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The expanding role of Neuropilin: regulation of vascular TGF β and PDGF signaling

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Abstract

Purpose of review—Long recognized for its role in regulation of vascular endothelial growth factor (VEGF) signaling, neuropilin (Nrp)1 has emerged as a modulator of additional signaling pathways critical for vascular development and function. Here we review two novel functions of Nrp1 in blood vessels: regulation of transforming growth factor (TGF) β signaling in endothelial cells and regulation of platelet derived growth factor (PDGF) signaling in vascular smooth muscle cells (VSMCs).

Recent findings—Novel mouse models demonstrate that Nrp1 fulfills vascular functions independent of endothelial VEGF signaling. These include modulation of TGF β –dependent inhibition of endothelial sprouting during developmental angiogenesis and PDGF signaling in VSMCs during development and disease.

Summary—Broadening our understanding of how and where Nrp1 functions in the vasculature is critical for the development of targeted therapeutics for cancer and vascular diseases such as atherosclerosis and retinopathies.

Keywords

Neuropilin; Angiogenesis; TGF β ; PDGF; vascular smooth muscle cell

INTRODUCTION

Traditionally, neuropilin (Nrp)1 has been described as a co-receptor that amplifies and modulates vascular endothelial growth factor (VEGF) signaling in endothelial cells (1–3). In the vasculature, Nrp1 is not only expressed by endothelial cells, but vascular smooth muscle cells (VSMC), pro-angiogenic tissue macrophages, and vascular precursor mesenchymal stem cells also express Nrp1 (4–7). It has been linked to multiple vascular signaling pathways, including those driven by fibroblast growth factor (FGF), transforming growth factor- β (TGF β), and platelet-derived growth factor (PDGF) (3). The observation that

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combined pharmacological blockade of Nrp1 and VEGF additively inhibits angiogenesis strongly suggests that Nrp1 can affect blood vessels in a VEGF-independent manner (8). Recent studies have begun to examine this expanded cardiovascular signaling repertoire of Nrp1. This review will describe and discuss recent advances in Nrp1 research with a specific focus on the role of Nrp1 in endothelial TGF β signaling and VSMC PDGF signaling.

1. NRP1: STRUCTURE AND FUNCTION

The neuropilins are single-pass transmembrane proteins composed of a large extracellular domain and a short cytoplasmic domain that lacks enzymatic activity (3,9). Nrp1, and its closely related family member Nrp2, share 44% sequence homology and a similar domain structure (10). Lymphatic endothelial cells preferentially express Nrp2, whereas Nrp1 is highly expressed by blood endothelial cells and VSMCs. The function and involvement of Nrp2 in vascular signaling are still poorly understood; thus Nrp1 will be the focus of this review.

The extracellular domain of Nrp1 contains distinct subdomains (a1, a2, b1, b2, and c) that enable ligand binding. The a1 and a2 subdomains allow for binding of Class 3 semaphorins (Sema3) and the b1 and b2 subdomains contain binding sites for VEGFs, placental-like growth factor (PLGF) and heparin (11,12). The c domain along with the transmembrane domain are involved in Nrp1 oligomerization. The cytoplasmic domain of Nrp1 contains three terminal amino acids (SEA) that encode a PDZ (PSD-95/Dlg/ZO-1)-binding consensus motif enabling binding to the PDZ domain of the adaptor protein synectin [GIPC and neuropilin-interacting protein (NIP)] (13). Synectin concurrently binds the motor protein myosin VI to drive endosomal trafficking (14,15). VEGFA₁₆₅ induces association of Nrp1, VEGFR2, and synectin to form a protein complex and drive intracellular trafficking of Nrp1 and VEGFR2 through specific endosomal compartments (16–18). Consequently, the cytoplasmic domain of Nrp1 is required for proper endocytic trafficking, and thus signaling of VEGFR2 (19). In contrast, Nrp1 presented in trans, for example by tumor cells can function to limit angiogenesis by impeding endothelial VEGFR2 trafficking (20). Whether Nrp1 regulates endocytic trafficking of additional endothelial proteins remains to be determined.

