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Possible novel targets for therapeutic angiogenesis

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An increasing number of studies about the molecular basis of angiogenesis are rapidly disclosing novel signal pathways involved in the blood vessel formation process. This review will focus on bone morphogenic proteins, Hedgehog, Notch, ephrins, neuropilins, neurotrophins and netrins. These recently discovered angiogenesis mediators are involved in vascular development during embryogenesis and, interestingly, they are shared between the nervous and vascular systems. They represent new potential targets in the vasculature and suggest novel therapeutic opportunities.

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Introduction

The concept of therapeutic angiogenesis evolved from pioneering work by Folkman, who documented the dependency of cancer growth upon neovascularization and suggested the existence of proangiogenic growth factors (GFs) [1]. The first identified proangiogenic GFs, which belong to the families of vascular endothelial growth factors (VEGF) and fibroblast growth factors (FGFs), were soon exploited by cardiovascular scientists to test the hypothesis that stimulating angiogenesis by therapeutically overexpressing GFs could improve perfusion and function in ischaemic situations, including limb ischaemia, myocardial infarct and cutaneous ulcers [2]. A large number of vascular GFs have now been identified. This article aims to give an overview on recently described angiogenic pathways, most of which were initially identified in embryonic vascular development and differentiation. Interestingly, these pathways impact on the development, survival and regeneration of both the vascular and nervous systems. Therefore, their pleiotropic capacity makes them interesting therapeutic targets.

Bone morphogenic proteins (BMPs)

BMPs belong to the transforming growth factor (TGF)-superfamily and signal through cell surface complexes of type I and type II serine/threonine kinase receptors. Once activated, these kinases form heterodimers and mediate intracellular signaling through Smad proteins. BMP activity is modulated by extracellular binding proteins, such as BMPER (BMP endothelial cell precursor-derived regulator) and noggin.

BMPs were initially described to induce ectopic bone formation and control axis development and organogenesis during embryogenesis [3]. Recent evidence highlights the central role of BMPs in vascular development. Several BMPs have been identified in mammals. BMP2/BMP4 group appears the most important for cardiovascular development. *BMP4*$^{-/-}$ mouse embryos die mostly around ED7.5 with defects in mesoderm formation and patterning. The few surviving embryos die at ED9.5 (time of vascular formation) and display a vascular phenotype with a reduced number of blood islands. These observations suggest that BMP4 is necessary for endothelial progenitor cell (EPC) differentiation [4]. Knocking out either *Smad5* or *Smad1* results in embryonic death around midgestation, due to several vascular defects [5–7]. BMPs are also involved in postnatal neovascularization. BMP4, via BMPER interaction, induces *in vitro* migration of endothelial cells (EC) and increases capillary network density in the *in vivo* chick embryo chorioallantoic membrane (CAM) and matrigel plug assays [8•]. BMP4-induced angiogenesis is mediated by ERK1/2 [9]. BMP2/4 may also be involved in vasculogenesis. In fact, Smadja *et al.* documented that BMP2/4 stimulates proliferation, migration and tube formation capacities of endothelial colony-forming cells (ECFCs), a bone marrow (BM)-derived population with a strong vessel-forming potential. Moreover, BMPs are required for human progenitor cell commitment to the endothelial lineage. Also, noggin (BMP endogenous antagonist) significantly attenuated ECFCs growth from mononuclear cell cultures [10].

Hedgehog (Hh)

Hh family was originally identified in *Drosophila* as a crucial regulator of cell-fate determination during embryogenesis. Hh members act as morphogens by regulating epithelial–mesenchymal interactions essential to limb, lung, gut, hair follicles and bone formation. There are three homologues of the *Drosophila* Hh genes in mammals: Sonic hedgehog (*Shh*), Desert hedgehog (*Dhh*) and Indian hedgehog (*Ihh*). Among them, Shh is the most widely expressed during development and Shh deficiency

induces embryonic lethality with multiple defects in early and mid gestation [11,12]. Ihh is not so broadly expressed and *Ihh*$^{-/-}$ mice survive until late gestation [13,14]. Dhh is expressed in the peripheral nerves, male gonads and EC of large vessels during development. *Dhh*$^{-/-}$ mice are viable, but display peripheral-nerve and male-fertility defects [15]. Hhs signal through interaction with the Patched-1 (Ptc1) receptor, which activates transcriptional factors belonging to Gli family.

