, or low doses of VEGF alone (V Low), i.e. the condition with the most efficient stabilization in the absence of PDGF-BB, as well as control myoblasts. Animals were treated systemically with 10mg/kg of anti-$NP1^A$ antibody by i.p injection three times a week (15), or with control IgG2A. In addition, mice were treated intraperitoneally with 25mg/kg Aflibercept or saline twice, 4 and 2 days before sacrifice at day 21, as in the previous experiments (Fig. 7A). Both doses induced similarly normal and pericyte-covered capillary networks, as expected (Fig. 7B-H). After 3 weeks Aflibercept treatment showed that 68% and 96% of the vessels induced by V Low and VIP high, respectively, were stable (Fig.7 C, E and I), confirming the previous results described in Groppa et al. 2015 (7) and in Fig. 2 above.

Treatment with the NP1[A] antibody significantly decreased the fraction of persistent vessels in both cases, by 60% with VIP High and by 30% with V Low (Fig. 7J). Interestingly, the vessel fraction that could stabilize independently of Sema3A/NP-1 signaling was the same in both conditions, accounting for about 30% of newly induced angiogenesis. These results demonstrate that Sema3A/Neuropilin-1 signaling on NEM is required for the accelerated vessel stabilization induced by PDGF-BB co-delivery.

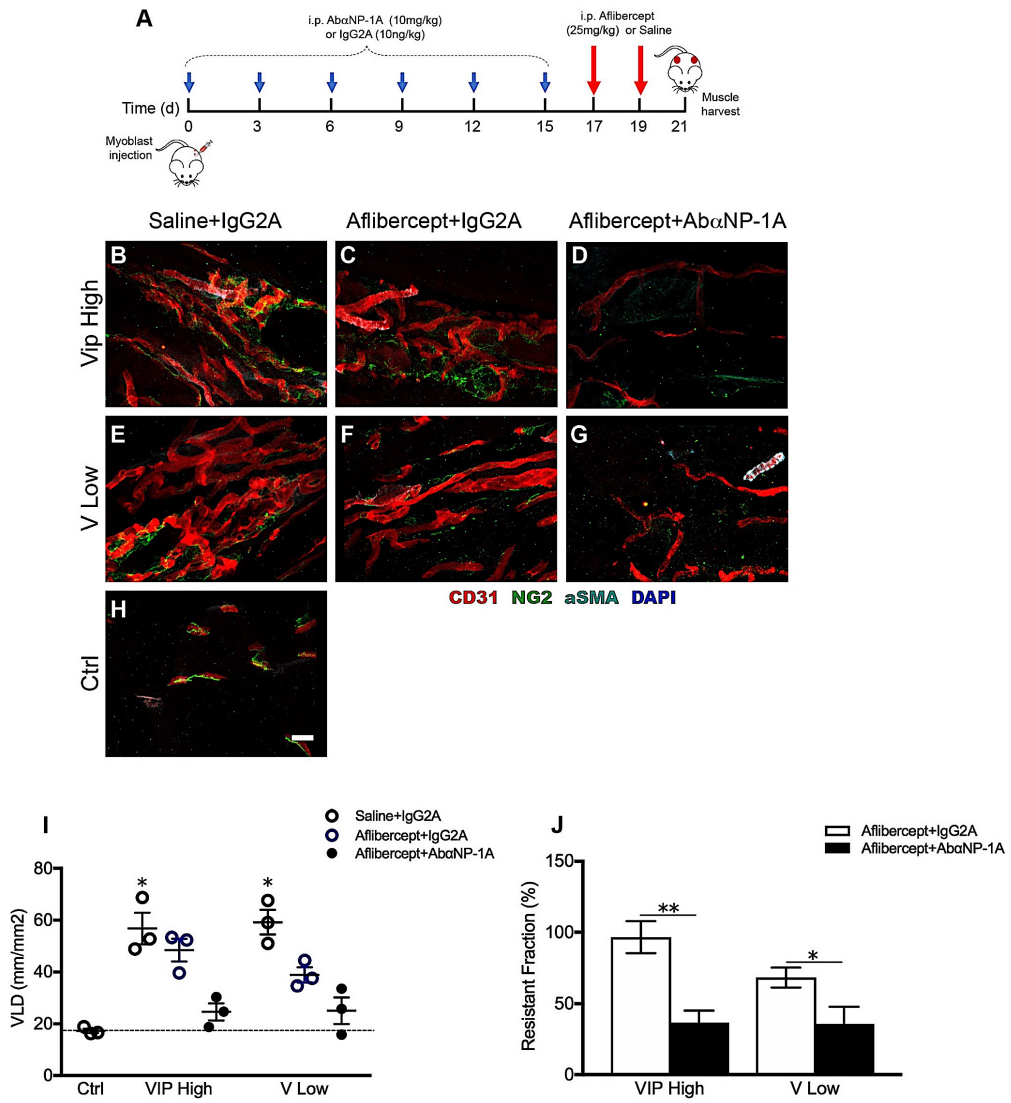

Figure 7. **Sema3A/NRP-1 signaling is required for vascular stabilization by PDGF-BB.** Mice were injected with VIP High, V Low and with control myoblast and treated with AbαNP-1A and Aflibercept as showed in the diagram (A). Mice were treated with the two blocking antibodies in order to assess the implication of Sema3A/TGF-β1/NEM axis in PDGF-BB stabilization effect. Immunostaining of the endothelium (CD31, red) and pericytes (NG2, green), α-smooth muscles cells (aSMA, cyan) on cryosections of muscles showed a decrease in VLD after VEGF-Trap and NP1 blocking (B-H). Scale bar= 20μm; Quantification of vessel length density (VLD) and resistant fraction at 3 weeks revealed a significant reduction of the fraction of stable vessels after the treatment with AbαNP-1A and Aflibercept compare to the treatment with Aflibercept and IgG2A (I-J). Data were represented as the mean ± SEM of individual's muscles. **P* < 0.05, ***P* < 0.001 by ANOVA Kruskal-Wailles with Dunn's multiple comparisons test.

