How neuropilin-1 regulates receptor tyrosine kinase signalling: the knowns and known unknowns

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Abstract

Essential roles of NRP1 (neuropilin-1) in cardiovascular development and in neuronal axon targeting during embryogenesis are thought to be mediated primarily through binding of NRP1 to two unrelated types of ligands: the VEGF (vascular endothelial growth factor) family of angiogenic cytokines in the endothelium, and the class 3 semaphorins in neurons. A widely accepted mechanism for the role of NRP1 in the endothelium is VEGF binding to NRP1 and VEGFR2 (VEGF receptor 2) and VEGF-dependent formation of complexes or NRP1–VEGFR2 holoreceptors with enhanced signalling activity and biological function. However, although some basic features of this model are solidly based on biochemical and cellular data, others are open to question. Furthermore, a mechanistic account of NRP1 has to accommodate research which emphasizes the diversity of NRP1 functions in different cell types and particularly an emerging role in signalling by other growth factor ligands for RTKs (receptor tyrosine kinases) such as HGF (hepatocyte growth factor) and PDGF (platelet-derived growth factor). It is uncertain, however, whether the model of NRP1–RTK heterocomplex formation applies in all of these situations. In the light of these developments, the need to explain mechanistically the role of NRP1 in signalling is coming increasingly to the fore. The present article focuses on some of the most important unresolved questions concerning the mechanism(s) through which NRP1 acts, and highlights recent findings which are beginning to generate insights into these questions.

Introduction

NRP (neuropilin) 1 and the related molecule NRP2 are receptors for two unrelated types of polypeptide ligand: class 3 semaphorins, a subtype of a large family of secreted polypeptides essential mainly for targeting neurons to their destination tissues in development, and members of the VEGF (vascular endothelial growth factor) family, which play pivotal roles in embryonic and disease-associated angiogenesis [1]. This dual-ligand specificity is mirrored by the phenotypes of NRP1-deficient mice, which die in mid-embryonic development and display a spectrum of abnormalities in the formation of the cardiovascular system and in neuronal guidance. Recent findings suggest that the repertoire of NRP biological functions and the extracellular stimuli that act through this molecule, reaches beyond the semaphorin/VEGF, neuronal/endothelial dichotomies mapped by the developmental phenotypes of genetically altered mouse models. One emerging aspect of the growing biological diversity of NRP1 actions, and which is a focus of the present article, is the role of NRP1 in signalling via RTKs (receptor tyrosine kinases) for PDGF (platelet-derived growth factor) and HGF (hepatocyte growth factor), in addition to its better known contribution to VEGFR (VEGF receptor) signalling. Furthermore, despite the steady increase in our knowledge of NRP1 functions and its target cells and tissues, the mechanism(s) via which NRP1 mediates the functions of different ligands in diverse cell types remains enigmatic. The present article focuses on recent work that has begun to generate insights into several of the key questions relating to NRP1 function in cell signalling.

NRP1 and NRP2 are transmembrane glycoproteins of up to 923 and 926 amino acid residues respectively, with 44% homology. The extracellular region contains two CUB (C1r/C1s, urchin embryonic growth factor and bone morphogenetic protein 1) domains (usually known as a1/a2) essential for semaphorin binding, two Factor V/VIII homology (b1/b2) domains, required for VEGF and semaphorin binding, and a MAM (meprin, A5 and receptor tyrosine phosphatase μ) domain (c), thought to be important for NRP1 homodimerization or oligomerization [1] (Figure 1).

Key words: angiogenesis, endothelium, neuropilin-1, platelet-derived growth factor (PDGF), receptor tyrosine kinase, vascular endothelial growth factor (VEGF).

Abbreviations used: BRVO, branched retinal vein occlusion; E, embryonic day; EC, endothelial cell; EGF, epidermal growth factor; ERK, extracellular-signal-regulated kinase; FAK, focal adhesion kinase; Flk-1, fetal liver kinase 1; GIPC1, GAIP (G_α-interacting protein)-interacting protein C-terminus 1; HCASMC, human coronary artery vascular smooth muscle cell; HGF, hepatocyte growth factor; HSC, hepatic stellate cell; HUVEC, human umbilical vein endothelial cell; KDR, kinase insert domain-containing receptor; NRP, neuropilin; PDGF, platelet-derived growth factor; PDGFR, PDGF receptor; PDZ, PSD-95 (postsynaptic density 95), Dlg (discs large) and ZO-1 (zonula occludens 1); PLCγ, phospholipase Cγ; RGC, retinal ganglion cell; RTK, receptor tyrosine kinase; Sema3A, semaphorin 3A; siRNA, short interfering RNA; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; VSMC, vascular smooth muscle cell.

