Gera Neufeld · Ofra Kessler *Editors*

The Neuropilins: Role and Function in Health and Disease

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Introduction and Brief Historical Overview

1

Gera Neufeld and Ofra Kessler

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The A5 neuron-specific cell surface antigen was initially identified in Xenopus embryos [1, 2] and characterized as a cell adhesion receptor [3] by the group of Hajime Fujisawa. The A5 antigen was subsequently renamed neuropilin [4]. A breakthrough in our understanding of the function of neuropilin occurred when it was characterized almost at the same time by the group of Alex Kolodkin and David Ginty and by the group of Marc Tessier-Lavigne, as a receptor for the axon guidance factor semaphorin-3A (sema3A) [5, 6], which is one of the seven axon guidance factors belonging to the class-3 semaphorins subfamily [7, 8]) (Fig. 1.1). The class-3 semaphorins induce the collapse of neuronal growth cones which is why they were initially named collapsins [9]. It was simultaneously found that yet another neuropilin family member was present in the human genome, and therefore neuropilin was renamed neuropilin-1 (NRP1) and the second family member named neuropilin-2 (NRP2) [5, 6]. NRP2 was also found to behave as a receptor for class-3 semaphorins although for different semaphorins than NRP1. Thus NRP2 was characterized as a receptor for sema3F and sema3G but not as receptor for sema3A [10]. The neuropilins were found to be required for signal transduction induced by six of the seven class-3 semaphorin family members, which during the development of the nervous

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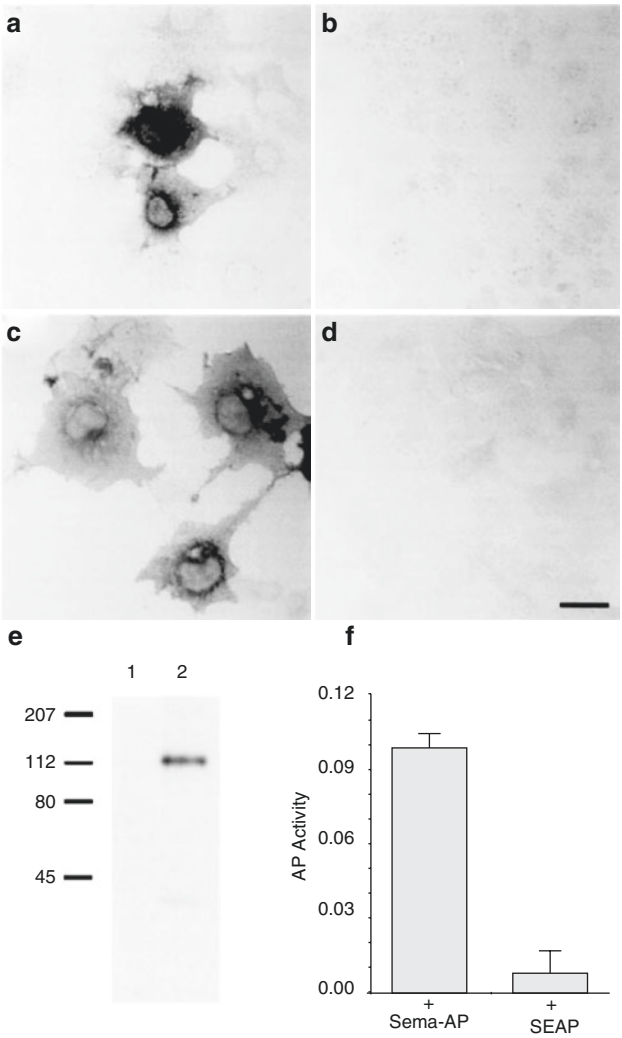

Fig. 1.1 Identification of neuropilin as a receptor for sema3A (From Kolodkin et al. [5]) (**a**–**d**) COS cells were transfected with an expression vector encoding neuropilin (**a**–**c**) or the empty vector (**d**). After two days, cells were incubated with Sema-AP (**a**) or SEAP (**b**) and then processed for alkaline phosphatase activity, or cells were fixed and subjected to immunocytochemistry using anti-neuropilin IgG (**c**, **d**). No neuropilin immunoreactivity was detected when COS cells expressing neuropilin were incubated with preimmune IgG (data not shown). Scale bar = 25 μm. (**e**) Anti-neuropilin immunoblot analysis of whole cell extracts prepared from COS cells that were transfected with the empty expression vector (lane 1) or an expression vector encoding neuropilin (lane 2). (**f**) Sema-AP binds directly to the extracellular domain of neuropilin. Either Sema-AP or SEAP was incubated with soluble myc-tagged neuropilin extracellular domain (myc-neuropilin^{ex}). Then, myc-neuropilin^{ex} was immunoprecipitated with an antibody directed against the myc epitope, and alkaline phosphatase activity in the immune complex was measured as described in experimental procedures and is reported as OD^{405}/second. Shown are the means ± SEM of three independent experiments

system function primarily as repulsive axon guidance factors [11]. The neuropilins possess a very short intracellular domain, and it was therefore assumed that they cannot transduce semaphorin signals on their own. Indeed, the next breakthrough in our understanding of the biological roles of the neuropilins came when it was realized that neuropilins form complexes with type-A plexin receptors to form functional class-3 semaphorin receptors [12, 13].

During binding/cross-linking experiments in which we aimed to characterize receptors for the different splice forms of vascular endothelial growth factor-A (VEGF-A), we noticed that the VEGF-A_{165} form binds to a receptor that is not recognized by the VEGF-A_{121} splice form (Fig. 1.2) [14]. This $VEGF_{165}$ binding receptor was subsequently identified as NRP1 in collaboration with the group of Michael Klagsbrun [15]. Subsequently, NRP2 too was also found to function as a receptor for specific splice forms of VEGF-A [16]. Both neuropilins were found to function as enhancers of VEGF-A signal transduction, to form complexes with the VEGFR-2 tyrosine-kinase receptor which is the receptor that is primarily responsible for the transduction of the pro-angiogenic signals of VEGF-A, and to play critical roles in embryonic angiogenesis [15–18]. At about the same time, it was realized that the neuropilins also function as receptors for additional members of the VEGF family [19–22]. VEGF-C and VEGF-D function as primary regulators of lymphangiogenesis, and it was observed that NRP2 also plays a critical role in the regulation of VEGF-C-induced lymphangiogenesis [23].

Surprisingly, the neuropilins were subsequently also found to function as receptors for additional growth factors such as TGF-β [24], galectin-1 [25], and hepatocyte growth factor (HGF)/scatter factor [26]. Furthermore, neuropilins were observed to form complexes with tyrosine-kinase receptors such as the platelet-derived growth factor (PDGF) receptor PDGFR-α [27, 28], the EGF receptor [29], and the hepatocyte growth factor (HGF)/scatter factor receptor MET [30]. In addition the neuropilins were found to form associations with receptors such as the cell-cell adhesion receptors L1-CAM and Nr-CAM [31, 32] as well as integrins such as integrin-β1 [33].

Taken together, these findings suggest that the neuropilins should perhaps be regarded as scaffold receptors that upon specific stimulation by diverse ligands associate with other cell surface receptors to modulate their signal transduction properties. Whether neuropilins can transduce signals on their own is still unclear. Because neuropilins can bind to so many different ligands and associate with so many diverse receptors, it is not surprising that they are expressed in many cell types and affect diverse biological processes, such as the development of the heart [34] or immune responses [35], and that they play important roles in the etiology of many diseases such as in cancer and autoimmune diseases as outlined in the following chapters.

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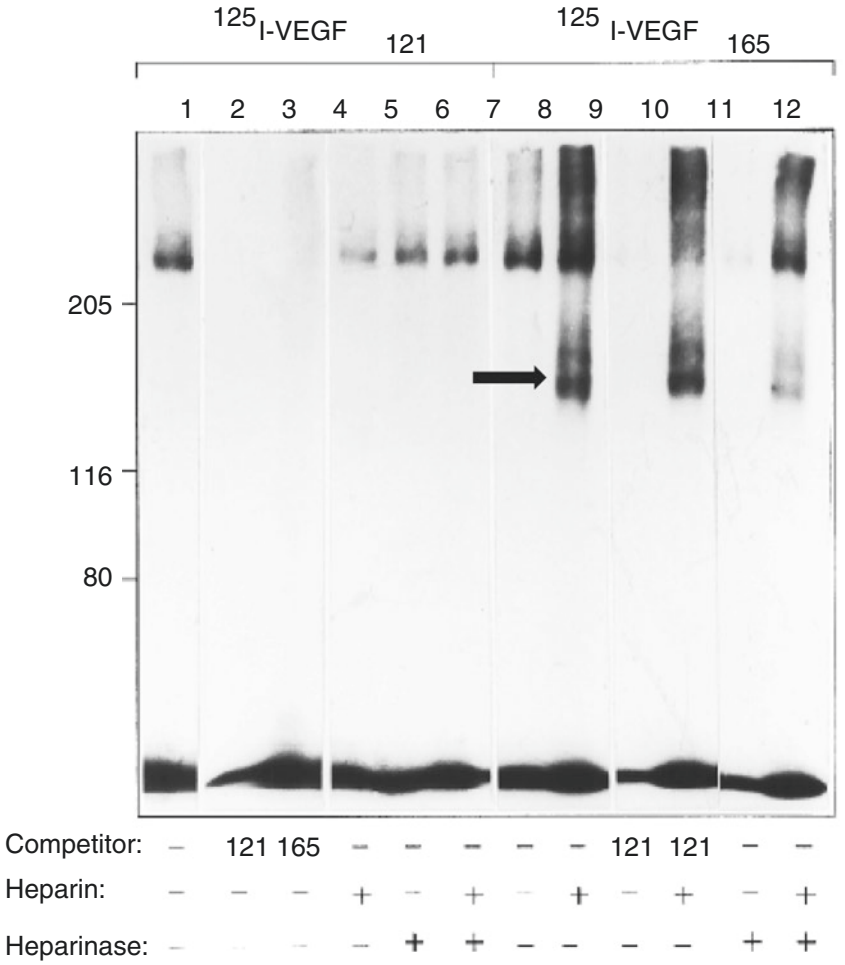

Fig. 1.2 Identification of a novel receptor that binds the VEGF-A_{165} form of VEGF but not the VEGF-A_{121} form of VEGF (From Gitay-Goren et al. [14]). HUE cells were grown to confluence on gelatin-precoated 10-cm dishes. Cells were washed once with phosphate-buffered saline at 37 °C and were incubated in binding buffer (20 mM HEPES, pH 7.2, 0.1% gelatin in Dulbecco's modified Eagle's medium) for 1 h at 37 °C with (*lanes 5, 6, 11,* and *12*) or without (*lanes 1–4* and *7–10*) 0.05 unit/ml heparinase 1. The cells were subsequently washed twice with cold phosphate-buffered saline, and 2.4 ml of cold binding buffer containing 10 ng/ml ^{125}I–$VEGF_{121}$ *(lanes 1–6)* or 5 ng/ml ^{125}I–$VEGF_{121}$ (*lanes 7–12*) were added to respective dishes. Other additions were heparin (1 μg/ml), *lanes 4*, *6*, *8*, *10*, and *12*; unlabeled $VEGF_{121}$ (2 μg/ml), *lanes 2*, *9*, and *10*; and unlabeled $VEGF_{165}$ (2 μg/ml), *lane 3*. The binding, the subsequent cross-linking of bound growth factor to the cells using 0,25 mM disuccinimidyl suberate, SDS-PAGE of cross-linked samples, and the visualization of cross-linked products were done as described. Equal amounts of protein from cell lysates were chromatographed in each lane. The arrow points at the VEGF binding receptor subsequently identified as neuropilin-1

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Part I

Molecular Mechanisms of Neuropilin Mediated Signal Transduction

Structure of Functional Neuropilin-Centred Class 3 Semaphorin and VEGF Receptors

2

E. Yvonne Jones

Contents

Abstract

Neuropilin functions as a co-receptor for multiple cell surface signalling systems; in particular it regulates VEGF and semaphorin signalling through their respective receptors. The molecular characteristics of neuropilin, the VEGF and semaphorin signalling complexes and their multiple interaction modes have been extensively investigated by structural and biophysical analyses. Much has been learned about the molecular mechanisms by which neuropilin acts as an interaction hub, but the complexities of neuropilin function still pose many questions.