Despite lacking inherent catalytic activity, Nrp1 partners with various cell receptors to modulate signaling of a number of intracellular proteins including phosphoinositide 3-kinase (PI3K)/Akt, ERK (extracellular signal-regulated kinase), p130^{cas} (Crk-associated substrate), Src (SRC Proto-Oncogene, Non-Receptor Tyrosine Kinase), and p38 MAPK (mitogen activated protein kinase) (2). As discussed in detail below, Nrp1 also modulates TGF β signaling.

2. NEUROPILIN-RELATED VASCULAR PHENOTYPES

Initial clues to the role of Nrp1 in the vasculature emerged from studies of multiple mouse models with altered Nrp1 expression and/or function (Table 1). Global Nrp1 deletion results in embryonic lethality by embryonic day (E) 14 and severe neuronal and cardiovascular defects (21,22). Mice with ectopic overexpression of Nrp1 also die embryonically and

display excessive capillary formation, as well as dilated and hemorrhagic blood vessels (7). Global Nrp2 knockout mice are viable, but display reduced lymphatic formation in the heart, dermis, and diaphragm during embryonic development (31). Mice with combined deletion of Nrp1 and Nrp2 die early at E8.5, suggesting genetic compensation between Nrp1 and Nrp2 (32).

Nrp1 has been deleted during embryonic development using several different endothelial-specific promoters. In all cases, lethality and varying degrees of impaired angiogenesis have been observed (24–26). Postnatal endothelial-specific deletion of Nrp1 impairs retinal angiogenesis (27).

Mice expressing Nrp1 with a mutated Sema3A binding site but unaltered VEGF-binding capacity, survive until birth and display normal cardiovascular development (25). Remarkably, mice harboring a Y297A mutation in the Nrp1 extracellular domain that specifically inhibits VEGF binding, but also causes Nrp1 hypomorphism, survive past birth demonstrating that VEGFA binding by Nrp1 is not required for a viable cardiovascular system (28). Mice expressing a different mutation (D320K) that also inhibits VEGFA binding but maintains normal Nrp1 expression are viable and fertile (29). They display normal angiogenesis in the developing cortex and only a mild delay in postnatal angiogenesis. Thus, VEGF binding by Nrp1 appears to be dispensable for angiogenesis. However, VEGF-binding by Nrp1 is required for arteriogenesis, as Nrp1 D320K VEGFA-binding deficient mice display reduced blood flow recovery following ligation of the common femoral artery and induction of hindlimb ischemia (29).

Likewise, mice that express a truncated form of Nrp1 lacking the cytoplasmic domain display normal angiogenesis, but impaired arteriogenesis (19,30). Endothelial cells isolated from these mice display reduced phosphorylation of the Y¹¹⁷⁵ VEGFR2 site that is responsible for phospholipase C- γ (PLC γ) due to decreased speed of VEGFR2 endosomal trafficking through the cytoplasm (19). As PLC γ is required for VEGFR2-induced activation of ERK signaling, this leads to decreased ERK1/2 phosphorylation and impaired arteriogenesis (33). Taken together, these studies demonstrate a critical role of Nrp1 in regulating VEGFR2-trafficking-dependent activation of ERK signaling, a process required for arteriogenesis (34).

3. NRP1-DEPENDENT REGULATION OF TIP CELL FORMATION

During sprouting angiogenesis, highly migratory and VEGF-responsive endothelial tip cells lead proliferative and lumenizing endothelial stalk cells to form angiogenic sprouts. Tip/stalk cell selection depends upon fluctuating VEGFR2 expression levels, governed by Notch lateral inhibition events (35,36). In the developing mouse hindbrain Nrp1 promotes tip cell formation (23). Postnatal deletion of endothelial Nrp1 causes a loss of tip cells and reduced vessel sprouting in the developing mouse retina (27). Assaying for tip cell selection in mice with incomplete endothelial-specific deletion of Nrp1 showed that endothelial cells that retained Nrp1 expression preferentially took the tip cell position (24,27). Nrp1 likely promotes tip cell selection by amplifying VEGFR2 signaling, since Nrp1 deficient endothelial cells display reduced VEGFR2 signaling (29). However, the exact mechanism by

which Nrp1 imparts the tip cell phenotype remains unclear. Based on the Nrp1 structural mouse mutants discussed above, we can assume that Nrp1 does not require its cytoplasmic domain, or VEGFA-binding capability to impart a pro-angiogenic endothelial phenotype. This leaves a possibility that Nrp1 achieves this regulation in a non-VEGF-dependent manner.