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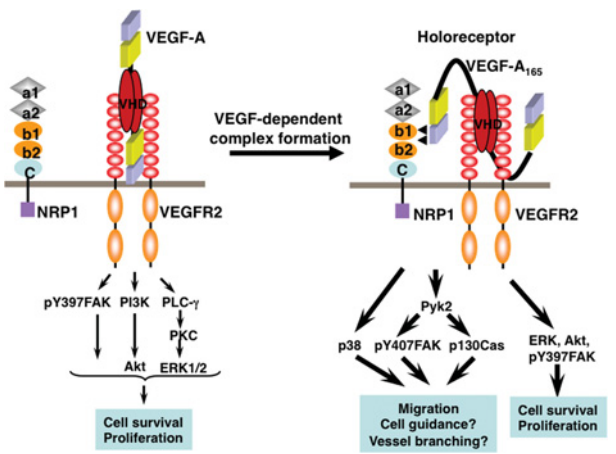
Figure 1 | Scheme for NRP1-dependent VEGF signalling

VEGF binding to VEGFR2 triggers activation of Akt, tyrosine phosphorylation of FAK at its major autophosphorylation site Tyr^{397}, and activation of PLCγ, leading to induction of the ERK cascade, and NRP1 appears to play little role in these signalling events and in cellular functions, such as cell survival and proliferation, that they mediate. Binding of the C-terminal domain of VEGF (yellow and mauve boxes) to NRP1 promotes complex formation with VEGFR2, and this holoreceptor mediates the mobilization of signalling via $p130^{Cas}$ tyrosine phosphorylation and, to some extent, p38 kinase, which play important roles in cell migration. NRP1 may be particularly important for vessel branching and patterning. VHD, VEGF homology domain.



NRPs have a single hydrophobic transmembrane region and small cytoplasmic domains (44 and 43 amino acid residues in NRP1 and NRP2 respectively), which has no defined function, but possesses a C-terminal SEA (Ser-Glu-Ala) motif implicated in associating with the PDZ [PSD-95 (postsynaptic density 95), Dlg (discs large) and ZO-1 (zonula occludens 1)] domain-containing protein synectin [1]. The major VEGF ligand for NRP1 is VEGF-A_{165}, which contains exons 7 and 8, but lacks exon 6 [2]. VEGF-A_{121}, which lacks exon 7, generally exhibits reduced biological activity compared with VEGF-A_{165}, and this has been attributed to its inability to bind NRP1. VEGF-A_{121} has, in fact, been reported to bind NRP1 *in vitro*, probably through its exon 8-encoded domain, but appears unable to promote NRP1–VEGFR2 heterocomplex formation [3].

NRP1-null mice die between E (embryonic day) 12 and E13.5 with a spectrum of cardiovascular and neuronal defects [4–6], and evidence from various mutant mouse models indicates that most, if not all, of these effects reflect tissue-specific actions of VEGF-A_{165} binding to NRP1 in the endothelium, and Sema3A (semaphorin 3A) binding to neuronal NRP1, which appear to be segregated with relatively little cross-talk between the two ligands [7]. Cell culture and *in vivo* studies show that in ECs (endothelial cells) and neurons NRP1 is most strongly linked to regulation of cell migration: inhibition or chemorepulsion in neurons stimulated by Sema3A [8], and stimulation or chemoattraction in VEGF-treated ECs [9–13].

NRP1 regulation of VEGF signalling

In the endothelium, NRP1 is a co-receptor for the major VEGF RTK, VEGFR2 [KDR (kinase insert domain-containing receptor) in humans, Flk-1 (fetal liver kinase 1) in mice]. NRP1 co-expression with VEGFR2 enhances VEGF binding to VEGFR2, VEGFR2 phosphorylation and VEGF-induced signalling and migration [8,10,14,15], and a physical association between NRP1 and VEGFR2 has been demonstrated by covalent cross-linking and co-immunoprecipitation [8,11,14–18]. However, the mechanism(s) underlying these effects have not been established. Thus it has not been shown definitively whether NRP1 complexation with VEGFR2 increases the affinity of VEGFR2/KDR for VEGF-A [11], with some investigators finding little effect of NRP1 on VEGF affinity for VEGFR2 [14], and others finding that NRP1 enhances VEGFR2 affinity for VEGF [19]; neither has it been demonstrated that NRP1 augments the intrinsic catalytic activity of the VEGFR2 kinase domain. Moreover, there is disagreement in the literature as to whether NRP1–VEGFR2 complexation has a stringent requirement for VEGF binding, with some groups reporting VEGF-induced NRP1 association with VEGFR2 [8,10], and others observing predominantly constitutive VEGF-A-independent complex formation [14,19]. We addressed the role of VEGF binding to NRP1 in NRP1–VEGFR2 complexation by examining complexation between exogenously expressed V5-tagged NRP1 and endogenous VEGFR2 in HUVECs (human umbilical vein endothelial cells), and these results show that complex formation occurs in a largely VEGF-dependent manner [13]. Furthermore, the finding that NRP1–VEGFR2 complex formation is inhibited by adenoviral overexpression of a Y297A NRP1 b1 domain mutant unable to support high-affinity ^{125}I-VEGF-A_{165} binding, lends further weight to the conclusion that complex formation is dependent on VEGF binding to NRP1. These and earlier cross-linking findings are consistent with a model in which the cysteine knot motif of VEGF-A_{165} located in the core VEGF homology region binds to VEGFR2, whereas the exon 7/8-encoded C-terminal moiety of VEGF-A_{165} forms contacts with the b1 domain of NRP1, bridging the receptors to form a tripartite complex. Deletion of the NRP1 C-terminal PDZ domain-binding motif diminished NRP1–VEGFR2 co-immunoprecipitation [20], suggesting that intracellular interactions are also important for heterocomplex formation, although it is unclear whether this involves a direct physical interaction between the NRP1 cytosolic domain and VEGFR2, or is mediated via another NRP1-binding molecule (see below).