2.1 Introduction

Human neuropilin 1 and 2 (Nrp1 and Nrp2) mediate multiple biological effects though their interactions with the ligands and receptors of the semaphorin and vascular endothelial growth factor (VEGF) signalling systems. The role of neuropilin in these systems is that of a regulator or modulator. Although the neuropilins have the N-terminal ectodomain, single transmembrane region and C-terminal

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cytoplasmic region typical of type 1 cell surface receptors, they lack the classic enzymatic domains required for direct signal transduction, merely containing a cytoplasmic PSD-95/Dlg/ZO-1 (PDZ)-binding motif. Thus, signalling activity is dependent on the binding of the semaphorin or VEGF ligands with their cognate cell surface receptors. Albeit the neuropilins serve as co-receptors, their influence on signalling outcomes can be profound. This chapter reviews our current understanding of how the structural characteristics and interactions of the neuropilins determine biological outcomes in semaphorin and VEGF signalling.

2.2 Neuropilin: Structure of a Co-receptor

The neuropilin ectodomain consists of five distinct domains classically referred to as the a1, a2, b1, b2 and c domains. Crystal structures have been determined for the a1-a2-b1-b2 regions of Nrp1 and Nrp2 [4, 33]. These structures reveal the a1 domain is flexibly linked to a tightly clustered unit of a2-b1-b2 domains (Fig. 2.1). The a1 and a2 domains have the β-sandwich topology of CUB domains. The b1 and b2 domains also have a β-strand-based fold and belong to the family of coagulation factor 5/8 type C domains. To date no structures have been determined for the c domain; however, sequence analysis indicates that it has the β-sandwich-type fold of a meprin/A5/mu (MAM) domain, similar to the structurally characterised N-terminal domain of the receptor protein tyrosine phosphatase RPTPμ [5, 6]. This modular composition of the neuropilin ectodomain, based on structurally stable β-strand folds, provides multiple possible binding surfaces for interactions with ligands and receptors (Fig. 2.1).

Early studies sought to dissect the functional contributions of the a, b and c domains of neuropilin. The interactions of class 3 semaphorins with neuropilins were mapped to the a1-a2-b1-b2 segment [29, 39] as were VEGF-neuropilin interactions [61]. The c (MAM) domain was reported to mediate neuropilin homodimerisation [10, 19, 47]. Interestingly, the MAM domain in RPTPμ contributes to receptor-receptor adhesive interactions [6]; however, the interaction characteristics of the neuropilin MAM domain have not been further characterised. Subsequent structural and functional studies have focused attention on the roles of protein binding sites on the neuropilin a1 and b1 domains. In addition, the interaction properties of the membrane-spanning α helix have been investigated.

2.3 Neuropilin and VEGF Signalling

VEGF signalling occurs through VEGF receptors (VEGFRs), which are type 1 single membrane-spanning receptors belonging to the receptor tyrosine kinase superfamily. VEGFR ectodomains consist of seven Ig-like domains (D1-D7). The VEGFs are homodimeric ligands that can be cleaved to generate an N-terminal dimer which retains VEGFR-binding activity and two monomeric C-terminal tails that bind heparin [34]. The subunits of the N-terminal dimer have cystine knot growth factor-type

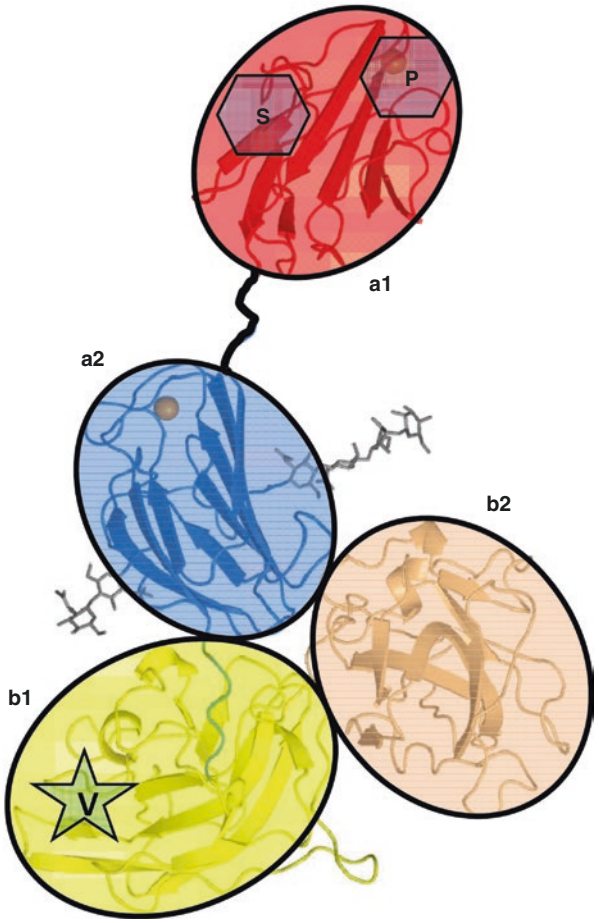

Fig. 2.1 Neuropilin structure and binding sites. Structure of Nrp1 *a1-a2-b1-b2* with interaction sites on the *a1* domain indicated by hexagons (*S* semaphorin binding, *P* plexin binding) and on the b1 domain indicated by a star (*V* VEGF binding) (Adapted from Janssen et al. [33])

architectures and are tightly interfaced with inter-subunit disulphide bonds [46]. The C-terminal tail comprises two, flexibly linked, subdomains [14]. As is common for the receptor tyrosine kinases, ligand binding results in VEGFR dimerisation with consequent activation of the cytoplasmic kinase domains [41]. The VEGF dimer cross-links two VEGFR ectodomains to generate the 2:2 complex (Fig. 2.2a). Structural analyses have detailed the various contributions of VEGFR domains D1 to D7. VEGFR D2 makes a major contribution to VEGF binding [70]. The ligand binding site also includes contributions from the D2-D3 inter-domain linker region and D3 [42], consistent with earlier mapping of domain function [15]. Low-resolution (negative stain electron microscopy and small-angle solution X-ray scattering, SAXS) studies of full-length VEGFR ectodomains in complex with VEGF suggest that VEGF-mediated VEGFR dimerisation at the membrane distal D2-D3 site triggers direct VEGFR-VEGFR interactions involving the middle segment of the ectodomain (D4-D5) as well as the membrane proximal domain, D7 [36, 43, 56]. Functional data support the importance of these homotypic interactions for

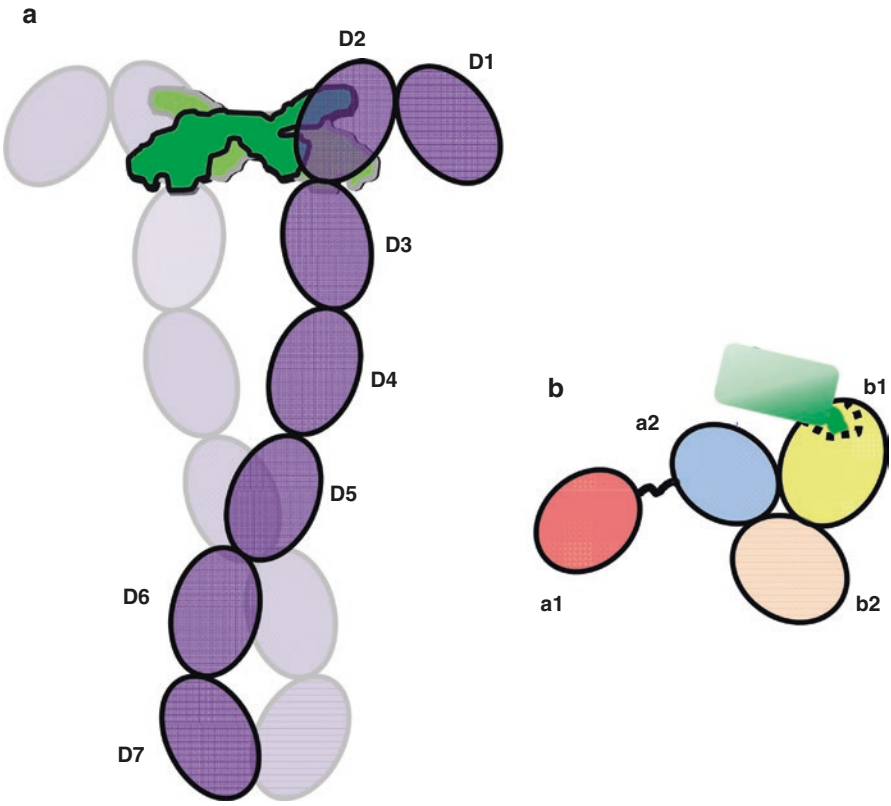

Fig. 2.2 VEGF-VEGFR structure and interaction with neuropilin. (**a**) A composite model of the 2:2 VEGF-VEGFR complex. The VEGF N-terminal dimer is shown in *green* and the VEGFR ectodomains in shades of mauve. (**b**) Schematic of VEGF C-terminal tail region (*green*) binding to neuropilin (domain colouring as in Fig. 2.1)

receptor activation and crystal structures of D7 and D4-D5 have detailed D7-D7 and D5-D5 interaction interfaces [43, 72]. Thus a series of structural studies has built up a model for the extracellular arrangement of the dimeric VEGF ligand and two VEGFR ectodomains in the 2:2 signalling complex (Fig. 2.2a). Neuropilin can modulate this core VEGF-VEGFR signalling complex in a number of ways.

In terms of structural analyses, the VEGF-neuropilin interaction is the best characterised mechanism for interplay between neuropilin and the VEGF-VEGFR system (Fig. 2.2b). The ability of neuropilin to interact directly with VEGF is primarily dependent on the isoform or proteolytically activated state of the ligand. Nrp1 binds specific VEGF-A isoforms, classically VEGF-A_{165} [51, 61]. Nrp2 has been shown to bind to proteolytically activated VEGF-C [53] as well as to particular VEGF-A isoforms [21]. These binding specificities can be understood in terms of the atomic level details of the neuropilin-VEGF interface. Structural studies have revealed that a surface groove in the b1 domain of neuropilin selectively binds linear epitopes that

have a C-terminal arginine [67]. The neuropilin-binding isoforms of VEGF-A and the proteolytically activated form of VEGF-C all have in common a tail region ending in a C-terminal arginine. The specificity of the interaction is fine-tuned by some additional side-chain specific interactions with the neuropilin b1 domain, but the multiple interactions of the C-terminal arginine render this residue the key structural determinant for ligand binding [51]. Typically VEGF may be expected to bind to cell surface-attached neuropilin; however, secreted splice variants have been identified [17, 55]. A secreted Nrp1 isoform comprising a1-a2-b1-b2 has been shown to be able to act as an antagonist of VEGF-A_{165} [17]. A splice variant of Nrp2 truncated within the b2 domain forms a disulphide-linked dimer, each subunit presenting a functional VEGF-binding site, prompting the suggestion that this covalently stabilised dimer may serve as a particularly potent inhibitor of VEGF-C binding to cell-attached Nrp2 [53].

The VEGFR and neuropilin binding sites on VEGF are independent. VEGF can therefore serve as a cross-linker to recruit neuropilin into a heterocomplex with VEGFR [61, 62]. Interestingly, formation of the neuropilin-VEGF-VEGFR complex appears possible either with neuropilin and VEGFR attached to the same cell (i.e. in *cis*) or with neuropilin and VEGFR attached to different cells (i.e. in *trans*), albeit *cis* complex formation is kinetically favoured [38, 62]. The ability to form *cis* and *trans* complexes clearly requires a substantial degree of freedom in the relative orientation of the VEGF-bound neuropilins and VEGFRs. This orientational flexibility might be facilitated by the nature of the VEGF-neuropilin interaction; rather than involving an extended molecular surface, only the C-terminal tail region of VEGF is required to bind to the b1 domain of neuropilin.