Several evidences suggested the participation of Hhs in vascular development. *Shh*$^{-/-}$ zebrafishes reveal disorganization of EC precursors and lack to form the dorsal aorta or axial vein. *Shh*$^{-/-}$ mice display an abnormal vascularization in developing lung. Conversely, transgenic *Shh* overexpression in the dorsal neural tube in mice induces hypervascularization of neuroectoderm [16]. Shh is required for arterial differentiation. *Shh*$^{-/-}$ zebrafish embryos fail to express *ephrin-B2a* within their vasculature, while exogenous Shh induces ectopic formation of arteries by promoting *VEGF* expression [16]. Pola *et al.* showed that recombinant Shh promotes a robust neovascularization in ischaemic hindlimbs. Shh-induced angiogenesis is characterized by large-caliber vessels and is mediated by fibroblasts producing a combination of potent angiogenic factors (VEGF, Angiopoietins) [17]. By contrast, Shh inhibition downregulates VEGF and impairs post-ischaemic angiogenesis [18]. In mice with myocardial infarction (MI), *Shh* gene transfer upregulated VEGF, Angiopoietins, IGF-1 and SDF-1 and promoted neovascularization, partially by enhancing the recruitment of BM-derived EPCs in the infarcted area [19••]. Finally, Shh reportedly mitigated diabetic neuropathy by increasing the number of both epineural/perineural and endoneural capillaries and thus improving nerve blood flow in rats [20].

Notch

Notch signaling is a highly conserved pathway, implicated in cell-fate decisions and differentiation of epithelial, neuronal, bone, blood, muscle and EC. In mammals, four Notch receptors (1–4) have been described. Notch receptors interact with transmembrane ligands expressed on neighbour cells. Notch ligands are encoded by the Jagged (*Jag1* and *Jag2*) and Delta-like (*Dll11*, *Dll3* and *Dll4*) gene families. Ligand binding induces gamma-secretase-mediated Notch cleavage and the subsequent translocation of the Notch intracellular domain (NICD) in the nucleus, where it interacts with RBP-J proteins to function as a transcription factor for downstream target genes, including that of the Hes/Hey family.

The contribution of Notch to vascular biology has been appreciated only recently. Notch1 and Notch4 are predominant in EC, whereas Notch1 and Notch3 are present in vascular smooth muscle cells (VSMC). Of the five ligands, Dll1, Dll4 and Jag1 are prevalently expressed in EC, while Jag1 and Jag2 and, to some degree Dll1, are also found in VSMC.

Mutations in Notch signaling alter vascular development, at multiple steps and to various degrees. Loss-of-function studies documented the importance of Notch at the stage of vascular remodelling, when the primitive plexus evolves into a hierarchic network. *Notch1*$^{-/-}$ murine embryos die by ED9.5 with defects in somitogenesis and severe cardiovascular anomalies [21,22]. Endothelium-specific *Notch* deletion is lethal at ED10.5, displaying profound vascular abnormalities in placenta, yolk sac and embryo [23]. *Notch3*$^{-/-}$ mice are viable and fertile, but they show enlarged arteries with abnormal distribution of elastic laminae, suggesting the importance of *Notch3* for the differentiation and acquisition of VSMC arterial identity [24]. *Notch4* inactivation did not generate observable vascular defects. However, *Notch1*/*Notch4* double knockout mice exhibit a more severe vascular phenotype than *Notch1*$^{-/-}$, indicating that Notch1 and Notch4 may have overlapping roles in vascular remodelling and morphogenesis during development [22]. *Dll4*$^{-/-}$ embryos die at E9.5. Similar to *Notch1*$^{-/-}$, they fail to properly remodel the primitive vascular plexus. In addition, their phenotype has other similarities with *Notch1*$^{-/-}$, including stenosis of the large arteries and defective arterial branching [25–27]. *Jag1*$^{-/-}$ mice die by E10.5, failing to remodel the primary vascular plexus to form the large vitelline blood vessels, a process that normally occurs by angiogenesis.