NRP1 complexation with VEGFR2 is not essential for VEGFR2 kinase activation. Thus different approaches to selective inhibition of VEGF binding to NRP1 using cells expressing VEGFR2 without NRP1 [21], treatment with specific NRP1-blocking antibody [10], specific peptide

antagonists of NRP1 binding [12,16] or NRP1-specific siRNA (short interfering RNA) [12,13] demonstrate unequivocally that VEGF is able to signal via VEGFR2 independently of NRP1. However, several lines of evidence indicate that NRP1 may be required for maximum VEGFR2 activity and/or tyrosine phosphorylation. Antibodies that block VEGF binding to NRP1 had little effect on VEGFR2 phosphorylation [10], and a Y297A mutation in the NRP1 b1 domain that blocks VEGF binding to NRP1 also did not significantly reduce VEGFR2 tyrosine phosphorylation, but targeted NRP1 knockdown using siRNA caused a 50% inhibition in VEGFR2 phosphorylation at Tyr^{1175} [13], a residue essential for VEGFR2-mediated activation of PLCγ (phospholipase Cγ)/ERK (extracellular-signal-regulated kinase), cell proliferation and normal embryonic development in mice. One explanation for this is that NRP1 may be important for VEGFR2 phosphorylation at selected sites, without affecting intrinsic kinase activity, although the mechanism involved in any putative site-directed receptor phosphorylation is unclear. Alternatively, NRP1 could be important for stability of VEGFR2 at the cell surface, and, consistent with this notion, NRP1 knockdown reduces VEGFR2 expression [22]. Therefore reduced VEGFR2 activity in the absence of NRP1 could simply reflect reduced VEGFR2 expression, possibly due to enhanced endocytosis and degradation through lysosomal and/or proteasomal pathways. If NRP1 is important for VEGFR2 activation or auto/transphosphorylation at specific tyrosine residues, it needs to be explained how an association between these molecules increases or targets intrinsic VEGFR2 catalytic activity. It is possible that a complex between the NRP1 cytosolic domain and VEGFR2 kinase region might create a high-activity VEGFR2 conformation, although evidence for this is lacking.

Studies of effects of NRP1 inhibition on post-VEGFR2 signalling have begun to reveal a more specific role of NRP1 in VEGF signalling and cellular function. Some essential functions of VEGF such as cell survival and proliferation appear to be either wholly or largely independent of NRP1, and, consistent with these observations, activation of the PLCγ/ERK and Akt pathways, which play major roles in proliferation and survival functions of VEGF respectively, are also largely unaffected in the absence of NRP1 or when VEGF binding to NRP1 is selectively abrogated by siRNA, blocking antibody or using b1 domain mutants unable to bind VEGF [10,12,13,16] (Figure 1). For example, antibodies that selectively block VEGF binding to NRP1 prevent NRP1 complexation with VEGFR2 and reduce VEGF-stimulated migration to approximately 50% of control responses, but also had modest effects on p38 kinase activation and permeability [10]. Similarly, NRP1 siRNA-mediated knockdown and non-VEGF-binding b1 domain mutants also reduced VEGF-induced migration by 50–80%, but had little effect on activation of ERK, Akt and HSP27 (heat-shock protein 27) phosphorylation at Ser^{28} [12,13]. VEGFs that bind VEGFR2 and NRP1, including VEGF-A_{165} and the VEGF-E-NZ2 subtype, induced similar increases in PLCγ tyrosine phosphorylation and activation of ERK and Akt as $VEGF_{121}$, which is unable to promote VEGR2–NRP1 complexation, but were more angiogenic and stimulated more p38 kinase activity than VEGF-A_{121} [23]. We identified an important role for NRP1 in mediating VEGF-induced tyrosine phosphorylation of $p130^{Cas}$, an adaptor protein with a key role in regulating actin filament dynamics and cell migration [24]. NRP-targeted siRNA, NRP1-blocking antibody and a specific antagonist of VEGF binding to NRP1 all inhibited VEGF-induced $p130^{Cas}$ tyrosine phosphorylation and $p130^{Cas}$ knockdown, and expression of a non-tyrosine phosphorylatable $p130^{Cas}$ mutant inhibited VEGF-induced migratory responses [12]. $p130^{Cas}$ associates with several other signalling molecules, including the related non-receptor tyrosine kinases, FAK (focal adhesion kinase) and Pyk2. Interestingly, although VEGF is able to stimulate FAK phosphorylation, we found that FAK knockdown does not affect $p130^{Cas}$ tyrosine phosphorylation, whereas Pyk2 knockdown significantly reduced the response [12]. Furthermore, although NRP1 knockdown did not affect FAK tyrosine phosphorylation at its major autophosphorylation site, Tyr^{397}, both NRP1 knockdown and expression of an NRP1 Y297A mutant deficient in VEGF binding blocked VEGF-induced FAK tyrosine phosphorylation at Tyr^{407} [13], a site that is regulated by Pyk2 [25]. These findings (summarized in Figure 1) suggest that NRP1 selectively mediates VEGFR2 signalling via a Pyk2/$p130^{Cas}$ pathway important for EC migration. Mice deficient in $p130^{Cas}$ die *in utero* primarily from cardiovascular defects and exhibit impaired actin filament assembly; $p130^{Cas}$ also appears to be expressed predominantly in the cardiovascular system at the time in embryonic development that lethality occurs in $p130^{Cas}$-null mice [26]. These findings suggest a link between NRP1 and $p130^{Cas}$ signalling which may play an important role in developmental angiogenesis. Future work on NRP1-specific signalling pathways should focus on $p130^{Cas}$ and associated signalling networks involving Pyk2 and other $p130^{Cas}$-associated protein as potential candidates for specific mediators of NRP1 functions in cell motility and angiogenesis functions of VEGF.