The interplay of neuropilin with VEGF and VEGFR is further complicated by the potential for interactions with the glucosaminoglycans (GAGs) of heparan sulphate proteoglycans (HSPGs) and heparin. Both VEGF and neuropilin bind heparin, and heparin has been reported to enhance the VEGF-neuropilin interaction [16]. The heparin binding site on neuropilin has been mapped to a positively changed surface extending over the b1 and b2 domains [67]. VEGFRs also bind heparin, consistent with the notion that cell surface-attached HSPGs can promote the formation and stability of signalling complexes [11–13, 20, 49, 71]. The HSPGs can be presented in *cis* (i.e. on the same cell surface as the VEGFR) or in *trans* on neighbouring cells [31]. Most recently detailed biophysical analyses have shown that VEGF, VEGFR and neuropilin bind synergistically to HSPG, suggesting that the multiple binding modalities of HSPG GAG chains modulate the composition, stability and output of VEGFR-based signalling complexes [65].

The mechanism of action of neuropilin function in VEGF-VEGFR signalling is still not fully understood. As discussed above the extracellular interactions with HSPG and neuropilin can serve to modulate VEGF-VEGFR binding and hence signalling output [25]. However, the PDZ-binding domain in the neuropilin cytoplasmic region can also influence complex formation with VEGFR [54]. Furthermore, although Nrp1 and Nrp2 do not bind $VEGF_{121}$, Nrp1 has been shown to be able to modulate $VEGF_{121}$-induced VEGFR2 signalling, and Nrp2 has been found to interact with VEGFR1 on endothelial cells stimulated by this ligand isoform [22, 59].

Mice have been generated that have Nrp1 bearing a mutation in the b1 groove that selectively abolishes VEGF binding [18]. Intriguingly, these studies suggest that, for developmental angiogenesis, the VEGF-neuropilin interaction is not central to a co-receptor function in the signalling complex, but rather than Nrp1 regulates the cell surface expression of VEGFR2 [18].

2.4 Neuropilin and Class 3 Semaphorin Signalling

Classically, semaphorin signalling is mediated by members of the plexin family of cell surface receptors [64]. Semaphorin ligands are secreted or cell surface attached, either by a single membrane-spanning helix or a GPI anchor. The class 3 semaphorins are the secreted members of the semaphorin family in mammals, namely, Sema3A, Sema3B, Sema3C, Sema3D, Sema3E, Sema3F and Sema3G. Whilst the cell surface-attached classes of semaphorins are able to trigger signalling by direct binding to their cognate plexin receptors, the class 3 semaphorins are not. All of the Sema3s interact with neuropilin and, apart from Sema3E, require a holoreceptor complex, comprising plexin and neuropilin, for signalling [24, 63, 64].

The semaphorins are now well characterised structurally. The eponymous sema domain has at its core a seven-blade β-propeller topology; however, the main chain fold is elaborated by some extensive insertions in the loops linking the β-strands in the so-called blades of the propeller (Fig. 2.3a) [3; 45]. The various classes of semaphorins vary in domain composition, but, immediately after the N-terminal sema domain, all contain a PSI (for plexin-semaphorin-integrin) domain. The PSI domain is a cysteine knot and interfaces with the sema domain to form a relatively rigid unit of some 700 residues (Fig. 2.3a) [45]. The semaphorins are functionally active as dimers [37; 40]. A sema domain to sema domain interface lies at the heart of semaphorin dimerisation, although inter-subunit interactions and disulphide bonds from additional, class-specific domains can also make important contributions to dimer stability [60]. The class 3 semaphorins comprise a sema domain, PSI domain and Ig-like (β-sandwich) domain followed by a basic, C-terminal region. The Ig-like domain contributes an additional homotypic interaction, and the basic tail provides inter-subunit disulphide bonds to stabilise Sema3 dimers [2; 37; 40; 33].

The plexins are type 1 cell surface receptors with N-terminal ectodomain, single transmembrane α-helix and C-terminal cytoplasmic region. The ectodomain contains an N-terminal sema domain, followed by multiple PSI and IPT (Ig-like, plexins, transcription factors) domains. The cytoplasmic region chiefly comprises a distinctive GTPase-activating protein (GAP) domain. The plexin GAP domain is unusual in that it has a Ras GAP-type topology but preferentially functions as a GAP for Rap [68]. The plexin GAP architecture is also novel in having inserted into a loop, midway through the GAP topology, a Rho GTPase-binding domain that protrudes as distinct additional domain [28; 66]. Structural and functional analyses suggest that the plexin GAP domain requires activation by conformational changes that are triggered in the juxtamembrane region as a result of extracellular ligand

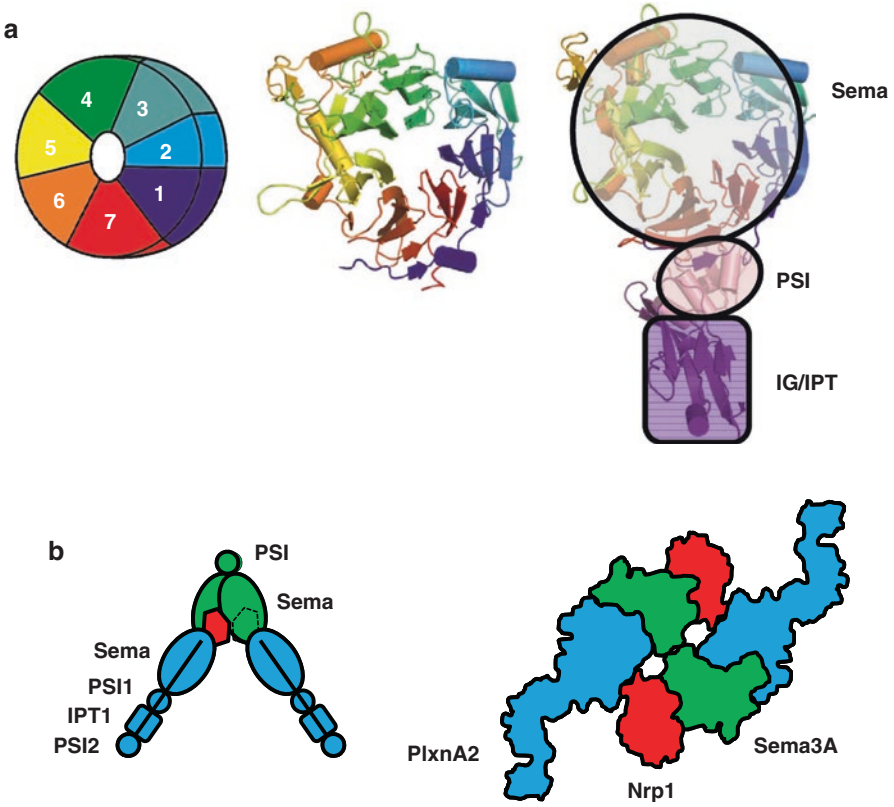

Fig. 2.3 Semaphorin-plexin structure and interaction with neuropilin. (**a**) The sema domain seven-blade β-propeller. The left panel shows a schematic of a seven-blade β-propeller. The middle panel depicts the Sema3A sema domain. The right panel shows a representative sema-PSI-Ig/IPT structure (based on the structure of Sema4D sema-PSI-Ig) (The panels are adapted from Siebold and Jones [60]). (**b**) Schematics (orthogonal views) of the Sema3A-PlxnA2-Nrp1 2:2:2 complex. Only the Nrp1 a1 domain (*red*) is visible

binding and consequent receptor dimerisation [8; 68; 69]. Interestingly, in silico studies indicate that the transmembrane α-helix and juxtamembrane regions of plexins have dimer, and in some cases trimer, forming propensities [73].

The interaction of semaphorin ligand with plexin receptor is entirely through sema-to-sema domain binding [32, 44, 48]. The sema domain of one semaphorin subunit binds 'face to edge' with the sema domain of one plexin ectodomain. This sema-to-sema interaction is repeated by the second subunit of the semaphorin dimer resulting in a 2:2 complex in which the semaphorin ligand cross-links two plexin receptors. The position of the sema-sema interface and 2:2 arrangement of the semaphorin-plexin complex are conserved across classes for cell surface-attached semaphorins and their cognate plexins, consistent with this architecture being central to receptor activation [32, 60]. However, although class A plexins can be

activated by class 6 semaphorins through engagement in the canonical 2:2 complex, these same plexin receptors must be associated with neuropilin for activation by the secreted class 3 semaphorins.

Neuropilin can interact directly with class 3 semaphorins as well as mediating holoreceptor-based complex formation with Sema3s and plexin As. In vitro studies, using purified Sema3A (minus the basic C-terminal region) in surface plasmon resonance (SPR)-based assays, showed direct Sema3A-Nrp1 interaction, but no measureable Sema3A binding to plexin A2 (PlxnA2) [33]. The ability of neuropilin to mediate a stand-alone interaction with class 3 semaphorins is consistent with the recent observation that premature repulsion of axons is prevented by the expression of neuropilin on neighbouring cells. The neuropilin acts as a developmentally regulated 'molecular sink', reducing the local levels of secreted semaphorin encountered by the axon growth cones [30]. SPR assays have also shown direct binding between Nrp1 and PlxnA2 ectodomain segments [33]. Thus for the semaphorin-plexin system, neuropilin does interact directly with both the ligand and the receptor in the extracellular region. The crystal structure of Sema3A-PlxnA2-Nrp1 reveals a 2:2:2 complex at the heart of which is the canonical 2:2 arrangement of dimeric semaphorin ligand cross-linking two plexin receptors (Fig. 2.3b; [33]). Although the interaction of the class 3 semaphorin with the class A plexin is too weak to form a stable 2:2 complex in isolation, two copies of the a1 domain of neuropilin 'glue' the classical ligand-receptor arrangement together. Each Nrp1 a1 domain makes an interaction with the sema domain of one Sema3A subunit and the sema domain of the PlxnA2 that is bound to the other Sema3A subunit. Thus the neuropilin a1 domain specifically acts as a cross-brace to stabilise the 2:2 complex involving semaphorin dimer and two plexins, rather than locking together the 1:1 interaction between a single semaphorin subunit and plexin ectodomain. This simple cross-brace mode of action provides a satisfying mechanism by which neuropilin can function as a co-receptor gating the formation of activated plexin signalling complexes. Interestingly, the architecture of the 2:2:2 complex can serve to homodimerise or hetero-dimerise PlxnAs consistent with functional data indicating that sometimes two different plexins are required to transduce class 3 semaphorin signals [9, 57].