The effects of Sema3A/NP1 interference on the Sema3A/NEM/TGF-β1 feedback loop were evaluated after a 1-week course of treatment with the anti-$NP1^A$ antibody in the presence of high VEGF and PDGF-BB, as described (7). The inhibition of Sema3A/NP1 signaling essentially abolished NEM recruitment induced by VIP High compared to controls (Fig. 8A-E). As expected, the loss of NEM recruitment caused a down-regulation of TGF-β1 and also of Sema3a expression in total muscles after $NP1^A$ treatment (Fig. 8F-G). The loss of Sema3A expression after the inhibition of Sema3A/NP1 binding was confirmed at the protein level, quantified by immunostaining on frozen sections (Fig. 8H-M). Taken together, these data suggest that PDGF-BB co-delivery accelerates stabilization of VEGF-induced angiogenesis by stimulating Sema3A expression and activating the Sema3A/NEM/TGF-β1 axis and the previously described TGF-β1/Sema3A positive feedback loop (7).

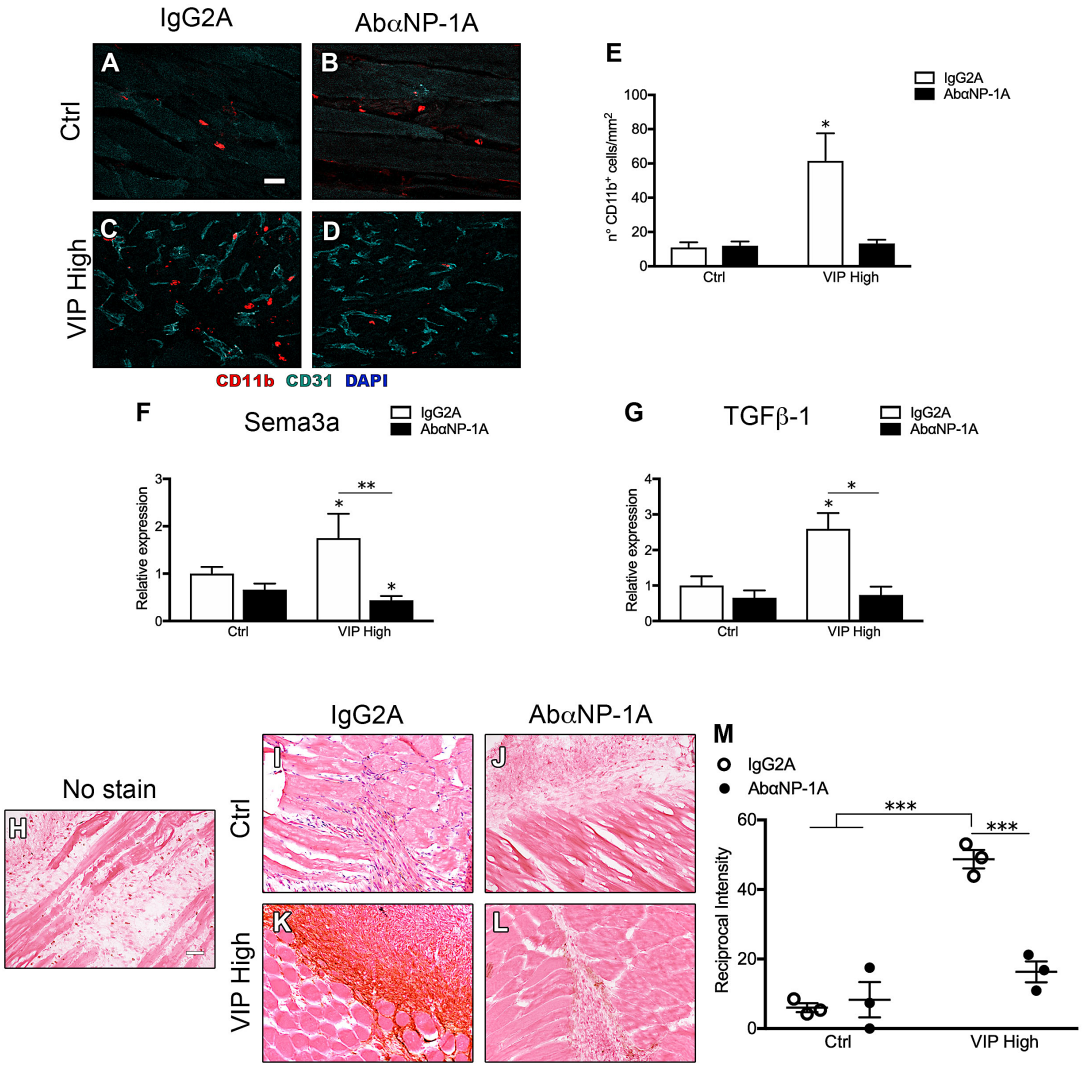

Figure 8. **Sema3a/Neuropilin-1 biding is required to maintain Sema3a/TGF-β1/NEM axis in presence of high doses of VEGF and PDGF-BB.** Immunofluorescent staining for NEM (CD11b, red) and endothelium (CD31, cyan) at 1 week on cryosections from muscles injected with VIP High and control cells. Mice were treated with AbαNP-1 or with control IgG2A (A-D). Scale bar= 20μm; A reduction in NEM recruitment in the area of neo-angiogenesis was observed and confirmed by quantification of CD11b⁺ cells/mm² (E). Decrease in *Sema3a* and *TGFb1* expression in total muscle after AbαNP-1 treatment (F-G). Immunohistochemistry of Sema3A on frozen muscle sections confirmed total protein reduction in the tissues after blocking of Sema3a/NP-1 binding (H-L). Quantification of Sema3A protein intensity in the tissue (M). Scale bar= 50μm; All data are represented as mean ± SEM of individual's muscles. NEM and ICH Sema3A quantification were subjected to ANOVA Kruskal-Wailles with Dunn's multiple comparisons test. $*P < 0.05$, $***P < 0.0001$. RT-PCR data were instead subjected to ANOVA with Bonferroni multiple comparisons test, after data normalization by logarithmic transformation. $*P < 0.05$, $**P < 0.001$.