One potential means of this regulation is suggested by a small pool of data that demonstrates that Nrp1 can regulate cell adhesion in a VEGF-independent manner. In particular, Nrp1 promotes endothelial cell adhesion to laminin and fibronectin in part through regulation of integrin $\alpha 5 \beta 1$ (37,38). Integrin signaling also modulates Nrp1 function in endothelial cells. For example, integrin $\alpha v \beta 3$ inhibits Nrp1 angiogenic function and integrin $\beta 3$ regulates Nrp1-dependent focal adhesion remodeling, and thus endothelial cell migration (39,40). Since cell adhesion is required for filopodia formation and extension, integrin-Nrp1 interactions can, in part, explain non-VEGF-dependent effects of Nrp1 on tip cell formation. A second potential means of this effect is Nrp1-dependent regulation of TGF β signaling that will be considered in detail below.

4. NRP1 REGULATION OF TGF β SIGNALING

4.1 The TGF β signaling pathway

TGF β signaling components are expressed by endothelial cells and play a critical role in vascular development and vessel homeostasis (41). Ligands of the TGF β superfamily including TGF- β , bone morphogenic proteins (BMPs), and activin, bind heteromeric complexes composed of type 1 (TGF β R1) and type II (TGF β R2) serine/threonine kinase receptors (42). Mammals express four types of type 2 receptors and seven type 1 receptors (Alk1–7) (41). While TGF β R2 and Alk5 are ubiquitously expressed, Alk1 is an endothelial-specific TGF β R1 (43,44). Endothelial cells also specifically express the type III receptor (TGF β R3) endoglin, which acts as a TGF β co-receptor to promote ligand binding and TGF β signaling (45–47).

Ligand binding by TGF β R2 induces recruitment and activation of TGF β R1. Receptor regulated (R)-SMAD cytoplasmic proteins are then recruited to the plasma membrane and phosphorylated by TGF β R1 to activate canonical TGF β signaling. Specific heteromeric complexes of phosphorylated (p)SMADs bind SMAD4 (the mammalian co-SMAD) and translocate to the nucleus to mediate gene transcription (48). In endothelial cells, it is generally held that Alk1 induces phosphorylation of SMAD1/5/8 and Alk5 induces activation of SMAD2/3 (41,49,50).

Activation of TGF β receptors can also initiate SMAD-independent signaling. Non-canonical TGF β signaling includes pathways driven by P13K, ERK, mTORC (mammalian target of rapamycin), p38, JNK (Jun amino-terminal kinase), and RHO GTPases (51)

4.2 TGF β -related vascular phenotypes

Global deletion of TGF β R2, Alk5, Alk1, or endoglin causes embryonic lethality around E10.5 due to yolk sac vascular insufficiency (50,52–54). Mice with constitutive endothelial-specific deletion of TGF β R2 or Alk5 phenocopy their respective global mutants,

demonstrating that endothelial TGF β signaling is required for embryonic viability (55). SMAD4 deletion also results in early embryonic lethality by E10.5 due to cardiovascular defects (56).

Deletion of TGF β R2 or Alk5 induced at later developmental time points in order to circumvent early lethality, leads to brain hemorrhages and angiogenic defects (57–60). Combined deletion of endothelial SMAD2/SMAD3 or SMAD1/SMAD5 also causes brain hemorrhages (61,62). These findings demonstrate that TGF β signaling not only regulates vessel formation, but also ensures vessel stability.