Neuropilin contains additional potential binding sites for both the semaphorin ligand and the plexin receptor. The importance of the a1 domain for neuropilin functions involving semaphorin-plexin signalling has been demonstrated in vivo [23]; however, early studies mapping domains involved in Sema3 binding to neuropilin identified both the sema domain (the site of the a1 domain interaction) and the basic C-terminal region [29]. Class 3 semaphorins contain furin cleavage sites in their basic C-terminal regions, which on proteolysis generate Sema3s bearing a similar C-terminal motif to that used by VEGF for binding to the neuropilin b1 domain [2; 50; 52]. Thus Sema3s can use the same 'C-terminal epitope-to-b1 groove' interaction mode as VEGFs, and potentially can compete with them to bind neuropilin [26, 52], albeit this is not necessarily the only mechanism by which class 3 semaphorins can inhibit VEGF activity [27]. The contributions of the sema and basic C-terminal region to neuropilin binding appear to be additive, consistent with simultaneous

engagement through the two binding modes [29]. The crystal structure of Sema3A-PlxnA2-Nrp1 was determined using a form of Sema3A lacking the basic C-terminal region, the N-terminal four domain segment of PlxnA2 (sema-PSI1-IPT1-PSI2) and the a1-a2-b1-b2 segment of Nrp1. Only the Nrp1 a1 domain was visible in the crystal structure, consistent with the flexible linker between a1 and a2 allowing the a2-b1-b2 unit to sample multiple positions relative to the rest of the complex. This flexibility appears sufficient to allow the neuropilin b1 domain to bind the basic C-terminal region of Sema3 in addition to a1 domain interactions with the core semaphorin-plexin complex. Furin cleavage at various points in the basic tail of class 3 semaphorins (including complete removal) therefore provides two mechanisms by which ligand function can be modulated: firstly, the generation or removal of neuropilin b1 domain binding ability and, secondly, the loss of intermolecular disulphide bridges that contribute to Sema3 dimer stability [2; 37; 40; 50; 52]. Notably, the combination of two flexibly linked binding sites provides neuropilin with an intriguing ability to act as a hub involved in multiple interactions bridging the semaphorin and VEGF systems. Further work is required to explore the molecular interactions that underlie the reported effects on plexin signalling of associations with VEGF receptors [9, 35].

The transmembrane α-helix in plexin and neuropilin also provides a possible region for receptor-receptor interactions, both homophilic and heterophilic. A number of in silico and biophysical analyses support this possibility [1, 58; 7]. The most recent in silico studies have begun to shed light on the interaction properties of the plexin transmembrane plus juxtamembrane region [73] however, our understanding of the interplay of interactions in the full-length transmembrane receptors is in its infancy.

Conclusions

Structural studies have detailed the molecular interaction modes of the neuropilin a1 and b1 domains as well as the overall architecture of the ectodomain. These analyses, combined with biophysical characterisations of binding properties plus functional assays, have started to provide detailed insights into the mechanisms by which neuropilin functions as a co-receptor in the VEGF-VEGFR and semaphorin-plexin signalling systems. Excitingly, the availability of detailed information on interaction sites can guide the design of mutant neuropilins to dissect the functional contributions of selected interactions in vivo (e.g. [18]). The combination of mechanistic insights, gained from detailed molecular level studies, and in vivo analyses offers a route to understanding, and learning how to manipulate, the modes of action of this multipurpose co-receptor.

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Neuropilins as Signaling Hubs, Controlling Tyrosine Kinases and Other Cell Surface Receptors

3

Sabrina Rizzolio and Luca Tamagnone

Contents

Abstract

Neuropilin-1 and neuropilin-2 form a small family of transmembrane molecules found in vertebrates. In addition to their established function as cell surface co-receptors for semaphorins and vascular endothelial growth factors (VEGFs), in association with plexins and VEGF receptors, neuropilins have been found to interact with many other transmembrane receptor molecules (such as EGFR, Met, IGF1-R and PDGF-R tyrosine kinases, TGFb receptor, Hedgehog, integrins, etc.) and elicit a range of intracellular signaling cascades. Thus neuropilins appear to act as signaling hubs on the cell surface, contributing to diverse

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signaling cascades in response to extracellular cues. Beyond their role in neurovascular embryo development, both neuropilins have been widely implicated in adult functions, especially in cancer. In this context, they are found not only in cancer cells but also in cells of the tumor microenvironment, in particular endothelial cells of tumor vasculature and tumor-associated macrophages. Thus, neuropilin function in cancer is multifaceted, because it implicates diverse receptor signaling complexes in different cell populations. In this chapter, we will mainly focus on the role of neuropilin-1 and neuropilin-2 in the regulation of associated signaling molecules in human tumors, beyond plexins or VEGF receptors.

3.1 Introduction

Neuropilins (Nrp1 and Nrp2) are well-known cell surface co-receptors for semaphorins and vascular endothelial growth factors (VEGFs), in complex with plexins and VEGF receptor tyrosine kinases Flt1-VEGFR1, Flk1/KDR-VEGFR2, and VEGFR3 [1–4].

The two neuropilins share similar structure [5]: the extracellular portion contains two complement-like binding domains (a1 and a2 domains), two coagulation factor V/VIII homology-like domains (b1 and b2 domains), and a meprin-like domain (c domain); a single transmembrane domain is followed by a short cytoplasmic tail terminating with a consensus sequence that interacts with PDZ (PSD-95/Dlg/ZO-1 homology) domains. Extracellular "a" and "b" domains are implicated in ligand binding, while "c" domain mediates neuropilin homo- and heterodimerization, which seems to be essential for function. Protein domains responsible for interaction with transmembrane molecules found in complex with neuropilins have not been elucidated.

Nrp1 and Nrp2 show different binding specificity for class 3 semaphorins [6]. Nrp1 homodimers bind with high-affinity Sema3A, while Nrp2 binds Sema3B, Sema3C, and Sema3F, which are also bound with lower affinity by neuropilin-1; Sema3C was shown to bind both neuropilin homodimers and heterodimers. As receptors for both VEGF family members, Nrp1 can bind VEGF-A, VEGF-B, VEGF-E, and placental growth factor 2 (PlGF2), while Nrp2 was reported to interact with VEGF-C and with isoform 145 of VEGF-A. There are contradictory data on the signaling competence of the small conserved cytoplasmic tail of neuropilins. According to many reports, neuropilins essentially provide a ligand-binding platform, while intracellular signaling is mediated by associated plexins or VEGFRs. Other findings, however, suggest an independent signaling function of the intracellular domain of neuropilins [7]. For instance, a cytosolic Nrp1-interacting scaffold protein, GIPC (also known as synectin), was involved in Nrp1-dependent function in angiogenesis [8]. Thus, by interacting with GIPC and potentially additional PDZ domain-containing proteins, neuropilins could regulate receptor complexes in the plasma membrane [9, 10]. Notably, the cytoplasmic tails of Nrp1 and Nrp2 are largely divergent, which raises major questions on whether they may interact with different adaptors or signal transducers.

3.2 Neuropilin Expression and Functional Role in Human Cancers

Neuropilins are well expressed in a wide variety of cancer cell lines and human tumors (summarized in Table 3.1), while commonly low (or absent) in the corresponding normal tissues. There is currently no evidence of functionally relevant genetic changes in NRP1 or NRP2 genes associated with human tumors. However, clinicopathological data often state a correlation between elevated expression of one of the neuropilins and advanced-stage tumors with poor prognosis [55]. For instance, high levels of Nrp1 significantly correlate with a poor outcome in patients with breast cancer and acute myeloid leukemia and correlate with invasive behavior and metastatic potential in gastrointestinal carcinomas, gliomas, non-small cell lung cancers, prostate carcinomas, medulloblastomas, melanomas, hepatocellular carcinomas, oral squamous carcinoma, and osteosarcoma [16, 43, 45, 53, 56]. Nrp1 expression is enhanced in ovarian cancer compared to normal counterparts and to tumors of low malignant potential. In non-small cell lung cancers, patients co-expressing both Nrp1 and Nrp2 have a worse prognosis. Also the expression of Nrp2 is variably correlated with tumor progression and prognosis in human cancer [55], for instance, Nrp2 levels in tumor cells correlate with poor prognosis in breast cancer, colorectal carcinomas, bladder cancer, renal cell carcinomas, pancreatic cancer, gastric carcinomas, invasive breast carcinomas, and osteosarcoma [35, 46, 52]. Notably, in gastrointestinal carcinoid tumors, the *loss* of Nrp2 expression correlates with tumor progression [57].

Table 3.1 Neuropilins expression in tumors

Tumor type	Nrp1	Nrp2	References
Bladder cancer	x	x	[11, 12]
Breast cancer	x	x	[13–15]
Colorectal cancer	x	x	[16–20]
Esophageal cancer	x		[21]
Gall bladder cancer	x	x	[22, 23]
Glioma	x	x	[24–28]
Neuroblastoma	x	x	[29, 30]
Non-small cell lung cancer	x	x	[31–34]
Pancreatic cancer	x	x	[35–40]
Prostate cancer	x	x	[41, 42]
Medulloblastoma	x	x	[43, 44]
Osteosarcoma	x	x	[45–47]
Melanoma	x	x	[48, 49]
Hepatocellular carcinoma	x	x	[16, 35]
Acute myeloid leukemia	x		[50]
Ovarian carcinoma	x		[51]
Gastric cancer		x	[52]
Oral squamous carcinoma	x		[53]
Pituitary adenoma	x		[54]

From the mechanistic viewpoint, it has been shown in renal carcinoma cells that Nrp1 expression helps to maintain an undifferentiated phenotype, as its silencing results in cell differentiation with the acquisition of epithelial markers [58]. In fact, upon Nrp1 downregulation, aggressive cancer cells display reduced migratory and invasive ability in vitro and decreased tumor forming ability in vivo [58–62]. Overexpression of Nrp1, instead, protects from hypoxia- and detachment-induced apoptosis in different tumor cell lines [63, 64]. In general, Nrp1 expression seems to favor and sustain tumor cell viability and proliferation [51, 58–60, 65]. Other reports, however, suggest that it might have the opposite function. For example, the elevated expression of Nrp1 was associated with a more favorable prognosis in colon cancer patients [66]. Nrp1 overexpression in pancreatic carcinoma cells lacking any VEGF receptors has been shown to inhibit cell migration, anchorage-independent growth, and tumor incidence in vivo [67]; however, in another pancreatic cell line, Nrp1 expression promotes tumor growth in vivo and increases tumor cell migration and susceptibility to chemotherapeutic agents in vitro [64]. These discrepancies are currently unresolved. They might reflect cell-type-specific responses and/or the involvement of different signaling pathways. In animal models, Nrp1 expression in cancer cells is furthermore reported to promote tumor angiogenesis, through still debated mechanisms [68]. For instance, according to some studies, Nrp1 exposed by tumor cells might act in trans on the endothelium, by enhancing VEGF-dependent signaling [31, 69–71]. Interestingly, a secreted splice variant isoform of Nrp1 (sNrp1) appears to act as VEGF-A165 antagonist, and it was shown to inhibit tumor growth; in fact, tumors xenografts overexpressing recombinant sNRP1 are characterized by extensive hemorrhages, damaged vessels, and apoptotic tumor cells [72–74].

Nrp2 overexpression promotes colon cancer cell survival and proliferation in vitro and tumor growth in vivo [17]. Conversely, Nrp2 knockdown in pancreatic adenocarcinoma cells or gastric and colorectal carcinomas reduces VEGFR1 signaling, cell survival, migration, invasion, and growth under anchorage-independent conditions in vitro, as well as tumor growth and metastasization in vivo [18, 36]. Nrp2 silencing in prostatic carcinoma cells downregulated VEGFR1/Src/AKT signaling pathway and the levels of the proangiogenic factor Jagged-1, thus diminishing tumor growth and vascular area [18, 36]. Nrp2 silencing in osteosarcomas led to reduced tumor growth, angiogenesis, and metastasis formation [75]. Somewhat differently, Nrp2-depleted invasive renal carcinoma cells formed less metastatic tumors, but did not show differences in primary tumor burden or vascularization [35]. The treatment with an Nrp2-specific blocking antibody reduced the metastatic dissemination of mammary carcinoma and glioma cell lines [76]. Moreover, the growth of triple-negative Nrp2-expressing breast tumors in vivo was significantly inhibited by Nrp2 blocking antibodies, likely due to reduced cancer cell proliferation and not to inhibition of angiogenesis [13]. Nrp2 is anyway also well expressed in the tumor endothelium, e.g., of advanced gastric carcinomas (as well as normal endothelium cultivated with gastric cancer cell lines), where it was shown to mediate cell proliferation and migration in response to VEGF [77]. Moreover, Nrp2 has been associated with lympho-angiogenesis and metastatic dissemination through the lymphatic system [76].