PDGF-BB maintains endothelial Sema3a expression despite high VEGF

We have recently found that endothelial cells are the main source of Sema3A production during VEGF-induced angiogenesis and that endothelial Sema3A is down-regulated by increasing VEGF doses (7). To investigate the effect of PDGF-BB co-delivery on endothelial Sema3A, its expression was measured from endothelial cells isolated *ex-vivo* from tissues exposed to different VIP doses. FACS-sorted $CD31^+$ cells expressed similar levels of *Sema3a* in all conditions (Fig. 9A). Therefore, the robust increase in Sema3A detected in the VIP High tissues was not observed in $CD31^+$ cells, suggesting that endothelium might not be the only source of Sema3A in these conditions. However, it should also be noticed that the loss of endothelial *Sema3a* expression that would be expected in the presence of increasing VEGF doses was prevented by PDGF-BB co-delivery, suggesting that PDGF-BB may also regulate Sema3A expression directly in endothelial cells.

In order to investigate this hypothesis, Human Dermal Microvascular Cells (HDMEC) were stimulated for 24 hours *in vitro* with two different high doses of VEGF alone (10 and 40 ng/ml, respectively), or together with PDGF-BB at a fixed 1:4 ratio (2.5 and 10 ng/ml of PDGF-BB, respectively), or with the same doses of PDGF-BB alone. *Sema3a* expression was quantified by qRT-PCR after cell stimulation. Both high levels of VEGF caused *Sema3A* down-regulation *in vitro* to less than half of the control levels, as previously described (7). However, increasing doses of PDGF-BB alone significantly up-regulated *Sema3a* compared to controls, showing that PDGF-BB can directly regulate Sema3a expression by endothelial cells (Fig. 9B). Furthermore, co-stimulation with VEGF and PDGF-BB restored *Sema3a* expression above control levels despite high VEGF, consistently with the results obtained with *ex vivo* isolated endothelium (Fig. 9B).

These data suggest that VEGF and PDGF-BB exert an opposite regulation on Sema3A expression in endothelial cells. In order to start investigating the mechanism of this interaction, we asked whether PDGF-BB may interfere with VEGF-R2 activation by VEGF. HDMEC were treated with 40 ng/ml of VEGF alone or together with 10 ng/ml of PDGF-BB and VEGF-R2 phosphorylation at the key tyrosine residue Tyr1175 was quantified by ELISA. The results (Fig. 9C) show that PDGF-BB co-delivery did not interfere with VEGF-R2 phosphorylation by VEGF, suggesting that VEGF and PDGF-BB might regulate endothelial Sema3A expression indirectly through downstream signaling cascades.