Several studies document a primary function of Notch signaling in regulating arteriovenous differentiation of EC during vascular development. Notch signaling-deficient zebrafish embryos exhibited a loss of expression of arterial markers from arterial vessels with an accompanying expansion of venous markers into normally arterial domains. Zebrafish embryos in which Notch signaling had been ectopically activated presented the reverse phenotype: suppression of vein-specific markers with ectopic expression of arterial markers in venous vessels [28]. Mutations in Notch members are responsible for certain late-onset hereditary vascular anomalies in humans. Alagille syndrome (AGS) is an autosomal dominant disorder that has been attributed to *Jag1* mutations. AGS patients exhibit abnormal blood vessels, arterial stenosis and heart disease, in addition to hepatic lesions and skeletal defects [29]. CADASIL is a disease characterized by strokes, migraines and progressive dementia. It has been linked to a *Notch3* mutation, resulting in progressive degeneration of the VSMC layer surrounding cerebral and skin arterioles [30,31].

A crucial role of Notch has also been demonstrated in postnatal angiogenesis. EC-selective *Notch1* deletion impairs post-ischaemic angiogenesis in limb muscles.

In this model, Notch1 angiogenic action is regulated by VEGF, as VEGF, via Akt, increases presenilin proteolytic processing, gamma-secretase activity, Notch1 cleavage and Hes1 expression in EC [32•].

Ephrins and Eph receptors

The ephrin ligand/Eph receptor family is widely expressed in embryonic and adult tissues. Its functions are best characterized in the nervous system, where it is involved in patterning the developing hindbrain rhombomeres, axon pathfinding and guiding neural crest cell migration [33].

Eph receptors are tyrosine kinases, divided in two subclasses, A (EphA) and B (EphB), depending on the type of interaction with the epharin ligands. In general, EphA bind to GPI anchored ephrin ligands (ephrin-A), while EphB bind to ephrin ligands containing transmembrane domains (ephrin-B) [34]. Ephrin–Eph-mediated signaling functions bidirectionally; after cell contact-mediated binding of the ligand, the Eph tyrosine kinases become clustered and phosphorylated, which leads to recruitment of signaling effectors and activation of signal-transduction cascades.

The involvement of ephrin–Eph molecules in vascular development is considerable. In fact, they have an essential role in the maturation and remodelling of the arterial–venous plexus. Ephrin-B2 expression specifically in the arterial and EphB4 in the venous endothelium represent one of the earliest known molecular distinctions between arteries and veins. Disruption of either gene leads to failure in the remodelling of the primary capillary plexus and in the formation of major embryonic vessels [35–37]. Interaction between ephrin-B2 and EphB4 at the arterial–venous interface is required to provide repulsion signals for the establishment and maintenance of boundaries between these vessels [35,37]. Furthermore, ephrin/Eph interaction between mesenchymal cells and EC mediates repulsive guidance for migrating EC during the formation of intersomitic vessels [37,38]. *In vitro* studies support the angiogenic nature of ephB/ephrin-B signaling. In cultured microvascular EC, EphB4 activation by ephrin-B2 triggers sprout formation as efficiently as VEGF or angiopoietin-1 [37]. Moreover, ephrin-B2 expression in cultured human microvascular ECs and arterial ECs can be induced by VEGF, bFGF and HGF (hepatocyte GF) [39].

The role of Ephrins/Eph receptors in therapeutic angiogenesis has been only partially explored. EphrB2 expression is higher at the site of reparative neovascularization in murine ischaemic muscles [40]. Importantly, intraperitoneal administration of ephrin-B2 in MI mice enhanced EC proliferation and increased capillary density in the periinfarcted area [40]. Moreover, EphB4 activation, via an ephrin-B2–Fc chimeric protein, enhanced the proangiogenic capacity of EPC in a mouse model of hindlimb ischaemia. In fact, ischaemic nude mice undergone ephrin-B2–Fc-treated EPC injection displayed increased capillary and vessel densities as well as improvement in blood flow recovery compared with the untreated mice [41•].