The mechanisms driving neuropilin overexpression in advanced tumors are poorly understood. Notably, a number of environmental signals have been found to regulate neuropilins' expression in tumor cells. Hypoxia, a key regulator of VEGF expression in tissues, leads to increased expression of Nrp1 in neuroblastoma cells but decreased expression in astrocytoma cells [78, 79]. Insulin-like growth factor-1 (IGF-1) and epidermal growth factor (EGF) induce Nrp1 expression in colon cancer cells [68, 70]. In fact, EGFR signaling can upregulate Nrp1 expression in various tumor cells [68, 70, 78, 80], possibly through phosphatidylinositol-3 kinase/Akt and p38/MAPK signaling pathways. In transgenic mice carrying an activated form of the Ras GTP exchanger SOS, it was observed the upregulation of both VEGF and its receptors VEGFR1, and neuropilin-1; in these conditions, skin tumors are formed, dependent on VEGF signals for tumor cell proliferation acting in a cell-autonomous and angiogenesis-independent manner [81]. Moreover, upon malignant transformation of ovarian epithelium with K-Ras oncogene, Nrp1 expression increases [51]. Glycoprotein NMB (GPNMB) is one potential target highly expressed in TNBCs, whose expression in the breast cancer epithelium is predictive of poor prognosis and recurrence-free survival [14]. Notably, Nrp1 expression is elevated in breast cancers overexpressing GPNMB and that its increased expression depends on Akt activity [14]. In turn, Nrp1 mediates GPNMB-induced primary tumor growth, by promoting Akt activity and VEGF/VEGFR2 signaling [14]. Pancreatic cancer cells express higher levels of IL-6 than normal counterparts. IL-6 boosts the secretion of multiple Th2-type cytokines, as well as the expression of VEGF165 and Nrp1 in pancreatic cancer cells [37], and these factors seem to contribute to cell proliferation and angiogenesis.

Nrp2 was found to be a transcriptional target of the canonical Wnt signaling pathway, since TCF4 can bind its promoter region, while Wnt inhibitors downregulated Nrp2 expression in osteosarcomas [75]. On the other hand, Nrp2 expression is under negative control by PAX8, a transcription factor important for thyroid development and differentiation. In fact, PAX8 can bind Nrp2 gene promoter and repress its transcription; in thyroid cancer cells, this leads to reduced proliferation, migration and invasiveness, and reversion of the mesenchymal phenotype [82]. The activity of the tumor-promoting cytokine IL-8 in human pancreatic cancer is associated with the upregulation of VEGF and Nrp2 expression [38]. On other hand, Nrp2 and its ligand Sema3F have been identified as transcriptional targets of p53 in colon carcinoma cell lines, potentially consistent with a negative regulation of cancer cell growth by the Sema3F/Nrp2 axis [83].

3.3 Neuropilins Control Tyrosine Kinases and Other Signaling Receptors on the Cell Surface

While a growing body of evidence supports the relevance of both neuropilins in tumor growth and malignant progression, the implicated molecular mechanisms are still largely unknown. As co-receptors for VEGFs, neuropilins could elicit VEGFR tyrosine kinase activity in cancer cells and cells of the tumor microenvironment (e.g., endothelium, tumor-associated macrophages, etc.) [32, 63, 70, 84]. Moreover, by binding to

neuropilins, secreted semaphorins could compete for VEGF binding sites, as well as trigger plexin-dependent signaling cascades [32, 48, 85–87]. The above aspects are discussed in more detail in other chapters of this book (*Refer to article by* [88]). However, accumulating experimental data suggest that neuropilins' function in cancer is likely not limited to acting as co-receptors for semaphorins and VEGFs, but depends on their broader role as partners of other tyrosine kinases and integrin receptors.

In fact, neuropilins can be seen as signaling hubs on the cell surface, due to their functional interaction with a range of transmembrane receptors, including receptor tyrosine kinases (such as EGFR, IGF1R, Met, TGFbR, etc.), integrins, and cell adhesion molecules. Furthermore, neuropilins have been found to enhance the functional response to multiple secreted factors, such as hepatocyte growth factor, platelet-derived growth factor, fibroblast growth factor 2 (bFGF), and transforming growth factor beta. Notable examples of these signaling cascades regulated by neuropilins are discussed below.

3.3.1 EGF-EGFR Signaling Axis

It has been shown that while Nrp1 silencing impairs growth and viability of cancer cells of different origin, Nrp1 extracellular domain alone is sufficient to rescue this phenotype and trigger the phosphorylation of EGFR and the downstream effectors AKT and MAPK, both in vitro and in vivo [65]. EGFR pathway is frequently activated in human tumors, by gene overexpression or ligand-dependent signaling, and it is pivotally implicated in sustaining cancer cell viability and growth [89, 90]. Interestingly, it was found that Nrp1 can physically interact with EGFR on the cell surface; moreover, in response to EGFR ligands, this association is strengthened and accompanied by formation of receptor clusters, dependent on Nrp1 expression [65]. EGFR oligomerization and clustering is followed by internalization and signaling in endosomal compartments [91, 92], especially relevant for eliciting Akt activity [93]. Notably, Nrp1 depletion impairs EGFR endocytosis and almost abrogates ligand-induced AKT phosphorylation. Moreover, constitutive EGFR activation sustained by autocrine TGFα signaling is dependent on Nrp1 expression in cancer cells [65].

Recently Nrp2 has also been found to regulate surface receptor endocytosis and intracellular trafficking; in fact, Nrp2 depletion in human prostate and pancreatic cancer cells resulted in the accumulation of early endosomes and delayed early-to-late endosome maturation [94]. In particular, Nrp2 depletion impaired ligand-induced endocytic transport of cell surface EGFR and its intracellular degradation, arresting functionally active EGFR in endocytic vesicles [94].

3.3.2 IGF1-IGF1R Signaling Axis

It was reported that Nrp2 regulates the expression of Bmi-1, a transcriptional repressor of the Polycomb group implicated in prostate cancers [95]. In particular, in response to VEGF, Nrp2 signaling upregulates Bmi-1, and this in turn suppresses IGF-1R transcription. Conversely, Nrp2 depletion leads to IGF-1R upregulation and

sustains IGF-1 dependent tumor growth [95]. This Nrp2-dependent regulation of IGF-1R features an appealing target in prostate cancer. In fact, the combined blockade of Nrp2 and IGF-1R resulted in the complete inhibition of tumor formation and triggered caspase-dependent apoptosis [95]. Interestingly, in prostate cancer patient-derived xenografts, high expression of Nrp2 is typically found in nonresponders to the anti-IGF-1R blocking antibody A12. Conversely, when Nrp2 is depleted, the therapeutic response to A12 is significantly increased [95]. These data highlighted Nrp2 as a putative predictive biomarker for anti-IGF-1R therapy.

3.3.3 HGF-Met Signaling Axis

It was reported that the overexpression of Nrp1 in pancreatic cancer cells promotes tumor invasion and HGF-induced c-Met signaling [39]. According to other studies, Nrp1 can associate with c-Met; moreover both Nrp1 and Nrp2 can bind the Met-ligand HGF and enhance HGF-induced endothelial cell migration and proliferation [96]. Nrp1 expression in glioma cells enhances the activation of autocrine HGF/c-Met signaling pathway leading to glioma progression (associated with increased tumor cell survival, proliferation, and angiogenesis) [24]. Intriguingly, in one study the Nrp1 ligand VEGF-A165 has been reported to trans-activate Nrp1/c-Met complex independent of VEGFRs [41]. Thus Nrp1 seems to be linked to the HGF-Met signaling axis, although the molecular details of this interaction have not been clearly elucidated.

3.3.4 PDGF-PDGF-R Signaling Axis

It was reported that Nrp1 promotes the motility of vascular smooth muscle cells (VSMC) in response to PDGF secreted by breast cancer cells [97]. Conversely, Nrp1 silencing impairs vascular smooth muscle cells migration, PDGF-induced PDGF-Rα activation, and p130Cas phosphorylation [98]. Notably, in vascular smooth muscle cells, Nrp1 is modified by chondroitin sulfate O-linked glycosylation, and this promotes PDGF signaling, as point mutation of the implicated residue (Ser612) impairs PDGF-BB-induced migration of VSMCs [98]. Notably, both neuropilins are significantly upregulated following arterial injury, and their silencing reduces neointimal regenerative hyperplasia in vivo, together with the phosphorylation of PDGFα and PDGFβ receptors [99]. A complex formed by Nrp1 and PDGF-R also regulates the migration of mesenchymal stem cells (MSC) and might control the role of these cells in neoangiogenesis and tissue remodeling [100], as well as their recruitment in tumors and differentiation into pericytes [101].

3.3.5 TGFb-TGFbR Signaling Axis

Nrp1 can interact with both ligands and receptors of the TGFb receptor family. In particular, it was reported to bind all three receptors for TGFb, as well as latent and active transforming growth factor (TGF)b1, and even promote the activation of the

precursor latency-associated peptide (LAP)–TGFb1 [102]. In breast cancer cells, Nrp1 acts as a TGFb co-receptor by augmenting canonical Smad2/Smad3 signaling triggered by TGFb-RI [103]. Nrp2 too was found to bind directly TGFb1. Moreover, its elevated expression in colon cancer cells correlates with Snail1, Twist1, and Gli1 levels, transcription factors implicated in epithelial–mesenchymal transition, and a typical program induced by TGFb1; in fact, Nrp2 overexpression empowers TGFb1 signaling, EMT phenotype, and the migratory and invasive ability of cancer cells [17]. Similar findings were observed in lung cancer model [33]. Notably, Nrp2 levels are upregulated upon TGFb1-driven EMT in lung cancer cells, featuring a servomechanism to reinforce TGFb1-signaling cascade in cancer [17]. Prostate cancer cells depleted of Nrp1 are resistant to EMT induction by TGFb since they are unable to inactivate GSK3beta [104]. The role of neuropilins in TGFb1 signaling is thoroughly discussed in another chapter of this book (*Refer to article by* [105]).

3.3.6 Hedgehog Signaling Axis

Abnormal hedgehog (Hh) signaling has been implicated in 30 % of medulloblastomas, the most common childhood brain tumors. Neuropilins silencing reduces Hh signaling in medulloblastomas and other tumors, by downregulating the expression of Hh effector Gli1 [44, 106, 107]. Nrp2 (but not Nrp1) silencing inhibits proliferation in vitro and decreases tumorigenesis in vivo potentiating Hh signaling inhibition alone, thus suggesting that Nrp2 has also Hh-independent functions and features a putative relevant therapeutic target in medulloblastoma [44].

Nrp2-Hh signaling cross talk is also important in aggressive, triple-negative breast cancers (TNBCs) [13]. In fact, TNBC tumor-initiating cells (TICs) are characterized by high expression of a6b1 integrin [108] in complex with Nrp2 [42]. The fraction of TNBCs enriched in integrin a6b1 and/or Nrp2 is more prone to form mammospheres [13, 109], while Nrp2 perturbation dramatically decreases the ability to form mammospheres. This is due to a VEGF-Nrp2-integrin-a6b1 signaling cascade that upregulates the Hh effector Gli1 to induce the expression of Bmi-1, a major transcription factor controlling stem cell renewal [13, 42, 95]. As reported in other models, Gli1-Hh signaling furthermore induces Nrp2 expression and integrin-a6b1 and FAK activity, thus suggesting an autocrine positive signaling loop [13].

Nrp1 too was implicated in association with Hh signaling. In fact, the expression of both sonic hedgehog and the effector Gli1 decreased upon Nrp1 knockdown in aggressive renal carcinoma cells, where this signaling cascade was found to maintain cancer cells in undifferentiated state [58]. Nrp1 was furthermore found to promote hedgehog signaling by directly recruiting the effector molecule phosphodiesterase-4D to the plasma membrane [107]. Notably, sonic hedgehog signaling was found to induce PLGF secretion by stromal cells in medulloblastomas, and this in turn can bind Nrp1 and promote cancer cell renewal and tumor growth [43].