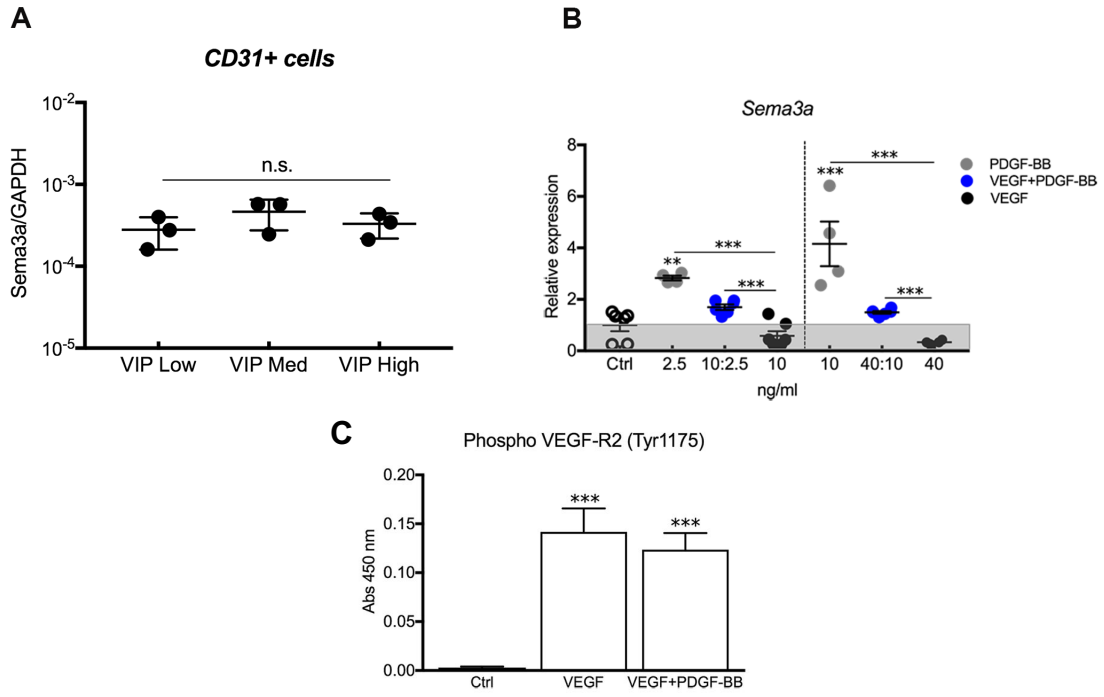

Figure 9. **Sema3a is restored in endothelial cells by PDGF-BB despite high VEGF levels.** CD31-positive cells were isolated by FACS sorting from muscles injected with VIP clones. Gene expression data relative to GAPDH showed that Sema3A was similarly expressed by endothelial exposed to increasing doses of VEGF and PDGF-BB (A). Primary human dermal microvascular endothelial cells (HDMEC) were stimulated with increasing doses of VEGF, PDGF-BB alone and VEGF+PDGF-BB for 24 hours. Sema3A expression was quantified by RT-PCR and normalized for non-treated cells. VEGF inhibited Sema3A expression whereas PDGF-BB up-regulated it. Co-expression of PDGF-BB restored Sema3A expression by endothelial cells (B). Data represent the mean ± SEM of individual values. All data were subjected to ANOVA with Bonferroni multiple comparisons test, after data normalization by logarithmic transformation. ***$P < 0.0001$. HDMEC were treated with VEGF alone or in combination with PDGF-BB, VEGF-R2 phosphorylation was quantified by ELISA. PDGF-BB co-stimulation did not affect VEGFR phosphorylation by VEGF (C). Data represent the mean ± SEM of individual values. Data were subjected to ANOVA with Bonferroni multiple comparisons test, after data normalization by logarithmic transformation. ***$P < 0.0001$.

PDGF-BB dose-dependently increases non-endothelial sources of Sema3a

Gene expression data from *ex vivo*-sorted endothelial cells, as well as from *in vitro* studies, demonstrated that PDGF-BB prevents Sema3A down-regulation by high VEGF levels. Nevertheless, endothelial Sema3A expression was not significantly increased with increasing VIP doses, contrary to the total Sema3A amount quantified in total muscles (Fig. 4). In order to identify potential non-endothelial sources of Sema3a expression and their relative contribution compared to endothelium, we performed fluorescent in situ hybridization (FISH) on frozen section to co-detect *Cd31* mRNA and *Sema3a* mRNA. The total amount of cells expressing both *Sema3a* (red signal) and *Cd31* mRNA (green signal) or only *Sema3a* was quantified in tissues harvested one week after implantation of VIP Low, Medium and High myoblast clones. As shown in Fig. 10, the majority of the *Sema3a* transcript was detected in EC (about 200-500 cells/mm^2) compared to non-endothelial cells, ranging from about 50 to 150 cells/mm^2. However, the amount of Sema3a-expressing endothelial cells did not show a clear dose-dependent trend (Fig. 10M, p=n.s.), confirming the *in vitro* and *ex vivo* results above. On the contrary, the non-endothelial fraction of *Sema3a* expressing cells was clearly expanded by increasing VIP doses (Fig. 10N; $p<0.05$), accounting for about ⅓ of the total Sema3a-expressing cells (≈150 cell/mm^2) in the presence of high levels of VEGF and PDGF-BB, which induce the fastest vascular stabilization. Furthermore, we observed from FISH images that the non-endothelial cells expressing *Sema3A* were located in proximity of endothelium (Fig. 11), suggesting a peri-vascular identity.