Neuropilins (NRP)

NRP-1 and NRP-2 are transmembrane proteins initially identified as receptors for class-3 semaphorin subfamily. They are involved in neuronal cell guidance and axonal growth during development of the nervous system, exerting a repulsive/inhibitory effect on neuronal growth cones. Neuropilins are also involved in angiogenesis and cardiovascular functions and they can bind to certain isoforms of VEGF. These observations place the NRPs at the heart of the cross-talk between the nervous and the vascular systems.

Both NRP1 and NRP2 are expressed in EC [42,43], where NRP1 enhances $VEGF_{165}$ binding to VEGF receptor 2 (VEGFR2), thus improving EC migration [44]. By contrast, sema3A inhibits VEGF-induced EC migration and sprout formation due to competition with $VEGF_{165}$ for binding to NRP1 [45]. *NRP* null mutation is lethal in mice. $NRP^{-/-}$ embryos presented severe neuronal alterations as well as deficiencies in neuronal vascularization, aortic arch malformations and diminished and disorganized yolk sac vascularization [46]. By contrast, *NRP1* trasngenic murine embryos showed hypercapillary formation, dilated blood vessels, haemorrhage and malformed hearts and limbs [42]. $NRP2^{-/-}$ mice are viable, with normal development of larger blood vessels, but displaying a severe reduction of small lymphatic vessels and capillaries [47]. Double $NRP1^{-/-}/NRP2^{-/-}$ mice had a more severe vascular phenotype than each single knockout. They died very early in utero and exhibited defective blood vessel development, including a lack of blood vessel branching in yolk sacs, failure in capillary formation and avascular embryos [48].

Little is known about the effect of Neuropilins in therapeutic angiogenesis. An *in vivo* study reported that newly formed vessels in healing dermal wounds abundantly express NRP1 and that mice treated with anti-neuropilin-1 antibodies exhibit a decrease in vascular density within these wounds. *In vitro*, VEGF-induced cord formation and EC migration was inhibited by antibodies directed against NRP1 [49]. Additional investigations are necessary to fully understand Neuropilins potential for therapeutic angiogenesis.

Neurotrophins (NTs)

NTs are a family of highly conserved proteins consisting of four different members: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), NT-3 and NT-4/5. NTs actions are mediated via binding to two

classes of receptors: tropomyosin-like kinases (trkA, trkB and trkC), which are tyrosin-kinases, and $p75^{NTR}$, a member of the TNF receptor family. Trk receptors exhibit ligand specificity with NGF binding to trkA, BDNF and NT 4/5 to trkB and NT-3 to trkC. Conversely, $p75^{NTR}$ binds all mature NTs with equal but low affinity, while it has higher affinity for pro-NTs [49,50]. NTs are best known for their actions on the nervous system [51]. Nevertheless, EC constitutively express trks and respond to NTs with improved survival, proliferation and migration [52]. Studies performed on genetically modified mice underlined the importance of NTs/trk signaling for cardiovascular development. *BDNF*$^{-/-}$ mice exhibited impaired EC survival and EC cell to cell contacts in intramyocardial arteries and capillaries, leading to intraventricular wall haemorrhage, depressed cardiac contractility and early postnatal death [53]. *NT-3*$^{-/-}$ mice showed defective great vessels and developmental delay in the primitive myofibril organization of the *truncus arteriosus* [54,55]. Transgenic mice overexpressing a truncated trkC receptor acting as a dominant negative displayed similar cardiovascular defects [56].

NTs exert potent effects on postnatal angiogenesis. NGF, by promoting EC proliferation, induces angiogenesis in both the cornea pocket [57] and the CAM assays [58]. The potential of NTs for therapeutic angiogenesis was demonstrated for the first time by our group using recombinant NGF in a mouse model of limb ischaemia. Repeated NGF injections in the ischaemic adductors reduced EC apoptosis, increased capillary and arteriole densities and improved blood flow recovery [59,60]. Furthermore, NGF supplementation stimulates therapeutic angiogenesis in diabetic cutaneous wounds, thus promoting cicatrization [61]. NGF-induced angiogenesis is mediated by the VEGF-Akt-eNOS signaling and MMP-2 upregulation [59,62,63]. Importantly, both NGF and trkA are upregulated by limb ischaemia to mediate the native angiogenesis response, as demonstrated by the anti-angiogenic effect of a NGF blocking antibody in this condition [59].