3.3.7 Integrin-Dependent Signaling Pathways

Nrp1 specifically promotes α5β1 integrin-mediated endothelial cell adhesion to fibronectin, which is crucial for vascular development [110]. In fact, Nrp1 interacts with α5β1 at adhesion sites independent of VEGF and Sema3A regulation; moreover, its cytoplasmic domain (by recruiting GIPC adaptor with C-terminal motif) mediates the internalization and recycling of active integrin in Rab5-positive early endosomes, thus supporting endothelial cell migration [110].

As demonstrated in melanoma, αvβ5 integrin seems to be a critical effector of Nrp1 to mediate tumor cell invasiveness and the formation of vessel-like structures (a.k.a. vasculogenic mimicry) [111].

An autocrine signaling loop mediated by VEGF and Nrp1 leads to Gli1 and Bmi-1 dependent transcriptional control of integrin α6B splice isoform, which is necessary for maintenance of breast cancer stem cell properties [109].

Interestingly, integrin-β3 can control Nrp1 activity by its recruitment in focal adhesions, for instance, it can negatively regulate VEGF-mediated angiogenesis by limiting the interaction between Nrp1 and VEGFR2 [112]. In fact, in the presence of αvβ3 integrin complexes, Nrp1 contributed minimally to VEGF-induced angiogenic processes in vivo and in vitro; while β3-integrin deficiency enhanced the formation of Nrp1/VEGFR2 complexes and promoted Nrp1-dependent migration of endothelial cells in response to VEGF. On the other hand, the simultaneous depletion of integrin and Nrp1 inhibited tumor growth and angiogenesis by impairing paxillin activation and focal adhesion remodeling [113].

Nrp1 can also promote tumor growth in vitro and in vivo by increasing the stiffness of extracellular matrix [114]. In fact, it was found that Nrp1 promotes fibronectin fibril assembly by myofibroblasts, by mediating integrin α5β1 activation, intracellular GIPC, and Abl tyrosine kinase signaling. Further, consistent with the functional interaction between Nrp1 and integrins on the cell surface, the extracellular matrix component fibronectin was found to stimulate actin remodeling, endothelial cell migration, and angiogenesis through the recruitment of intracellular Abl tyrosine kinase to Nrp1- and Abl-dependent paxillin phosphorylation [115].

As mentioned above, Nrp2 is in association with α6β1 integrin in breast tumor-initiating cells [42], and this complex interacts with Hh signaling cascade leading to Gli activation (via FAK kinase activity), Bmi-1 expression, and enhanced stem cell renewal.

It was shown that Nrp2, highly expressed by most aggressive renal carcinoma cells, interacts *in trans* with integrin α5 on the surface of endothelial cells; this interaction mediates extravasation and metastasis in xenografts of renal cell carcinomas and pancreatic tumors [35]. Moreover, Nrp2 was found to support lymphangiogenesis (and lymphatic metastasis of colorectal carcinomas cells) not only by VEGF-C/VEGFR3-dependent but also VEGF-C/VEGFR3-independent mechanisms; in particular, by eliciting the association of integrins α9 and β1 in lymphatic cells [19]. Nrp2/integrin α9β1 complexes in turn activate FAK and Rac1 effectors to promote migration and sprouting of lymphatic endothelial cells [19].

3.3.8 Intracellular Effectors

The abovementioned data qualify neuropilins as versatile signaling platforms on the cell surface, potentially capable of regulating a range of receptors in cancer cells, as well as in cells of the tumor microenvironment. Furthermore, neuropilins have been associated with major intracellular effectors, putatively implicating their competence to autonomously trigger signaling pathways in response to the cognate ligands.

For example, it was found that upon Nrp1 knockdown in cancer cells, the pro-survival PI3K-AKT signaling pathway is strikingly attenuated [58, 65], consistent with reduced cell viability, which is partly rescued by silencing the AKT negative regulator PTEN [65]. Notably, Nrp1 has recently been reported to interact and regulate PTEN function in T regulatory lymphocytes (Treg), which play an important role in restraining antitumor immune response [116]. In fact, it was shown that Sema4A-dependent ligation of Nrp1 on the surface of Treg blocks PTEN at the immunological synapse and promotes T_{reg} function.

It was recently shown that also the Nrp2-ligand Sema3F can suppress PI-3K and Akt activity; moreover, this was associated with mTOR/Rictor complex (TORC2) disassembly and mTOR reduced activity. The reported recruitment of PTEN to Nrp2 could partly explain its regulatory effects on this signaling cascade [117]. Another Nrp2 ligand, VEGF-C, was found to inhibit instead the mTORC1 complex; in cancer cells, this was associated with the activation of autophagy, a self-digestive process that can protect cancer cells from death. These data suggest a link between the VEGF-C/Nrp2 axis and cancer cell survival to chemotherapy-induced stress [118].

Nrp1 has been furthermore associated with the downstream activation of transcriptional regulator NF-kB. For instance, Nrp1 silencing or treatment with Nrp1 blocking antibodies diminished NF-kB signaling in breast cancer cell lines and hampered mammosphere formation [119]. Conversely, Nrp1 overexpression promoted NF-kB activation and EMT in oral squamous cell carcinomas, with enhanced invasive and metastatic properties and the acquisition of stem cell features [53]. It is presently unclear how NF-kB activity is regulated downstream to Nrp1.

Finally, VEGF-induced Nrp1-GIPC interaction in keratinocytes (independent of VEGFRs activity) resulted in subsequent GIPC-Syx association. Syx is a RhoGEF that induces RhoA activity, positively regulating cell proliferation in skin cancers [120].

Conclusions and Open Questions

Nrp1 and Nrp2 are now in the focus as versatile transmembrane molecules exposed on the cell surface, capable of controlling the binding of diverse extracellular ligands to their respective receptor complexes, as well as regulating alternative intracellular signaling cascades. Especially in the cancer context, the role of both neuropilins appears to be widespread to all human tumor types and impinge on diverse cancer hallmarks. Thus, their recognized functions have gone far beyond the role of co-receptors for semaphorins and VEGF family members in embryo development. Yet, a number of questions need to be addressed. Of particular interest from the standpoint of this article, is still unexplained the sur-

prising capability of neuropilins to physically interact with a range of cell surface receptors, bearing significantly different protein structure. Moreover the role of the short intracellular domain of Nrp1 and Nrp2, possibly beyond its reported interaction with PDZ domains of scaffolding proteins, remains controversial and needs further elucidation.

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Crosstalk Between Cell Adhesion Molecules and the Semaphorin/Neuropilin/Plexin Signalling

4

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Abstract

Signalling by cell adhesion molecules (IgCAMs) plays diverse and fundamental roles in the formation, maturation and function of the nervous system. Investigations of their mechanisms of action during early steps of the wiring of neuronal circuits uncovered a contribution of the L1CAM subgroup of IgCAMs in axonal responses to Class 3 Semaphorins (Sema3s), which are secreted in vertebrates. L1CAMs were found to interact with Neuropilins (NRPs), the ligand-binding moiety of Sema3 receptor complexes. As such, L1-NRP *cis* interactions were shown to be required for some Sema3s to elicit a neuronal guidance response, while *trans* interactions were found to regulate the nature of the response. From these initial findings, additional contributions and molecular interplay with the Semaphorin signalling have been characterized, which expand the physiological and pathological contexts in which IgCAM/Semaphorin cross-talk might contribute.

4.1 Introduction

Semaphorins are a large family of membrane and secreted molecules present from virus to human. Semaphorins control many aspects of cell behaviours in various contexts from development to physiological and pathological conditions [1–3]. Nevertheless, Semaphorins are best known for their ability to control axon navigation during the wiring of neuronal circuits. Semaphorins have been grouped in eight subfamilies according to their structure and sequence similarities. All Semaphorins contain a Sema domain that is essential for Semaphorin dimerization and receptor binding. With the exception of the secreted Sema3ss (except Sema3E), most of the Semaphorins were shown to bind directly to a family of transmembrane proteins, the Plexins. The extracellular domain of Plexins contains specific protein-protein interaction motifs (Sema, PSI and IPT domains for Ig-like, Plexins and transcription factors), and their cytoplasmic domain is highly conserved. Nine members have been found and classified into four subfamilies, PlexinA (A1–A4), PlexinB (B1–B3), PlexinC (C1) and PlexinD (Dl). Sema3s, present in vertebrates, were found to interact with the Neuropilins (NRPs), NRP1 and NRP2. NRPs are transmembrane proteins initially identified as cell adhesion molecules regulating axon fasciculation in the *Xenopus* retinotectal system [4]. Although they possess a short cytoplasmic domain, this domain appeared to be dispensable for eliciting a Sema3 response, at least in neurons, suggesting the existence of NRP co-receptors [5]. Plexins A and B were found to bind to NRPs and to mediate Sema3 signalling [6–8]. Later Ig superfamily cell adhesion molecules (IgSFCAMs) mainly of the L1 subfamily (L1CAMs) were shown to interact with NRPs and to be essential for Sema3 signalling in neurons. L1CAMs are single-pass transmembrane receptors. Their extracellular domain contains six Ig-like domains and five fibronectin domains, engaging various homophilic and heterophilic interactions with IgCAMs and other adhesion and matrix proteins [9, 10]. The interactions between NRP1 and NRP2 with specific L1CAMs

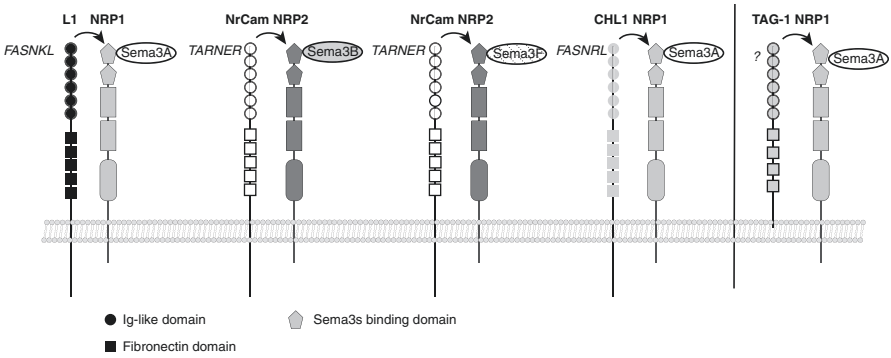


Fig. 4.1 Schematic representation of the interactions between IgCAMs and NRPs. L1, NrCAM and CHL1 are transmembrane proteins, and their extracellular domain is composed of six Ig-like domains and five fibronectin (FN) domains. The interaction with NRPs is mediated by a binding sequence located in the Igl domain. TAG-1 is a GPI-anchored protein which also comprises six Ig-like domains but only four FN domains. Its interaction domain with NRP1 has not yet been characterized

opened new perspectives on how the same Semaphorin could generate various responses and how different signals could be integrated during axon navigation. To date, NRP1 has been reported to associate with L1 [11–13] and CHL1 [14], whereas NRP2 interacts with NrCAM [15–17] (Fig. 4.1; Table 4.1). The amino acid sequence of L1 that binds to NRPs has been mapped to the first Ig-like domain. The CHL1 and NrCAM-binding sequence to NRP was then shown to locate at similar position [14, 16, 18] (Fig. 4.1). NRP1 was also found to interact with another IgCAM, TAG-1, which is GPI-anchored protein and thus lacks transmembrane and cytoplasmic domain [19] (Fig. 4.1).

This chapter reviews, in a first section, the different biological contexts in which L1CAM requirement for Sema signalling and L1CAMs-NRPs interactions have been characterized. Next in the second section, it presents the different signalling that L1CAMs can activate and how they contribute to Sema3 responses. Finally in the third section, it gives an overview of the modulations of the Sema3 responses by IgCAMs and their potential contribution to pathological conditions.