4.3 TGF β in sprouting angiogenesis

Recent reports implicate TGF- β signaling in tip/stalk cell specification. Postnatal deletion of endothelial TGF β RII results in impaired retinal angiogenesis characterized by a blunted angiogenic front with endothelial cell clusters, and impaired formation of the deep retinal vascular plexus (59). Endothelial deletion of SMAD1/5 altered embryonic hindbrain vessel sprouts, which were observed as broad clusters of endothelial cells with numerous ectopic filopodia (62). Expression analysis has revealed elevated levels of Alk1 and increased TGF- β signaling in stalk cells, as compared to tip cells (27,62,63). BMP9 treatment of cultured endothelial cells induces phosphorylation of SMAD1/5 in an Alk1-dependent manner and inhibition of BMP9 signaling in the mouse retina increases retinal vessel density, suggesting that ALK1 functions to inhibit endothelial cell sprouting(49,63,64). *in vitro* and *in vivo* cell competition assays revealed that endothelial cells deficient for Alk1, Alk5, SMAD4, or SMAD1/5 preferentially take the tip cell position (27,62,63). Taken together, these data suggest that TGF β signaling in endothelial cells inhibits tip cell behavior and promotes the stalk cell identity

4.4 Nrp1 interaction with TGF β receptors and ligands

In cell free assays, Nrp1 binds active TGF β as well as its latent form, which is non-covalently associated with latency-associated protein (LAP) (65). Both latent and active TGF β compete with VEGFA₁₆₅ for Nrp1 binding (65). In cultured breast cancer cells, Nrp1-mediated activation of LAP-TGF β induced SMAD activation (65). It has been proposed that Nrp1 activates LAP-TGF β via a RKFK sequence located in the Nrp1 extracellular b2 subdomain (65). Nrp1 complexes with TGF β R1, TGF β R2, and TGF β R3 and similar to endoglin, Nrp1 can promote TGF β R1/2 dimerization (66,67). Nrp1 and endoglin both contain a PDZ-binding consensus motif, thus they may share similar functions in TGF β signaling.

4.5 Nrp1 regulation of TGF β signaling during angiogenesis

In angiogenic endothelial cells, Nrp1 appears to inhibit TGF β signaling. In the developing brain cortex, Nrp1-deficient endothelial cells expressed increased levels of pSMAD3, indicative of increased TGF β signaling (26). In line with these findings, deletions of Alk1 or Alk5 in Nrp1-deficient endothelial cells can rescue the tip cell phenotype (24,27). Cultured endothelial cells with Nrp1 knockdown display increased phosphorylation of SMAD2/3 when stimulated with TGF β (26,27). Nrp1 structural mutants lacking the cytoplasmic domain or PDZ-binding consensus motif have no affect on SMAD2 activation,

demonstrating that the Nrp1 extracellular domain is required for TGF β signal inhibition (27). Nrp1 effect on SMAD1/5/8 remains ambiguous, as one group reported it unaffected in HUVEC with Nrp1 knockdown treated with BMP9 or TGF β , whereas another group reported increased TGF β -induced pSMAD1/5/8 in HUVEC with Nrp1 knockdown (26,27). Thus, Nrp1 regulation of TGF β signaling may be contextually dependent and it remains to be determined if Nrp1 differentially affects Alk1 and Alk5 signaling in endothelial cells.

A recently proposed paracrine signaling axis links Nrp1, TGF β and integrin signaling during developmental angiogenesis in the brain. In this model, activation of LAP-TGF β by neuroepithelial-derived integrin α v β 8 drives TGF β signaling in endothelial cells, a process inhibited by endothelial Nrp1 (26). Furthermore, integrin α v β 3 on neuroepithelial cell and endothelial Nrp1 can form a trans-cellular complex between these cells leading to promotion of angiogenesis (26).

Taken together, these findings demonstrate that Nrp1 promotion of angiogenic sprouting is two-fold; Nrp1 amplifies VEGFR2 signaling in tip cells and Nrp1 inhibits TGF β signaling in stalk cells (Figure 1).

5. NRP1 IN VSMC BIOLOGY

5.1 PDGF signaling in VSMCs

VSMCs make up the vessel wall of mature arteries and veins where they provide structural support and regulate vessel tone. VSMCs also contribute to the development of the vascular system where their recruitment is critical for vessel remodeling and stabilization. VSMCs alternate between two different phenotypes: a de-differentiated synthetic phenotype and a more differentiated contractile phenotype (68). In established vessels, disruption of the vessel wall can induce VSMC switch from a contractile phenotype to a synthetic phenotype (69).