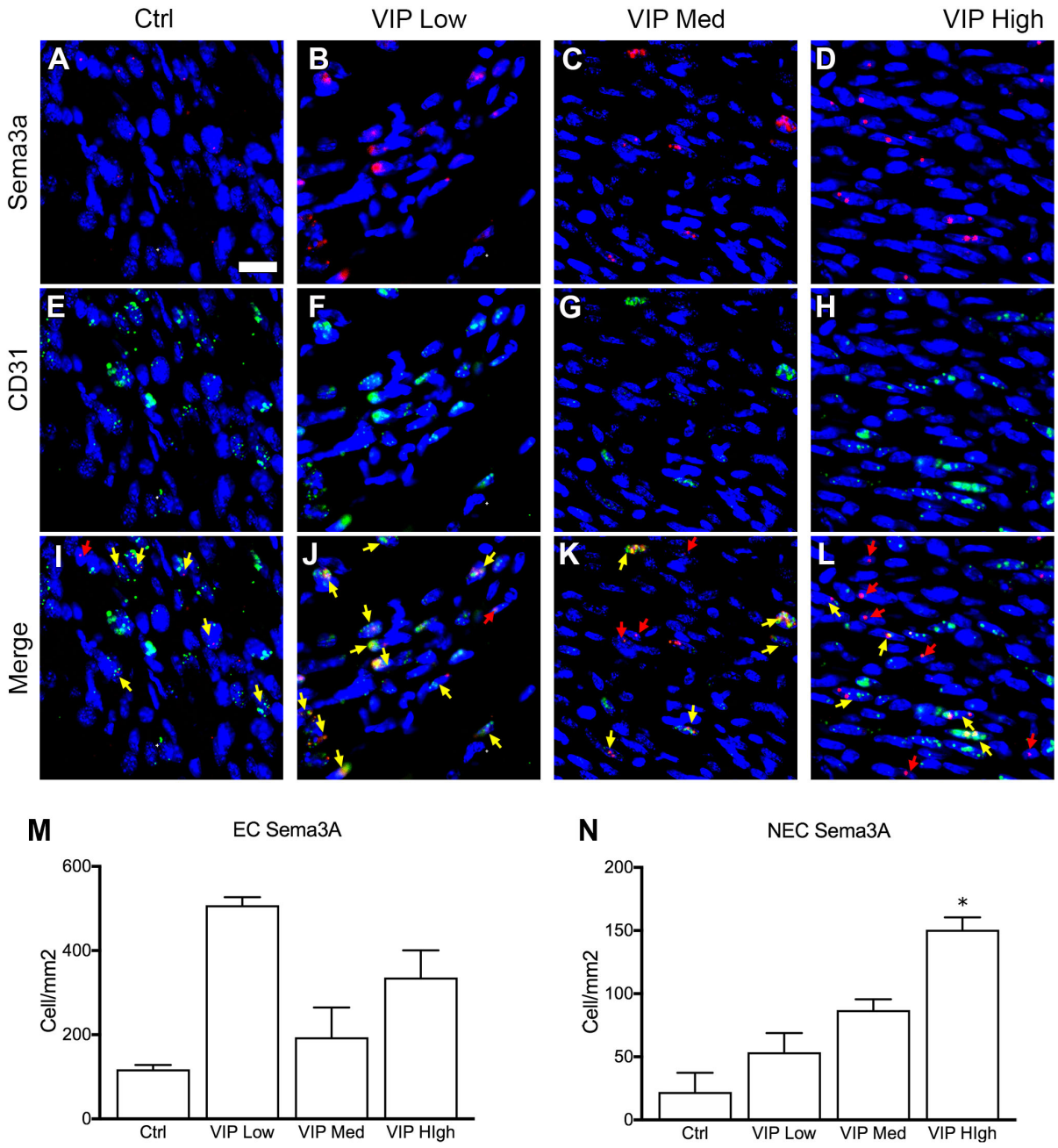

Figure 10. **Sema3a is expressed manly by the endothelium.** In situ hybridization for *CD31* transcript (green) and *Sema3a* transcript (red) on frozen muscles injected with increasing doses of VEGF+PDGF-BB. Arrows indicates cells expressing either transcripts (yellow) or only Sema3A (red) (A-L) Scale bar= 20µm; Quantification of the total number of endothelial cell (EC) or non-endothelial cells (NEC) expressing Sema3a in the area of effect (M-N). Data represent the mean ± SEM of 10 individual fields of view from independent muscles (n=3). *$P < 0.05$ Data were subjected to ANOVA Kruskal-Wailles with Dunn's multiple comparisons test.

In conclusion, these data show that PDGF-BB increases non-endothelial sources of Sema3a expression, but not endothelial ones. The cellular identity of non-endothelial Sema3A sources, as well as the effects of PDGF-BB on their levels of Sema3A expression and the underlying mechanisms, remain to be investigated.

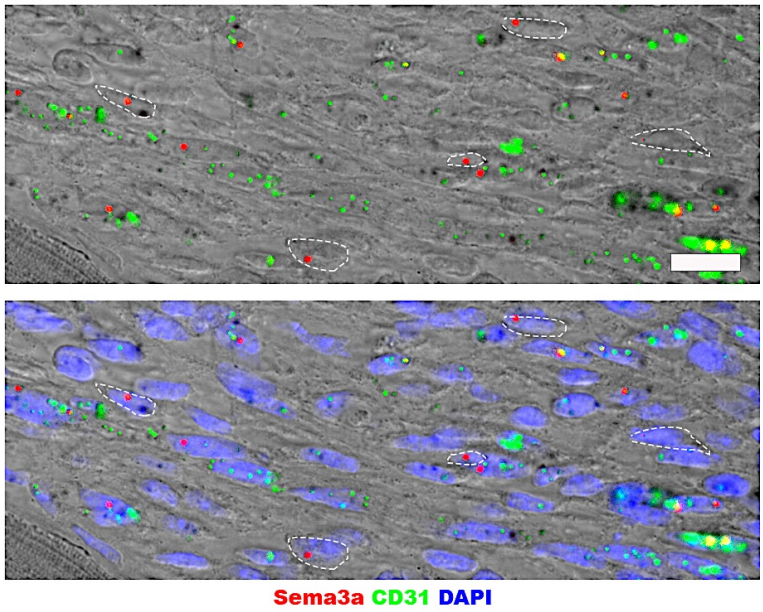

Figure 11. **Sema3A-expressing NECs are located in proximity of the vessels.** In situ hybridization (for *CD31* and *Sema3a)* images at higher magnification. NEC source expressing Sema3A seems to be located near CD31-positive cells (marked by the dash line). Size bar=20μm

Discussion

Therapeutic angiogenesis by VEGF gene therapy aims to promote the formation of functional vessels properly connected to the pre-existing vasculature. VEGF therapeutic potential is challenged by the need to control both dose and duration of expression. Moreover, it has been shown that sustained and uncontrolled levels cause the growth of angioma-like tumors, but transient delivery shorter than about 4 weeks is insufficient for stabilization and persistence of induced vessels (4). We previously demonstrate that VEGF and PDGF-BB balanced co-expression from a single bicistronic vector switch the formation of vascular tumors induced by VEGF overexpression into normal capillaries overcoming VEGF dose limitation (8). Recently it was found that increasing VEGF doses impair vessels stabilization by inhibiting the endothelial Semaphorin3A/Neuropilin1-expressing monocytes (NEM)/TGF-β1 paracrine axis (7).

Here we found that PDGF-BB dose-dependently accelerates stabilization of VEGF-induced vessels, without affecting pericyte recruitment, but rather by stimulating the Sema3A/NEM/TGF-β1 signaling axis (Fig. 12). Our data showed that PDGF-BB co-delivery significantly improved stabilization of vessels induced by uncontrolled levels of VEGF (Fig. 1). Moreover, when analyzing the dose effects of PDGF-BB we observed that PDGF-BB co-expression did not change the effects at low and medium VEGF levels, but it greatly accelerated vascular stabilization at high VEGF (90% of stable vessels at 3 weeks vs 0% with VEGF alone) (Fig. 2).