Can VEGF regulate cell function via NRP1 independently of VEGFR2?

It is generally accepted that all biological functions of VEGF-A are mediated via either VEGFR1 or VEGFR2, and there is broad consensus that, in the endothelium, VEGF-A signals and functions predominantly via VEGFR2. VEGF signalling, which is dependent on NRP1, also requires VEGFR2, and we are unaware of VEGF-stimulated endothelial signalling events that occur independently of VEGFRs. Thus VEGF-induced $p130^{Cas}$ tyrosine phosphorylation, which we have shown to be selectively dependent on NRP1, is also completely blocked by VEGFR2 knockdown (I.M. Evans and I.C. Zachary, unpublished work). Nevertheless,

biological effects of VEGF have been reported in cells which express NRP1 and/or NRP2, but which often express little or no VEGFR2 and VEGFR1, including VSMCs (vascular smooth muscle cells) and various cancer cell lines [1,27–30]. However, the status of VEGFR expression in these cell types appears to be variable. Taking VSMC as an example, some studies show that pro-migratory effects of VEGF are in fact mediated via VEGFRs and NRP1 [27]; in other studies, VEGF stimulated cell migration, but receptor expression was not determined [28]; and in other reports, cells expressing NRPs, but lacking detectable VEGFRs, do not respond chemotactically to VEGF [29]. VEGF also stimulates chemotactic responses in neuronal explants expressing NRP1, although again expression of VEGFR2 in these cells appears to vary. Erskine et al. [31] reported that NRP1 is essential for the contralateral projection of retinal ganglion cell axons and found that this was dependent on the major NRP1 ligand $VEGF_{164}$, as indicated by a similar reduction in contralateral RGC (retinal ganglion cell) axonal projection in NRP1-null mice and in mice expressing $VEGF_{120}$, but lacking $VEGF_{164}$ and $VEGF_{188}$. Flk1 (mouse VEGFR2) was not detectable in RGCs at a developmental stage when axon outgrowth occurs (E12.5–E14.5), and a function-blocking Flk1 antibody did not inhibit $VEGF_{164}$-stimulated RGC axon outgrowth in explants of peripheral retinal tissue, indicating that, in these neurons, $VEGF_{164}$ may signal through NRP1 independently of Flk1. It is noteworthy, however, that another study has similarly reported that VEGF mediates chemoattraction of axons albeit in a different developmental context, the spinal cord ventral midline, but, in contrast, shows that these VEGF-responsive neurons do express Flk-1, and that Flk-1 is required for targeting of these axons *in vivo* [32]. The possibility that VEGF functions via binding to NRP1 independently of VEGFR2 is an intriguing one, but the signals and mechanism(s) involved remain to be elucidated.

Do NRP1 and NRP2 interact in VEGF signalling?