BDNF shares some angiogenic properties with NGF. BDNF is expressed in EC [62] and its expression is upregulated by hypoxia as well as limb ischaemia [64••,65,66] Endogenous BDNF promotes EC survival, whereas exogenously added recombinant protein stimulates *in vitro* angiogenesis, via VEGF/PI3K/Akt cascade [65]. *BDNF* gene transfer promoted blood flow recovery and increased capillary density in the mouse limb ischaemia model. Moreover *BDNF* overexpression promoted mobilization of BM-derived CD11b+ myeloid cells and haematopoietic Sca-1+ precursor cells, which express the trkB, thus suggesting that BDNF may contribute to vessel formation by vasculogenesis [64••]. Healthy capillary EC do not express $p75^{NTR}$, but receptor expression is induced by diabetes and ischaemia [60]. $p75^{NTR}$ expression promotes EC apoptosis and inhibits the angiogenesis process [67••]. We proved that $p75^{NTR}$ inhibition by local gene transfer of a dominant negative form of the receptor can be used therapeutically to restore proper reparative neovascularization in limb muscle following ischaemia in diabetic mice [67••].

Netrins

Netrins constitute a family of highly conserved secreted proteins structurally related to laminins. In mammals this family comprises three members, Netrin-1, Netrin-3 and Netrin-4. Netrins are bifunctional guidance cues in the developing central nervous system, attracting some axons, while repelling others. Attraction and repulsion are mediated by binding to receptors of the deleted in colorectal cancer (DCC) and uncoordinated five (UNC5) families. The DCC family consists of DCC and neogenin, while the UNC5 family comprises four members, UNC5A to UNC5D. Axon attraction is mediated by the DCC receptors, whereas repulsion requires signaling through the UNC5 receptor homodimers or with UNC5–DCC receptor heterodimers [68–71]. Netrins and receptors are expressed in several non-neural tissues, including EC, thus suggesting a broader role of these molecules in processes other than axon pathfinding.

The role of Netrins in the developing vascular system is uncertain, with numerous data reporting both pro-angiogenic and anti-angiogenic activities. Lu *et al.* showed that Netrin-1 is a negative regulator of capillary branching, acting via UNC5B. UNC5B is localized in developing blood vessels, and *Unc5B*$^{-/-}$ mice exhibit excessive branching at this level and excessive extension of filopodia in endothelial tip cells. Treatment of endothelial tip cells with Netrin-1 induces filopodia retraction in wild-type mice but not in *Unc5b*$^{-/-}$ mice, suggesting that Netrin-1 inhibition of vessel branching is mediated by UNC5B. Furthermore, knockdown of the netrin-1 orthologue in zebrafish generates abnormal branching in developing blood vessels [72]. By contrast, other studies suggested a pro-migratory and pro-mitogenic effect of Netrin-1 on primary EC and VSMC. According to Park *et al.*, Netrin-1 stimulates migration and proliferation of both EC and VSMC. Neogenin was found responsible for the effect in VSMC but not in EC, where *DCC*, *UNC5* or *Neogenin* were not detected. Moreover, Netrin-1-induced angiogenesis in both the CAM and corneal micropocket assays [73]. Furthermore, Nguyen *et al.* found that Netrin-1 enhanced arterial EC proliferation and migration, through a DCC-dependent increase in nitric oxide production and the feed-forward activation of ERK1/2–eNOS, [74]. Likewise, Wilson *et al.* reported that gene transfer with *Netrin-1* and *Netrin-4* facilitates post-ischaemic limb revascularization via an unknown Netrin receptor [75•].

Conclusions

This review tried to focus on some newly revealed families involved in the angiogenesis process. These

signaling pathways shape embryonic vascular development and are recapitulated in adult tissue, where they have a regulatory role on reparative angiogenesis. Interestingly, these pathways are shared between the nervous system and the vasculature. The two systems have much more in common than was originally anticipated. Indeed, as we have reported in this article, they use similar signals and principles to differentiate, grow and navigate towards their targets. Further investigations on these molecular mechanisms and their interconnections might give further hints for new approaches in therapeutic angiogenesis.

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