The physical association between pericytes and nascent vascular tubes is crucial for vascular maturation and stabilization (23). Disruption of endothelial-pericyte associations by VEGF overexpression or genetic ablation of PDGF-B/PDGFRβ signaling during development, results in abnormal vascular remodeling, instability and regression

(24, 25). Vessels induced by increasing doses of VEGF and PDGF-BB displayed similar pericyte coverage despite clearly different stabilization kinetics (Fig. 3). Although pericytes are essential for vessel maturity, their simple physical presence on endothelial structures is not sufficient to protect vessels from regression after VEGF withdrawal. Therefore pericyte-independent mechanisms exist to regulate the stabilization of newly formed vessels.

Several lines of evidence show that blood flow shear stress and blood pressure affect vascular remodeling and maturation (26, 27). As the establishment of blood flow can favor vessel maturation and consequently their stabilization, the rate of vascular perfusion was quantified at increasing doses of VEGF and PDGF-BB, but no differences could be observed among the conditions (Fig. 4).

In recent years, Sema3A role in angiogenesis has garnered growing interest, as it is important for endothelial cell migration and survival *in vitro* as well as for tumor-induced angiogenesis *in vivo* (28). Moreover, several reports have demonstrated that: I) Sema3A signaling through neuropilin results in a greater pericyte migration and vascular remodeling while normalizing tumor angiogenesis (29, 30); II) Sema3A is a chemoattractant for circulating monocytes called NEM (neuropilin-expressing monocytes), which have been shown to favor arterial formation in a paracrine fashion (6). Previous studies in our lab revealed that low doses of VEGF enable efficient vascular stabilization by allowing high expression of endothelial Sema3A, leading to robust NEM recruitment and high TGF-β1 levels, which on one hand stabilize endothelial structures by activating Smad2/3 signaling, and on the other hand start a novel positive feedback loop to stimulate further Sema3A expression, which maintains the stabilizing signals. Conversely, high doses of VEGF impair stabilization by inhibiting the Sema3A/NEM/TGFβ1 axis (7). Here we observed that PDGF-BB co-delivery restored

Sema3A and TGF-β1 expression that normally were abolished by high VEGF doses, suggesting that PDGF-BB may accelerate vascular stabilization also by modulating Sema3A expression. The expression trends of Sema3A and TGF-β1 in the presence of increasing doses of VEGF and PDGF-BB matched the vascular stabilization pattern previously observed in conditions of low VEGF (Fig. 4). In agreement with the levels of Sema3A expression, an increase recruitment of NEM was observed in the presence of high levels of VEGF and PDGF-BB (Fig. 5).

TGF-β1 is a pleiotropic cytokine, which can either inhibit EC migration and proliferation promoting vessel stabilization through ALK5 receptor signaling via Smad2/3, or simulate endothelium activation through TGF-β/ALK1 pathway and Smad1/5 phosphorylation (31). Moreover, TGF-β dose can balance the activation state of the endothelium via activation of ALK1 or ALK5: low doses of TGF-β triggers ALK1 activation and stimulate EC proliferation and migration, while high doses of TGF-β inhibit these responses via ALK5 (32). Consistently, in the presence of high doses of VEGF and PDGF-BB, which showed a faster vascular stabilization, TGF-β1 was highly upregulated and it promoted Smad2/3 phosphorylation and upregulation of Smad2/3 specific downstream gene PAI-1, thereby stimulating vessel stabilization (Fig. 6). Increased endothelial cells quiescence with VIP high was additionally confirmed by Ki67 staining.

To further confirm that the Sema3A/NEM/TGF-β1 axis is responsible for the PDGF-BB-induced acceleration of vessel stabilization we inhibited Sema3A-mediated recruitment of NEM by preventing Sema3A interaction with the co-receptor neuropilini-1 on NEM. The blocking antibody anti-NP1^{A} specifically interferes with the binding between NP-1 and Sema3A, but not with VEGF (14, 15). The condition with low VEGF levels, which has been shown to promote the fastest stabilization under conditions of

VEGF alone through the Sema3A/NEM/TGF-β1 axis, was used as positive control. Blocking of Sema3A/NP-1 binding drastically reduced vascular stabilization by both low VEGF alone and even more in the presence of high VEGF and PDGF-BB (Fig. 7). Nearly 70% of the vessels induced by VEGF and PDGF-BB co-delivery regressed, suggesting that the Sema3A/NEM/TGF-β1 axis is crucial for PDGF-BB mediated acceleration of vascular stabilization. Interestingly, however, both conditions (V Low and VIP High) exhibited a similar fraction of resistant vessels of about 30% after Sema3A/NP-1 blockade, despite starting from rather different stabilization rates (68% and 96%, respectively). This similar fraction of Sema3A- and NEM-independent vessel stabilization is regulated by a different mechanism and it is tempting to speculate that this may be pericyte-dependent, as in fact pericyte recruitment did not vary among these conditions. Moreover, *in vivo* neuropilin-1 blockade at 7 days caused a reduction in NEM recruitment, TGF-β1 and Sema3A expression, confirming that the TGF-β1/Sema3A positive feedback loop mediates also VIP-induced vascular stabilization (Fig. 8).