4.2 L1CAMs-NRPs Interactions in the CNS and PNS: From Axon Guidance to Synaptogenesis

The seminal works that led to the discovery that L1CAMs participate in Sema3 signalling were focused on Sema3A-mediated guidance of cortical axons. Since then, neurons from the peripheral nervous system were also demonstrated to use IgCAMs to respond to Sema3s during axon guidance. Thereafter, L1CAMs-NRPs interactions were reported during synapse formation, further broadening the spectrum of biological contexts in which they play a role. Interestingly, in *C. elegans*

Table 4.1 Summary of the interactions between IgCAMs and Neuropilins in the central and peripheral nervous system

	Co-receptor	Ligand	Functional evidence	Biochemical evidence	Binding sequence	System	Phenocopy between KO mice	References
L1	NRP1	Sema3A	Collapse, coculture	Co-IP in vitro and in vivo, binding assay	FASNKL	Corticospinal tract, DRG	No NRP1/L1	Castellani et al., 2000 Law et al., 2008
NrCAM	NRP2	Sema3B, Sema3F	Collapse, coculture	Co-IP in vitro and in vivo, binding assay, co-capping	TARNER	Anterior commissure Thalamocortical pathway Synaptogenesis	Sema3B/NrCAM NRP2/NrCAM Sema3F/NrCAM	Falk et al., 2005 Demyanenko et al., 2011 Demyanenko et al., 2014
CHL1	NRP1	Sema3A	Collapse	Co-IP in vitro and in vivo, co-capping	FASNRL	Thalamocortical pathway	NRP1/CHL1	Wright et al., 2007
TAG-1	L1/NRP1 + NRP1 alone	Sema3A	Collapse, coculture	Co-IP in vitro, binding assay		NGF-dependant sensory neurons (DRG)	NRP1/L1/TAG-1	Gu et al., 2003 Law et al., 2008; Dang et al., 2012
		Sema5B	Electroporation, neurite outgrowth assay			NGF-dependant sensory neurons (DRG)	undertermined-chick model	Liu et al., 2014

that lacks NRPs, the L1CAM LAD2 was shown to interact directly with Plexin and Sema2, contributing to axon repulsion by this Semaphorin. This revealed that interactions between L1CAMs and the Semaphorin signalling evolved prior to vertebrates [20].

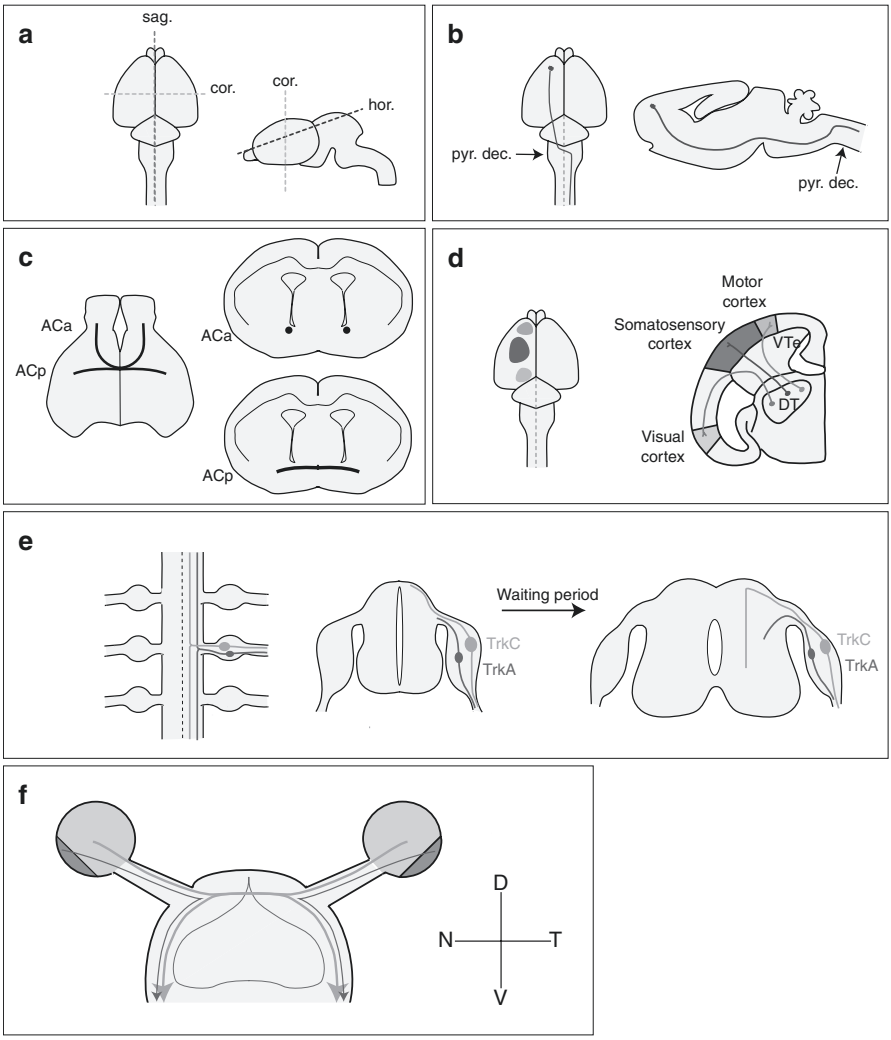
4.2.1 Corticospinal Tract Development

The corticospinal tract principally connects layer five neurons of the motor cortex to interneurons and motoneurons of the spinal cord. During development, corticospinal axons take a ventral route towards the medulla and cross the midline at the junction between the lower medulla and the spinal cord, a process referred to as the pyramidal decussation (Fig. 4.2b). Deletion of L1 in mutant mice leads to several guidance defects including errors in the decussation of corticospinal projections [13, 21, 22]. In situ hybridization experiments showed that Sema3A is expressed in the region between the medulla and the spinal cord, where and when the pyramidal tract operates this midline crossing [11]. Moreover, NRP1 is expressed by pyramidal cells of the cerebral cortex [18, 23]. Coculture assays (Fig. 4.3a) demonstrated in vitro that cortical axons are repelled by a ventral spinal cord-derived signal, supporting the view that during decussation, the change in axon trajectory from ventral to dorsal is controlled by a repulsive cue released by the ventral tissue. This repulsive response was mimicked when the ventral spinal cord tissue was replaced by cell aggregates secreting Sema3A and was lost when neurons were taken from L1-deficient mice. Finally, co-immunoprecipitation assays and binding assays revealed that L1 and NRP1 interact together both in vitro and in vivo [11]. Altogether, these data suggested that the L1-NRP1 complex might contribute to corticospinal axon decussation. This hypothesis was strengthened by the absence of corticospinal phenotypes in mutant mice expressing an L1 protein that cannot interact with L1 and integrin but still interacts with NRP1 [13]. However, analysis of NRP1 and Sema3A mutant mouse models questioned the functional relevance of L1/NRP1/Sema3A signalling in vivo. Indeed, corticospinal axons decussate normally in Sema3A-deficient mice at both early and late stages of development [24, 25]. In mutant mice expressing a form of NRP1 unable to bind Sema3s ($NRP1^{sema-/-}$), corticospinal axons were shown to cross the midline and extend dorsally, although the tract was significantly defasciculated [25]. Thus, if Sema3A contributes to guide corticospinal axons, its loss of function is likely to be compensated by other cues that contribute to pyramidal decussation [25–27].

4.2.2 Anterior Commissure Formation

The anterior commissure is one of the major brain commissures, which interconnects the left and right neurons from the piriform cortex, anterior olfactory nucleus and the amygdala. It is formed by two branches, the anterior and the posterior part, which converge to cross the midline. The anterior part of the commissure (ACa) is composed of axons from the anterior olfactory nucleus and the anterior piriform cortex. The

posterior part (ACp) contains axons from the posterior piriform cortex and the amygdala [15, 28–30] (Fig. 4.2c). The anterior and posterior parts form a compact bundle of axons, which run in the same dorsoventral position. The development of the anterior commissure is controlled by Sema3s because it is almost absent in NRP2 mice and Sema3F-deficient adult mice. When present the remnants of AC are defasciculated and mislocalized [31, 32]. The deletion of Sema3B, another ligand of NRP2, also disrupts the formation of the anterior commissure. This deletion results in defasciculation; aberrant positioning of both ACa and ACp, which no longer run in the same dorsoventral plane; and the lateral shift of the ACa [15]. Sema3B expressed in the sub-ventricular zone of the lateral ventricle was proposed to position the ACa through an attractive effect, which was confirmed by coculture assays of ACa explants



and Sema3B-HEK cell aggregates. Expression of Sema3B ventrally to the ACp was also reported to control the position of ACp, through an opposing repulsive effect. Interestingly, some of the defects of the Sema3 mutants, such as defasciculation, invasion of ventral territory by the ACp and lateral shift of the ACa, were recapitulated in NrCAM-deficient mice [15]. Consistently with the phenotypic similarities, coculture assays revealed that NrCAM is required for the attractive and repulsive responses exerted by Sema3B on axons from the ACa and ACp, respectively, as well as for the guidance effects exerted by the lateral ventricle border and the ventral territory [15]. Moreover, the collapse response of growth cones (Fig. 4.3b) that reveals the repulsive effect of Sema3F depends on NrCAM for both ACa and ACp axons. Finally, similarly to L1 and NRP1, NrCAM was shown by co-immunoprecipitation experiments to be associated with NRP2 in cell lines and in embryonic brain extracts. Binding assays (Fig. 4.3c) confirmed the interaction and demonstrated that the NRP2-NrCAM interaction involves NRP2 and NrCAM ectodomain and that the L1CAMs neither bind the Sema3 ligands nor the PlexinAs [15].

4.2.3 Navigation of Thalamocortical Axons

The thalamocortical pathway refers to the route taken by axons to interconnect the different nuclei of the thalamus with specific areas of the cerebral cortex. Recent studies have demonstrated that the ventral telencephalon (VTe) acts as an intermediate target ensuring the proper targeting of thalamic axons to the appropriate cortical area [33, 34]. Sema3A is expressed in the VTe in a high caudal to low rostral gradient [35, 36]. Furthermore, in NRP1$^{sema-/-}$ mice, in which the cell responses to Sema3A are specifically abrogated, thalamocortical projections are disrupted, causing a subset of somatosensory thalamic axons to turn caudally within the VTe [14].

Fig. 4.2 Reported biological contexts of IgCAM/Semaphorin crosstalks. (**a**) Schematic representation of the different planes of the brain. *cor* coronal, *sag* sagittal, *hor* horizontal. (**b**) Corticospinal pathway represented in front and side view. Neurons located in layer five of the cerebral cortex project ventrally towards the spinal cord and cross the midline in the caudal medulla, forming the pyramidal decussation (*pyr dec*). (**c**) The anterior commissure represented in horizontal and coronal view. The anterior commissure is composed of two branches, the anterior (*ACa*) and posterior part (*ACp*), which converge and cross the midline. The commissure interconnects the piriform cortex, anterior olfactory nucleus and amygdala. (**d**) Thalamocortical pathway represented in front and horizontal view. Neurons located in the dorsal thalamus (*DT*) project their axons through the ventral telencephalon (*VTe*) towards their specific area in the cortex. The different thalamocortical tracts are presorted in the VTe. (**e**) Axon trajectory of dorsal root ganglia (*DRG*) neurons represented in front and coronal view. All developing DRG afferents project to the dorsal root entry zone (DREZ – *middle panel*). During the waiting period, they extend longitudinally, in both rostral and caudal directions (*left panel*) before invading the spinal cord. NGF-dependent (TrkA$^+$) afferents project in the dorsal horn of the spinal cord, whereas NT3-dependent afferents (TrkC$^+$) project in the ventral horn (*right panel*). (**f**) Optic chiasm represented in horizontal section. Retinal ganglion cell (RGC) axons arising from the ventrotemporal crescent project ipsilaterally, whereas RGC arising from all other regions of the retina project contralaterally. *D* dorsal, *V* ventral, *T* temporal, *N* nasal