NRP2-deficient mice are viable and grossly normal, with some specific developmental aberrations in nerve targeting and fasciculation but no apparent embryonic cardiovascular defects, although adult mice display a reduction in small lymphatic vessels and capillaries, in addition to restricted defects in nerve patterning [33]. However, targeted disruption of both NRPs ($NRP1^{-/-}/NRP2^{-/-}$ mice) causes lethality significantly earlier in embryogenesis than in $NRP1^{-/-}$ mice (E8.5 compared with E12.5–E13.5) and results in a more severe vascular phenotype closer to that of the VEGF-A and VEGFR2 (Flk-1) knockouts [34]. Furthermore, mice lacking NRP1 or NRP2 but heterozygous for the other NRP ($NRP1^{+/-}/NRP2^{-/-}$ or $NRP1^{-/-}/NRP2^{+/-}$) are embryonic lethal at E10–E10.5, also earlier than $NRP1^{-/-}$ mice. One interpretation of these findings is that, whereas loss of NRP2 in the vasculature can be compensated for by NRP1 throughout development, loss of NRP1 can be compensated for by NRP2 only up to approximately E12.5, and that up to this time, one or other NRP is essential in mid-embryonic vascular development in the yolk sac and embryo, after which it appears that NRP1 continues to be essential, but NRP2 is largely redundant in the vasculature. This might reflect divergence later in embryogenesis in the expression of NRP1 and NRP2, with NRP1 playing a key role in later arterial development, whereas NRP2 may play a more restricted and developmentally less essential role in the venous and lymphatic endothelium. Since cardiovascular abnormalities are not a feature of either $Sema3A^{-/-}$ or $Sema3F^{-/-}$ mice, and the latter display developmental neuronal defects that closely phenocopy those in NRP2 knockouts [6,35,36], the more severe embryonic cardiovascular phenotype of $NRP1^{-/-}/NRP2^{-/-}$ mice is most likely to be due to the role of both NRPs in earlier VEGF-driven vascular development.

The severe phenotype of NRP1/NRP2-deficient mice suggests that they have overlapping functions, allowing NRP2 to compensate for loss of NRP1 in mid-embryonic vascular development. In support of a functional interaction between NRPs, NRP1 and NRP2 heterodimerize, and co-expression of NRP2 with a Y297A NRP1 mutant unable to bind NRP1 results in inhibition of VEGF binding to NRP2, even though the VEGF-binding domain of NRP2 is unaltered [13]. These data infer that in cells naturally co-expressing NRP1 and NRP2, much of the VEGF binding may be to heterodimers. However, since VEGF-A_{165} has a much higher affinity for NRP1 than NRP2, with K_d values of 0.2–0.3 nM and 5.2–8 nM respectively [11,13,37], the biological role of VEGF-A_{165} binding to NRP2 at physiologically relevant VEGF concentrations is not entirely clear. This raises the alternative possibility that the functions of NRP1 that are compensated for by NRP2 in mid-embryonic vascular development may be largely independent of binding to either of their major polypeptide ligands.

Is VEGF binding to NRP1 essential for NRP1 endothelial function?

Recently, we obtained a structure for the NRP1 b1 domain, which is mainly responsible for VEGF binding, co-crystallized with a small molecule antagonist (EG00229), which selectively inhibits VEGF binding to NRP1 [38]. Design and synthesis of EG00229 was based on the C-terminal hexapeptide of VEGF-A_{165} encoded by exon 8, suggesting that it would bind to at least part of the VEGF-A_{165}-binding site. Site-directed mutation of b1 residues involved in the binding groove for EG00229 revealed several residues essential for high-affinity VEGF-A_{165} binding in cells. Interestingly, overexpression of a non-binding NRP1 mutant (Y297A) caused a dominant-negative inhibitory effect on VEGF binding to wild-type NRP1, and also blocked VEGF-dependent complex formation between NRP1 and VEGFR2 and reduced VEGF-stimulated EC migration. Y297A NRP1 also partially inhibited VEGF stimulation of branching endothelial tubule formation in an organotypic co-culture model of angiogenesis [13]. These findings support the

conclusion that VEGF binding to NRP1 is essential for the biological role of NRP1 in EC migration and angiogenesis, at least in a human EC culture model.

However, it is evident that not all important vascular functions of NRP1 can easily be explained in terms of the VEGFR2 co-receptor paradigm. The discordance between the neural and vascular phenotypes of NRP1-null mice and those of mice lacking the major NRP1-binding VEGF isoform, VEGF-A_{164}, or both VEGF-A_{164} and Sema3A suggests that NRP1 may function independently of its two major types of ligands [7]. Furthermore, some endothelial cellular functions of NRP1, particularly cell adhesion, appear to occur via a fundamentally different type of mechanism. Thus adhesion of a variety of cell lines is supported by recombinant NRP1 protein via specific regions in the b1 and b2 domains, but was not blocked by Sema3A or VEGF-A_{165} [39]. Moreover, siRNA-mediated NRP1 knockdown disrupted EC adhesion to fibronectin, laminin or gelatin, whereas VEGFR2 silencing and overexpression of non-VEGF binding b1 domain Y297A NRP1 mutant were ineffective [13,40]. A region of NRP1 important for cell adhesiveness has been localized to amino acid residues 347–364 in the b1 domain and the homologous residues 501–547 in the b2 domain [39], a region that does not overlap substantially with the region of the b1 domain important for binding VEGF-A_{165} [38]. Blocking NRP1 antibodies also have no effect on EC adhesion to fibronectin [10], consistent with the independence of an adhesive function from VEGF or Sema3A ligand binding.