Sema3A is mainly expressed by endothelial cells and it regulates endothelial cells migration and vessel remodeling (33). However, it remains to be completely elucidated how PDGF-BB regulates Sema3A expression by endothelial cells. Interestingly, increasing doses of PDGF-BB alone caused an upregulation of Sema3A expression *in vivo* even in the absence of VEGF, suggesting a possible direct regulation of Sema3A expression by PDGF-BB (Fig. 4). Consistently, PDGF-BB dose-dependently stimulated endothelial Sema3A expression *in vitro*, whereas increasing doses of VEGF impair Sema3A expression. Additionally, *in vitro* VEGF and PDGF-BB co-stimulation of endothelial cells prevented loss of Sema3A expression despite high VEGF levels (Fig. 9). Based on these observations, we reasoned that PDGF-BB may compete with VEGF in the regulation of Sema3A

expression by endothelial cells. However, VEGF and PDGF-BB co-stimulation of endothelial cells *in vitro* did not interfere with VEGF-dependent VEGFR2 phosphorylation, although it can't be excluded that PDGF-BB may act through VEGFR2 (Fig. 9C). Recently, Mamer and coworkers identified and measured novel PDGFs to VEGF-R interactions utilizing surface plasmon resonance. They proved that PDGF-BB can bind VEGFR2 with a KD=40 nM, and this cross-family interaction can expand the role of these growth factors in angiogenesis. In a cross-family signaling system, ligands can compete to bind the same receptors and ligand concentration as well as ligand-receptor binding kinetics may determine which ligand(s) is dominating the signaling (34). Moreover, PDGF-BB can physically interact with NP1 (35). Thus, additional *in vitro* assays such as blocking of VEGFR2, PDGFRα or NP1 after stimulation with PDGF-BB are necessary to better elucidate Sema3A regulation of expression by endothelial cells.

Ex-vivo endothelial *Sema3a* expression data and fluorescent *in situ* hybridization for Sema3A revealed that endothelial cells are not the only source of the glycoprotein. In fact, increasing doses of VEGF and PDGF-BB promoted Sema3A expression by a population of non-endothelial cells (NEC) (Fig. 10). Further, FISH data showed that *Sema3a*-positive NEC were localized near the vessels in the areas of effects. Recently, it has been shown that satellite cells can express Sema3A in response to hepatocyte growth factor (36). However, the non-endothelial source of Sema3A that we observed and that is stimulated by PDGF-BB remains still to be identified.

In conclusion, we showed that PDGF-BB co-delivery both prevents loss of endothelial Sema3A expression induced by high doses of VEGF and expands a non-endothelial population of Sema3a-expressing cells. These combined actions boost NEM recruitment, leading to high level of TGF-β1 in the tissue. TGF-β1 acts through Smad2/3 signaling in endothelial cells to maintain the endothelium in a quiescent state, which

favors vessels stabilization, while further stimulating Sema3A production by endothelium in a pro-stabilization positive feedback loop. PDGF-BB plays an essential role in pericyte recruitment and vessel maturation. However, here we found a novel pericyte-independent function of PDGF-BB in promoting vessel stabilization by stimulating Sema3A expression to start the NEM/TGF-β1/Sema3A stabilizing feedback loop previously described. Therefore, PDGF-BB co-delivery represents a promising strategy for therapeutic angiogenesis as it allows to overcome both main limitations of VEGF delivery: 1) PDGF-BB normalizes VEGF-induced angiogenesis expanding VEGF therapeutic window; and 2) it accelerates vascular stabilization through Sema3A up-regulation, NEM recruitment and TGF-β1 release, enabling a safe short-term VEGF gene therapy.

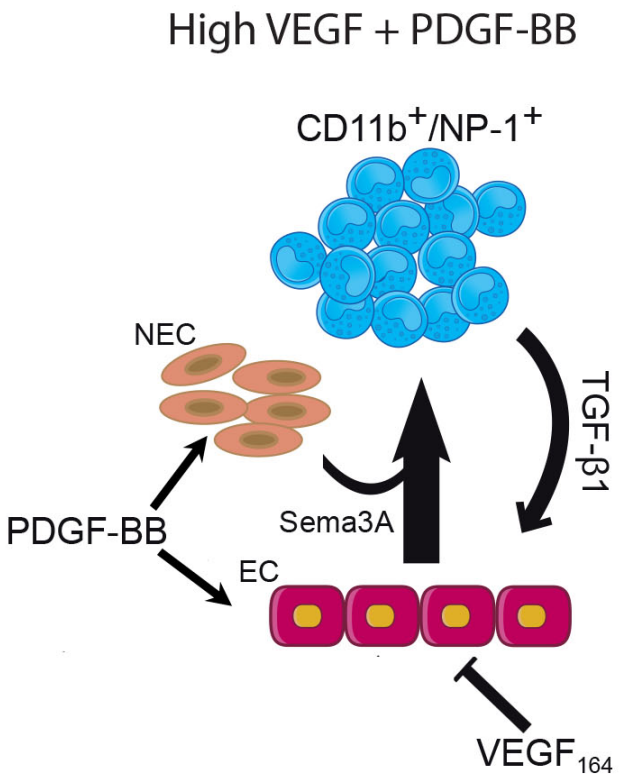

Figure 12. **PDGF-BB accelerates stabilization of VEGF-induced vessels through Sema3A/NEM/TGβ1 axis.** High levels of VEGF impair vascular stabilization, whereas PDGF-BB co-delivery restored Sema3A expression by endothelial cells and stimulate Sema3A expression by non-endothelial cells. Sema3A upregulation promote significantly NEM recruitment and TGF-β1 expression. High levels of TGF-β1 acts through ALK5 and Smad2/3 pathway to favor endothelium quiescence.

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Summary and Future prospective

VI. Summary and Futures Prospective

Peripheral artery disease (PAD) affects over 200 millions of adults and remains the leading cause of premature death worldwide. This condition is the results of atherosclerosis, in which atherosclerotic plaque can harden and narrow the arteries limiting blood flow to the limbs (1). PAD that can results in a most severe form called critical limb ischemia, characterized by pain during walking (claudication), gangrene and in some cases limb amputation (2). Although patients with PAD are treated with a combination of risk factor modification, such as anticoagulants, vasodilators, angioplasty and cathether-mediated procedures, these treatments cannot be applied to all patients. To overcome this limitation, therapeutic angiogenesis has emerged as a potential alternative to promote vascularization of poorly perfused ischemic tissues (3, 4). Therapeutic angiogenesis can be achieved via delivery of pro-angiogenic growth factors in the form of recombinant protein, gene therapy, or by cell-based delivery, such as administration of endothelial progenitor cells (5).