PDGF signaling is a potent inducer of the VSMC synthetic phenotype, as it drives VSMC proliferation, migration, and survival (70). PDGF-A and PDGF-B signal as homo- or heterodimers through two different PDGF-receptor tyrosine kinases, PDGFR- α and PDGFR- β to activate PLC γ , AKT, and ERK signaling pathways in VSMCs (71).

Endothelial-derived PDGF-BB activates PDGFR- β on VSMCs to induce their recruitment and vessel coverage. Accordingly, mice mutant for PDGF-B or PDGFR- β die perinatally and display severe hemorrhaging (72–74).

5.2 Nrp1 regulation of PDGF signaling

Nrp1 expression by VSMCs has been demonstrated in developing mouse embryos, adult vessels, and in cell culture (4,75,76). Nrp2 expression has also been documented in VSMCs (75). VSMC Nrp1 expression is elevated in response to FGF and PDGF, supporting a role for Nrp1 in regulating the VSMC mitogenic response (77–79). Elevated Nrp1 expression has also been observed in the VSMCs of neoplastic breast tumors (80). The hyperplastic neointima of mouse carotid arteries display elevated Nrp1 and Nrp2 levels following balloon angioplasty (76). Recent data suggests that neuropilin promotes the synthetic VSMC

phenotype since inhibition of Nrp1 or Nrp2 expression via adenovirus delivery of small hairpin (sh) RNA reduces neointima formation following angioplasty (76).

Early data showed that tumor cell-conditioned media containing PDGF-B can induce VSMC migration and this effect is blocked by Nrp1 deletion (77). More recently, it was demonstrated that Nrp1 knockdown impairs PDGF-B driven VSMC migration (75). In mesenchymal stem cells, which have the potential to differentiate into VSMCs, Nrp1 also promotes PDGF-dependent migration and proliferation (81). Inhibition of Nrp1 or Nrp2 expression in rat aortic SMCs impaired activation of both PDGFR- α and PDGFR- β signaling in response to PDGF-B (76). Thus, while Nrp1 can promote PDGF signaling, the mechanism by which Nrp1 achieves this effect is only beginning to be elucidated.

Co-immunoprecipitation experiments suggest that Nrp1 interacts, directly or indirectly, with PDGF-B (77). Nrp1 co-immunoprecipitates with PDGFR- α and PDGFR- β in mesenchymal stem cells treated with PDGF-AA or PDGF-BB, respectively, suggesting that Nrp1 may function as a co-receptor for PDGFs (81). In human coronary artery SMCs Nrp1 was shown to associate with PDGFR- α to activate signaling mediated by p130^{cas}, an adaptor protein involved in cell migration (75).

Nrp1 can undergo glycosylation via the addition of a heparin sulfate (HS) or chondroitin sulfate (CS) chain to an evolutionarily conserved serine residue (Ser⁶¹²) in its extracellular domain (78). This modification, an event specific to SMCs, increases Nrp1 molecular weight to approximately 250 kDa. Interestingly, Nrp1 is predominantly CS-modified, with an HS-modification being a markedly infrequent event (75,78). Glycosaminoglycan (GAG) modification of Nrp1 promotes PDGF signaling, as substitution of Ser⁶¹² with an alanine, a mutation that abolishes its glycosylation, impairs PDGF-B-induced migration of VSMCs (75). In addition, CS-modification of Nrp1 was demonstrated to inhibit VEGF binding, promote VEGFR2 degradation and impede VEGFR2 signaling (78). Thus, one could hypothesize that GAG modification of Nrp1 specifically in VSMCs supports PDGFB signaling while inhibiting the potential for ectopic VEGF signaling.

5.3 A cell-autonomous role of Nrp1 in VSMCs

Surprisingly, a recent publication demonstrated that SMC-specific deletion of Nrp1 using a cre-recombinase under control of the smooth muscle myosin heavy chain promoter (smMHC) produces viable and fertile mice that do not display overt cardiovascular defects (82). Analysis of these mice revealed impaired differentiation of SMCs in the colon and bladder to a contractile phenotype that contributed to impaired gut motility. This study implies that Nrp1 promotes, at least in non-vascular SMCs, the contractile phenotype, in contrast with the aforementioned data suggesting that Nrp1 promotes the VSMC synthetic phenotype. Nrp2 may compensate for Nrp1 to modulate PDGF signaling in VSMCs. It should also be noted that VSMCs are a diverse population of cells with multiple cellular origins, thus Nrp1 deletion by other SMC-specific promoters may result in cardiovascular defects. Furthermore, it remains to be determined if Nrp1 regulates TGF β signaling in VSMCs.