A role for NRP1 in EC matrix adhesion may be mediated via an interaction with integrins. Robinson et al. [41] reported that the $\alpha v\beta 3$ integrin associates with NRP1 in a VEGF-dependent manner through the $\beta 3$ cytoplasmic tail, and that $\beta 3$ negatively regulates angiogenesis by reducing NRP1 complexation with VEGFR2. An interaction between NRP1 and the major fibronectin receptor integrin $\alpha 5\beta 1$ appears to mediate $\alpha 5\beta 1$ endocytosis and is important for adhesion of human umbilical artery endothelial cells to fibronectin [42]. Although the NRP1 cytosolic domain was dispensable for association between NRP1 and $\alpha 5\beta 1$, it seems to be required for $\alpha 5\beta 1$ endocytosis, and the authors proposed that this is due to interaction between the SEA motif and synectin/GIPC1 [GAIP (G_α-interacting protein)-interacting protein C-terminus 1]. However, this model is based on inhibitory effects of synectin siRNA on adhesion and $\alpha 5\beta 1$ endocytosis, and, notably, the study did not provide new evidence for an association between NRP1 and synectin. NRP1 association with $\beta 1$ integrin is also implicated in pancreatic cancer cell growth, survival and invasion [43].

Does NRP1 participate in signalling via other RTKs?

An emerging, but still poorly understood, feature of NRP1 function is its role in cellular responses to non-canonical ligands, including several for RTKs, particularly HGF [12,44–46] and PDGF [29,47–49]. NRP1 overexpression promotes tumour progression by potentiating the effect of the HGF/c-Met pathway, and tumour cell invasion mediated by HGF/c-Met is dependent on NRP1 through an association with c-Met [44,45]. NRP1 and NRP2 can bind HGF and mediate HGF stimulation of EC migration and proliferation [46]. NRP1 was also reported to associate with PDGF-BB and to mediate migration of smooth muscle cells stimulated by tumour cell-derived PDGF [47].

Similar to effects of VEGF in ECs, $p130^{Cas}$ tyrosine phosphorylation also plays a major role in NRP1-dependent signalling by HGF and PDGF [12,29]. NRP1 overexpression in U87 glioma cells selectively up-regulated $p130^{Cas}$ tyrosine phosphorylation, without affecting ERK and Akt activity, and this was associated with increased invasion of these cells in a three-dimensional collagen gel [50]. Recently, we showed that $p130^{Cas}$ tyrosine phosphorylation and cell migration stimulated by HGF and PDGF in U87MG glioma cells are inhibited by NRP1 siRNA and by adenoviral overexpression of an NRP1 mutant with a deletion of its cytosolic domain (Ad.NRP1ΔC), whereas ERK, Akt and FAK Tyr^{397} phosphorylation were not altered [12]. HGF and PDGF-BB also promoted a striking co-localization of $p130^{Cas}$ and NRP1 in the plasma membrane in U87 cells, although co-immunoprecipitation was unable to detect a direct physical association between these molecules. NRP1 also mediates PDGF-BB and -AA stimulation of $p130^{Cas}$ tyrosine phosphorylation and migration in HCASMCs (human coronary artery vascular smooth muscle cells) [29]. In addition, a study of PDGF responses mediated via PDGFR (PDGF receptor) β in HSCs (hepatic stellate cells), showed that NRP1 knockdown inhibited PDGF-BB-induced chemotaxis and enhanced Rac1 activity via an association with the c-Abl tyrosine kinase, but with no detectable change in ERK and Akt activity [49]. Taken together, these results suggest that NRP1 is required for specific signalling events linked to enhanced cell migration downstream of PDGFR and c-Met activation in diverse cell types. Furthermore, in some cases, this appears to be mediated via the NRP1 cytosolic domain. One explanation for all these findings is that NRP1 is required for maximum activity of c-Met and PDGFRs. In support of this, c-Met phosphorylation has been shown to be partially dependent on NRP1 expression [46], and NRP1 overexpression augmented the level and longevity of PDGFRβ phosphorylation in HSCs [49]. In contrast, we found that levels of neither c-Met nor PDGFRβ tyrosine phosphorylation in U87MG cells were altered by either NRP1 siRNA treatment or expression of Ad.NRP1 wild-type or Ad.NRP1ΔC constructs [12]. In HCASMCs, which express both PDGFRβ and PDGFRα, NRP1 co-immunoprecipitates with PDGFRα and NRP1 knockdown significantly reduced PDGFRα activation, but had little effect on PDGFRβ [29]. Furthermore, Ball et al. [48] reported that NRP1 is involved in PDGF signalling and is associated with PDGFRα in mesenchymal stem cells. Although these findings suggest a possible role for NRP1 as a co-receptor for PDGFRβ or PDGFRα, dependent

on the cell type, and possibly important for PDGFR activity, it remains unclear whether PDGF (or HGF) acts physiologically as a ligand for NRP1. Evidence in favour of a PDGF ligand interaction with NRP1 are that NRP1 could be co-immunoprecipitated with PDGF-BB [47], and that NRP1 overexpression in HSCs enhanced the affinity of ^{125}I-PDGF-BB binding to PDGFRβ, whereas NRP1 knockdown reduced ^{125}I-PDGF-BB binding in HSCs [49]. However, in the absence of NRP1 overexpression, negligible co-immunoprecipitation of NRP1 with radiolabelled PDGF could be detected in HSCs [49]. Moreover, we were unable to detect clear specific radiolabelled PDGF-BB binding to NRP1-expressing porcine aortic ECs [29]. Indeed, whereas some evidence, mainly obtained from cell-free binding studies, suggests that NRP1 is a potential ligand for HGF and FGF-2 (fibroblast growth factor 2), as well as PDGF, definitive demonstration of specific high-affinity binding of endogenous cellular NRP1 to other polypeptide ligands for RTKs is so far lacking.