The delivery of Vascular Endothelial Growth Factor in ischemic tissues has been associated with improved collateral artery development, which is a key process for functional recovery (6). VEGF is the major player during the angiogenic process, as it mediates endothelial cell migration and proliferation becoming one of the most important molecular target for therapeutic angiogenesis (7). VEGF therapy has showed promising results in animal models but failed to prove significant therapeutic efficacy in clinical trials (8). Among the reasons for VEGF therapies failure we can identify two main issues: dose and duration of VEGF expression. In fact, it has been shown that its expression period should be sustained enough (4 weeks) to achieve the formation of

functional and stable vessels (9), however excessive expression of this potent growth factor rises safety concerns, as uncontrolled levels of VEGF can promote the formation of aberrant vessels and angioma-like tumors (10).

We previously found that balanced co-expression of VEGF and PDGF-BB can prevent the issues of excessive localized VEGF doses normalizing VEGF-mediated aberrant vascular growth (11). As PDGF-BB co-delivery showed to harness the potency of VEGF by preventing its toxic effects we decided to investigate whether PDGF-BB co-expression could also positively modulate the stabilization of VEGF-induced vessels.

Taking advantage of a myoblast gene-delivery system to provide heterogeneous levels of VEGF alone or with PDGF-BB we found that PDGF-BB co-expression not only normalized VEGF-induced vessels but significantly accelerate vascular stabilization. However, when analyzing the dose effects of VEGF and PDGF-BB co-delivery on the time-course of vessels stabilization we discovered that normal vessels induced by low, medium and high doses of VEGF and PDGF-BB presented similar pericyte coverage and blood perfusion but were stabilizing differently. In fact, the greatest stabilization effect was observed only at high doses of the two growth factors, demonstrating that simply pericyte coverage was not sufficient to achieve maximum vascular stabilization. Rather we found evidence for a pericyte-independent function of PDGF-BB in regulating the Sema3A/NEM/TGF-β1 signaling axis, which we previously identified to contribute to vascular stabilization after delivery of VEGF alone and to be disrupted by increasing VEGF levels (12).

Here we demonstrated that PDGF-BB restored Sema3A expression despite high levels of VEGF. Sema3A upregulation in turn promoted NEM recruitment, which exert their stabilizing effect by promoting endothelial quiescence through TGF-β1/SMAD2/3 signaling, achieving a stable vascular fraction of 90% already at 3 weeks with VEGF and

PDGF-BB co-expression. The abrogation of Sema3A binding on NEM by a specific blocking antibody resulted in a dramatic reduction of vessel stabilization, Sema3A and TGF-β1 expression as well as NEM recruitment confirming that PDGF-BB accelerate vascular stabilization by modulating Sema3A/NEM/TGF-β1 axis.

Interestingly, we discovered that in the presence of PDGF-BB co-delivery Sema3A was not produced only by the endothelium, and PDGF-BB dose-dependently expanded the non-endothelial population of Sema3A-expressing cells, while it did not affect the amount $Sema3A^+$ endothelial cells. Therefore, it will be interesting to determine which non-endothelial population/s are a PDGF-BB-responsive source of Sema3A. Therefore, the isolation of endothelial cells, pericytes, satellite cells and myeloid cells from muscles injected with different VEGF and PDGF-BB doses, and the subsequent analysis of Sema3A expression by qRT-PCR, will allow us to define quantitatively and qualitatively the source of the glycoprotein. On the other hand, little is known of how PDGF-BB restores and regulate Sema3A expression by endothelial cells. Therefore, it is necessary to assess the mechanism of PDGF-BB regulation of Sema3A expression in endothelial cells *in vitro*.

Several lines of evidence suggest that Sema3A can mediate pericyte recruitment, thereby reducing tumor growth and normalizing tumor angiogenesis (13). Furthermore, Jurisic *et al* showed that Sema3A signaling can also modulate lymphatic vessel maturation, as Sem3A is expressed by lymphatic endothelial cells and promotes perivascular cell migration though neuropilin-1. In vivo blockade of Sema3A binding on neurpilin-1 resulted in the formation of aberrant lymphatic vessels with impaired lymphatic flow (14). Thus, it will be interesting to study if Sema3A can modulate pericyte biology and investigate if the gene expression profile of pericyte is changing at increasing doses of PDGF-BB and Sema3A.

As a future prospective, PDGF-BB balanced co-delivery can be exploited to accelerate vascular stabilization providing a safe and efficient strategy to achieve short-term therapeutic angiogenesis for example using viral vector delivery. Importantly, the ratio between Sema3A and VEGF is crucial, in fact Sema3A overexpression at more than physiological levels through adeno-associated viral delivery (AVV) has been shown to inhibit VEGF-mediated angiogenesis (15). Therefore, Sema3A intrinsic up-regulation by PDGF-BB might allow to better control the effects of Sema3A only leading to prominent formation of normal and stable vascular structures.

In conclusion, here we demonstrated that PDGF-BB not only promotes pericyte recruitment during vessel maturation and stabilization, but it can also dose-dependently favor vascular stabilization independently of pericytes through Sema3A expression and the NEM/TGF-β1 axis. These results confirm that PDGF-BB co-delivery has the potential to overcome the limitations of VEGF gene delivery (dose and duration), providing a rational strategy to improve the clinical outcome of patients with limb ischemia.

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