CONCLUSION

Neuropilin is emerging as a multifaceted vascular regulator. Every cell of the vascular system expresses Nrp1 and Nrp1 functions in several signaling pathways critical for blood vessel development and function. By regulating TGF β signaling in endothelial cells and PDGF signaling in VSMCs, Nrp1 has the potential to not only contribute to angiogenesis, but also regulate important vascular maturation events. The function of Nrp1 in other vascular cell types, such as macrophages and pericytes remains to be determined. Furthermore, the mechanism by which Nrp1 modulates different signaling pathways is still an important focus of investigation.

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KEY POINTS

- Endothelial Nrp1 promotes the tip cell phenotype during sprouting angiogenesis
- Endothelial TGF β signaling promotes the stalk cell phenotype during sprouting angiogenesis
- Nrp1 inhibits endothelial TGF β signaling
- Nrp1 modulates PDGF signaling to promote the synthetic VSMC phenotype

Figure 1. Nrp1 modulation of the endothelial tip cell phenotype

(A) In tip cells, Nrp1 promotes endothelial sprouting by enhancing VEGFR2 signaling. (B) Canonical TGF β signaling inhibits endothelial cell sprouting and promotes the stalk cell phenotype. In tip cells, Nrp1 inhibits TGF β signaling.

Table 1

Vascular phenotypes of Nrp1 mutant mice

	Nrp1 Mutation	Vascular Phenotype	Reference
Nrp1 Gain of Function	Ectopic global overexpression	<ul style="list-style-type: none"> - Embryonic lethal mid to late gestation - Hyper-vascularization - Vessel dilation - Vessel hemorrhaging 	7
Nrp1 Loss of Function	Global Null	<ul style="list-style-type: none"> - Embryonic lethal at E14 - Impaired vascularization of the CNS - Abnormal great vessel patterning - Impaired yolk sac capillary formation - Reduced sprouting angiogenesis in the brain 	21, 22, 23
	Tie2Cre-driven constitutive endothelial deletion	<ul style="list-style-type: none"> - Embryonic lethal mid to late gestation - Vessel enlargement - Impaired vascularization of the CNS - Reduced vessel branching 	24, 25
	Alk1Cre-driven constitutive endothelial deletion	<ul style="list-style-type: none"> - Embryonic lethal around E16.5 - Brain hemorrhaging - Edema - Impaired vessel sprouting in the brain 	26
	Inducible PDGFBCre-driven embryonic endothelial deletion	<ul style="list-style-type: none"> - Reduced vessel branching of the subventricular vascular plexus 	24
	Inducible Cdh5Cre ^{ERT} -driven postnatal endothelial deletion	<ul style="list-style-type: none"> - Reduced retinal angiogenesis: r reduced sprouting, branching, and radial expansion 	27
Structural Mutants	Sema3A binding mutant	<ul style="list-style-type: none"> - Viable - No overt vascular defects 	25
	VEGFA binding mutant (hypomorph)	<ul style="list-style-type: none"> - Viable, but reduced postnatal survival - Reduced retinal angiogenesis - Reduced hindbrain angiogenesis - Reduced pathological angiogenesis 	28
	VEGFA binding mutant	<ul style="list-style-type: none"> - Viable - Normal cerebral angiogenesis - Altered retinal angiogenesis: decreased outgrowth, reduced artery number, normal vessel density - Impaired hindlimb re-perfusion following femoral artery ligation 	29
	Cytoplasmic domain deletion	<ul style="list-style-type: none"> - Viable - Normal developmental angiogenesis - Normal pathological angiogenesis 	19, 30

	Nrp1 Mutation	Vascular Phenotype	Reference
		<ul style="list-style-type: none">- Increased number of retinal arteriovenous crossings- Impaired developmental arteriogenesis- Impaired hindlimb re-perfusion following femoral artery ligation	

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