What is the role of the NRP1 cytosolic domain?

A PDZ domain protein called synectin, also known as NIP-1 (NRP-interacting protein-1) or GIPC1, associates in a yeast two-hybrid screen with the NRP1 cytosolic domain via the C-terminal consensus PDZ-binding SEA motif [51]. Several lines of evidence suggest a biologically important role for the NRP1 cytosolic domain, although whether a function of this region is mediated entirely or partly via an interaction with synectin is more problematic. Expression of NRP1 lacking the C-terminal SEA residues resulted in aberrant vessel formation in zebrafish, suggesting a role for this interaction in angiogenesis [52]. In addition, adenoviral overexpression of NRP1 lacking the whole cytosolic domain (Ad.NRP1ΔCyt) caused a striking inhibition of VEGF-induced p130^{Cas} tyrosine phosphorylation and cell migration in HUVECs, and similar effects were observed in U87 glioma cells treated with HGF and PDGF-BB [12]. Synectin has been reported to associate with NRP1 in human ECs, and is implicated in NRP1-mediated migration in response to EGF (epidermal growth factor) in ECs overexpressing a chimaeric EGF–NRP1 receptor [52,53], although, in contrast, we found that knockdown of synectin with siRNA markedly reduced synectin expression, but had no effect on VEGF-induced tyrosine phosphorylation of p130^{Cas} or growth-factor-induced phosphorylation of p130^{Cas} in U87 glioma cells [12]. It is also noteworthy that evidence for direct physical association between NRP1 and synectin in ECs is limited [12].

Synectin-deficient mice are viable and have no obvious developmental defects similar to those in NRP1 knockouts, although the adult mice are smaller and exhibit a restricted arteriogenic phenotype with reduced microvascular arteriolar density. In addition, analysis of ECs derived from synectin-null mice showed that growth-factor-stimulated migration of venous ECs was not impaired, whereas migration and angiogenesis of arterial ECs was reduced [54]. This indicates that if synectin plays any role in NRP1 function, it is restricted largely to postnatal arterial endothelial growth. Recent findings suggest that synectin might function in trafficking of VEGFRs. Intriguingly, synectin associates with myosin VI, which is unique among actin-based motor proteins in being able to migrate along actin filaments towards the minus end, and this interaction is important in mediating recruitment of myosin VI to endocytic vesicles following clathrin uncoating [55]. NRP1 trafficking was impaired in aortic ECs from synectin-null mice [56], and the synectin–myosin VI interaction also seems to be important for VEGFR2 endocytosis, although it was not examined whether this requires NRP1 [57]. A further twist is provided by the unexpected observation that aortic ECs from both synectin-null and myosin VI-null mice exhibit a severe reduction in VEGF-induced Tyr^{1175} VEGFR2 phosphorylation, PLCγ Tyr^{783} phosphorylation and ERK activation, which can be rescued by re-expression of synectin [58]. Furthermore, these authors proposed that the role of synectin/myosin VI in VEGFR2 signalling was independent of NRP1, although this conclusion is based on the finding that VEGF-D exhibited a similar defect in signalling and the assumption that VEGF-D binds to VEGFR2, but not to NRP1, whereas VEGF-D has also been reported to interact with NRP1 and NRP2 [58]. These findings suggest that the functions of synectin and NRP1 are largely separable and divergent, but that synectin, independent of NRP1, may have a distinct role in VEGF signalling via Tyr^{1175} and trafficking which is restricted to postnatal arteriolar growth. The myosin VI-null mice are a close phenocopy of synectin-deficient mice: viable, with no overt developmental or adult defect other than being smaller and with reduced arterial density in the renal and peripheral circulations. Why the defect in Tyr^{1175} signalling in synectin-null cells does not produce a more severe phenotype similar to the mid-embryonic lethality (E8.5–E9.5) in mice expressing a VEGFR2 Y1173F mutant [59] is unclear, but may result from the different effects of partial and complete loss of VEGFR2 Tyr^{1173} phosphorylation.

Mice expressing NRP1 with a deletion of the whole cytosolic domain (NRP1$^{cyto\Delta/\Delta}$) produce heterozygous and homozygous progeny at the predicted Mendelian ratios, and are viable, fertile, appear normal and exhibit no detectable defects in embryonic angiogenesis [60]. Furthermore, mice lacking the NRP1 cytosolic domain and also null for NRP2 are very similar, indicating that the NRP2 cytosolic domain does not compensate for loss of the NRP1 intracellular region. These mice do not phenocopy synectin-null mice, which display aberrant recruitment of pericytes during retinal vascularization not apparent in NRP1$^{cyto\Delta/\Delta}$ mice. These findings indicate that the NRP1 and NRP2 intracellular regions are not required for vascular development, at least in mice, and are consistent with the view that synectin does not mediate NRP1-dependent developmental functions. Analysis of neonatal retinal vascularization in NRP1$^{cyto\Delta/\Delta}$ mice detected an increased number of arteriovenous crossing

points, something that is also a feature in mice with genetically reduced VEGF-A_{164} expression in neural progenitors [61]. BRVO (branch retinal vein occlusion), a major cause of visual impairment in humans, results from an increased incidence of arteriovenous crossings in which the less muscular vein usually occurs between the thicker-walled artery and the retina, and the two vessels share a common adventitial layer. This results in mechanical distortion or compression of the vein, which is thought to result in further degeneration and remodelling of the vessel wall, possibly secondary to perturbed venous blood flow, ultimately leading to vein occlusion. Thus the $NRP1^{cyto\Delta/\Delta}$ mice might provide a novel model for investigation of the pathogenesis of BRVO. Although the restricted phenotype of $NRP1^{cyto\Delta/\Delta}$ mice suggests that this region is unimportant for murine development, it does not preclude the possibility that this region plays a more important role in humans and/or is important for disease-associated or other instances of postnatal angiogenesis. NRP1 is also involved in other vascular functions, such as vascular permeability [62], that may require the cytosolic domain.

Conclusions and perspectives

There is strong evidence that NRP1 plays an important role in VEGF-regulated endothelial function by acting as a receptor for VEGF and via ligand-dependent complex formation with VEGFR2. Recent data suggest that in cells co-expressing NRP1 and NRP2, VEGF binds to NRP1–NRP2 heterodimers, and this may be the functionally important unit which engages in VEGF signalling via VEGFR2 in some cases. It is unclear, however, whether involvement of NRP2 is able to alter the signalling properties of VEGFR2, a problem that has implications for understanding the role of NRPs in embryonic vascular development. It is also increasingly clear that the VEGF interaction with NRP1 is not required for some key cellular functions of VEGF, for VEGFR2 activation, and for much signalling distal to VEGFR2. Furthermore, although NRP1 is essential for embryonic cardiovascular development, prevention of VEGF binding to NRP1 does not inhibit angiogenesis as much as blockade of VEGF itself [10,13], indicating that NRP1 performs a specific functionally restricted role in VEGF-induced angiogenesis. Consistent with this view, a picture is emerging in which NRP1 is important for enhancing VEGF stimulation of specific signal transduction pathways, including $p130^{Cas}$ phosphorylation and p38 kinase, which play important roles in cell migration. It remains unclear precisely what functional role(s) NRP1 plays in angiogenesis. It seems unlikely that NRP1 is obligatory for endothelial migration itself, since NRP1 inhibition only partially blocks VEGF-induced migration. Instead, there are indications that NRP1 may be important for vessel branching and network modelling [13,23,60]. Given the role of NRP1 in the guidance of neuronal axons, it is attractive to speculate that it may play an analogous role in the targeting and patterning of blood vessels, rather than vessel growth or elongation.

Another unanswered question is how NRP1 mobilizes specific signalling pathways. It is plausible that NRP1 complex formation with VEGFR2 produces a holoreceptor with additional functionality, possibly involving enhanced site-specific VEGFR2 phosphorylation, although the exact mechanism involved needs to be elucidated. Whether the NRP1 cytosolic domain is able to associate with intracellular binding partners and thereby recruit them to the VEGF/VEGFR2 signalling axis remains unclear. The NRP1 cytosolic domain appears to be important for some cellular functions, such as NRP1–VEGFR2 complexation, and for signalling via $p130^{Cas}$, but this region is not essential for mouse development, although it seems to play a role in arteriovenous patterning at least in retinal vascularization. Regardless of its role, recent work militates against the hypothesis that NRP1 functions are mediated via an association of its cytosolic region with synectin. However, several other NRP1 protein–protein associations have been documented, although further work is required to delineate their functional role and that of the NRP1 cytosolic domain.

A mechanistic scheme for NRP1-dependent regulation of cell function also needs to account for the role of NRP1 in PDGF and HGF signalling, which has now been described in cell types as diverse as mesenchymal stem cells, VSMCs, tumour cells and HSCs. Currently, it remains unclear whether these growth factors are functionally relevant ligands for NRP1, or whether NRP1 acts as a c-Met or PDGFR co-receptor analogous to its role in VEGFR2 signalling. Since NRP1 is implicated in tumour growth [1] and in liver cirrhosis [49], elucidating the mechanisms underlying NRP1's role in signalling by PDGF and HGF as well as other biological contexts, is not only of biochemical interest, but also likely to be important for understanding the pathogenesis of human disease, and for developing new therapeutic approaches.

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