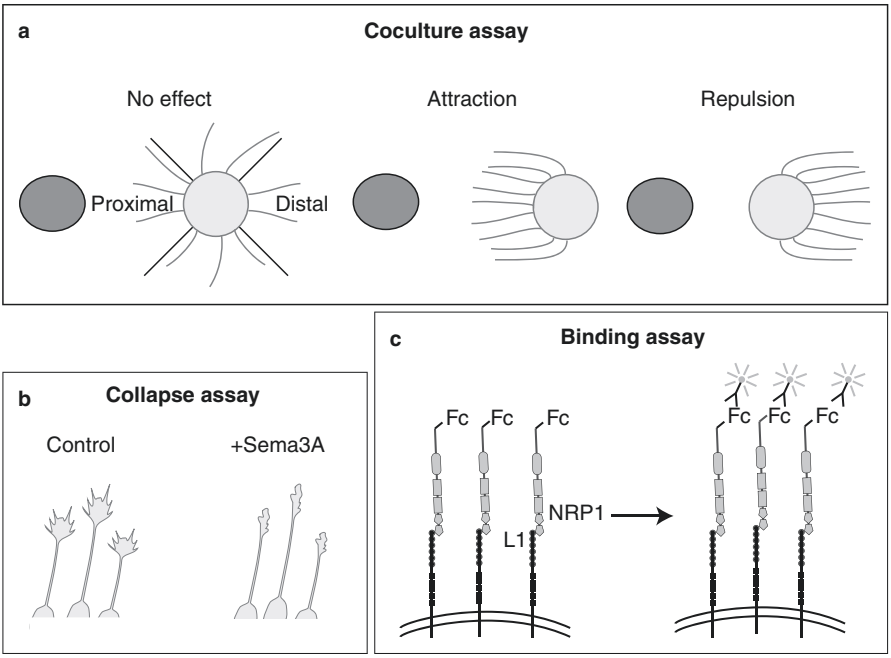


Fig. 4.3 Principal experimental assays used for studies of IgCAM/Semaphorin crosstalks. (**a**) Coculture assay: small pieces (explants) of neural tissue (*light grey*) are grown adjacent to an aggregate of COS7 or HEK-293 cells expressing a specific Semaphorin (*dark grey*). Depending on the Semaphorin effect, axons grow towards the cell aggregate, (*middle panel*) or away (*left panel*). To analyse the attractive or repulsive effects, the number and length of axons growing in proximal and distal quadrants to the Semaphorin source (*left panel*) are measured and compared by statistical analysis. In the coculture, the cell aggregate can be replaced by a tissue explant, to test its potential of attraction or repulsion. (**b**) Collapse assay: dissociated neurons or explants are exposed to a soluble form of Semaphorin during variable period of time, generally ranging from 5 to 60 min (here Sema3A). The cultures are fixed and stained, and the percentage of collapsed growth cones (*right panel*) is then counted and compared to a control (*left panel*). (**c**) Binding assay: COS7/HEK-293 cells transfected to express a given NRP or IgCam (here LI) are exposed to a soluble form of NRP or IgCam (here NRP1), often conjugated to an artificial epitope. After incubation and fixation, the culture is stained by immunocytochemistry. The intensity of staining is proportional to the binding kinetic of the recombinant protein to the transfected NRP/lgCam, and binding affinities can be calculated by Scatchard analysis

This error is phenocopied in CHL1 mutant mice, in which thalamocortical axons also turn caudally and misproject to the visual cortex. This suggested that CHL1 is needed for the control of the proper targeting of thalamocortical axons in response to Sema3A signalling. In support, an interaction between CHL1 and NRP1 was demonstrated both in vitro and in vivo by co-capping assay and co-immunoprecipitation. Furthermore, in vitro, collapse assays demonstrated that CHL1 is responsible for the Sema3A-induced growth cone collapse, and this response is mediated by its interaction with NRP1. Thus, the CHL1/NRP1 complex might be necessary for the responsiveness of thalamocortical neurons to the

gradient of Sema3A expressed in the ventral telencephalon, to ensure the proper targeting of these axons to the somatosensory cortex [14].

L1, which also binds to NRP1, is expressed in the dorsal thalamus and is localized on axons navigating in the VTe [37–39]. Nevertheless, L1-deficient thalamocortical axons do not exhibit the topographic errors observed in the CHL1 mutant mice, although their fasciculation is impaired [37, 39]. However in addition to the somatosensory caudal shift specifically observed in the CHL1 mutant, a caudal shift of motor thalamic axons occurs in mice having double deficiency for L1 and CHL1 [37]. This phenotype was not considered to result from alteration of the Sema3A signalling but rather from impaired thalamocortical responses to another guidance cue, EphrinA5 [37]. However, part of the topographic phenotype resulting from altered Sema3A signalling could be L1-NRP1 dependent. Indeed, L1 and CHL1 distributions along thalamic axons navigating in the VTe appear not strictly similar [37]. Moreover, some thalamic axons still respond to Sema3A although lacking CHL1, but their identity (motor or somatosensory) has not been not determined [14].

Similarly to Sema3A, Sema3F is also expressed in a high caudal to low rostral gradient in the VTe [17, 36]. Disruption of the Sema3F receptor, NRP2, leads to a caudal shift of a subset of somatosensory and motor axons within the VTe. In the same way, NrCAM mutant mice phenocopy this default, leading to the improper targeting of somatosensory and motor axons to the visual cortex. Collapse assays demonstrated that NrCAM is needed for responsiveness of thalamocortical axons to Sema3F. Finally in this system too, an interaction between NrCAM and NRP2 was shown both in vitro and in vivo. Thus, these studies established that NrCAM associates with NRP2 and ensures the correct responsiveness of thalamocortical axons to the gradient of Sema3F expressed in the VTe. This mechanism is necessary for proper targeting of the somatosensory and motor cortex [17].

4.2.4 Navigation of Somatosensory (DRG) Neuron Axons

The population of somatosensory neurons, forming the dorsal root ganglia (DRG), is composed of several subtypes. During development, the afferences of all DRG neurons project in the dorsal root entry zone (DREZ) where they bifurcate both rostrally and caudally and extend over several segments, forming the dorsal funiculus and Lissauer's tract. This period during which the axons extend longitudinally without invading the spinal cord is known as the "waiting period". It lasts about 48 h and ends with DRG axon collateral entry into the grey matter [40, 41]. Then, depending on the neuronal subtype, collateral branches target different regions of the spinal cord: NGF-dependent ($TrkA^+$) afferents project to the dorsal horn of the spinal cord, whereas NT3-dependent ($TrkC^+$) afferents project to the ventral horn [42] (Fig. 4.2e). Semaphorin3A and NRP1 have been proposed to regulate the waiting period by preventing premature entry of collateral branches into the spinal cord. Moreover, Sema3A is expressed in the ventral spinal cord by the time sensory axons extend into the spinal cord [43, 44]. Deletion of Semaphorin3A or its receptor

NRP1 in mice leads to an aberrant collateral projection of NGF-dependent axons to the ventral spinal cord [45, 46]. Interestingly, the loss of TAG-1 and L1 in mutant mice also leads to premature entry of NGF-dependent afferents to the dorsal horn of the spinal cord [12] suggesting that TAG-1 and L1 could contribute to the control of collateral extension of sensory neurons by Sema3A. In vitro, coculture assays and collapse assays (Fig. 4.3a, b) with tissue from mutant embryos showed that TAG-1 or L1 deletion results in a loss of responsiveness of NGF-dependent neurons to the repulsive effect of Sema3A [12]. In vitro, binding assays and co-immunoprecipitation assays (Fig. 4.3c) demonstrate that TAG-1 interacts directly with both L1 and NRP1 and the formation of a TAG-1/L1/NRP1 complex can also be observed [12, 19]. Thus, NGF-dependent neurons require both L1 and TAG-1 for their responsiveness to Sema3A signalling.

In addition to this, TAG-1 has been shown to be involved in the response of NGF-dependent sensory neurons to Sema5B in the model of the chick embryo [47]. As was the case with Sema3A, Sema5B is expressed in the spinal cord at the time of sensory afferent outgrowth. In vivo knockdown of Sema5B leads to premature entry of NGF-dependent afferents in the spinal cord. Together with some in vitro and in vivo experiments, it demonstrated that Sema5B inhibits NGF-dependent neuron outgrowth [47, 48]. Furthermore, in vitro inhibition of TAG-1 with a blocking antibody could reduce the inhibitory effect exerted by Sema5B, suggesting that TAG1 participates to the Sema5B effect [47]. The nature of the interactions linking TAG1 to Sema5B has not been characterized yet.

4.2.5 Role in Synaptogenesis

Extensive studies have established major contributions of L1CAMs to synapse formation and function [49–51]. More recently, Sema3s also emerged as regulators of the synaptic maturation and physiology, which made it a very interesting context to investigate the interplay between IgCAMs and Semaphorins [52–55].

Sema3F was found to control the number of postsynaptic elements or spines built on apical dendrites of cortical neurons in both the somatosensory and visual cortex [16, 55]. In the absence of Sema3F or NRP2, the density of spines increases. NrCAM-deficient mice exhibit the same phenotype [16]. Furthermore, heterozygotes for both NrCAM and Sema3F show an increase of the spine number that neither the NrCAM nor Sema3F heterozygote animals exhibit. In agreement with a genetic interaction, as suggested by the phenotype of the double heterozygotes, NrCAM was shown to be required for the Sema3F-induced reduction of the dendritic spine number in vitro [16]. These changes are associated with modifications of neurotransmission that can be measured with electrophysiological recordings. Indeed, Sema3F and NrCAM-deficient mice exhibit an increased frequency of excitatory postsynaptic potentials [16, 55]. Finally, an NrCAM-NRP2 complex was found in the postnatal brain, further supporting the role of NrCAM as a component of the Sema3F receptor in synaptogenesis [16].

4.3 Semaphorin Signalling via IgCAMs

Several findings support that IgCAMs of the L1 subfamily transduce Semaphorin3 signals. First, L1CAMs of the L1 subfamily are crucial for the response of specific axons and synapses [11, 15, 56, 57]. Second, the response to Sema3A requires the cytoplasmic domain of L1 further supporting the existence of L1CAM-dependent signal transduction [57]. Third, L1CAMs can mediate Sema3s signalling independently of Plexins. L1CAMs can form complexes with NRPs in the absence of Plexins [11, 15, 56, 57]. L1/NRP1 receptor complex was demonstrated to be sufficient to mediate collapse response of COS7 after Sema3A addition [56]. This raised the question of the signalling mechanisms that L1CAMs initiate to allow the responsiveness of the growth cone to Sema3s. Growth cone responses to axon guidance molecules depend on dynamic and coordinated changes to the cytoskeleton, cell adhesion complexes and membrane trafficking. IgCAMs of the L1 subfamily can control these aspects in several ways. Studies on L1CAMs underscored their ability to anchor to the actin cytoskeleton and to be internalized after homophilic and heterophilic interactions. L1CAMs also stimulate kinases and GTPases [58–60] (Fig. 4.4). In the following section, we review the different interactors and discuss their roles during cell responses to Sema3s.

4.3.1 IgCAMs Coupling to Actin Cytoskeleton

First, L1CAMs could regulate growth cone behaviour via their interactions with F-actin. The coupling of L1CAMs to the cytoskeleton has received a lot of attention as it was suggested to generate the mechanical forces that pull the growth cone forward. Myosin-dependent retrograde flow of F-actin is thought to generate forces when adhesion molecules, which bind to F-actin and immobilized extracellular ligands, impede F-actin movement. This hypothesis referred to as “the clutch hypothesis” was pioneered by work on the *Aplysia* IgCAM; however, both NrCAM and L1 were shown to be coupled to actin retrograde flow [61–67].

4.3.1.1 Interactions with PDZ-Containing Proteins

NrCAM was shown to interact via its C-terminus with the PDZ proteins, Dlg3/SAP102, Dlg/SAP90/PSD95 and SAP97 [68, 69] (Figs. 4.4c and 4.5). These proteins belong to the synapse-enriched MAGUKs and act as scaffolds to localize and organize receptors such as glutamate receptors and adhesion molecules at the synapse. PSD95 is a key component of the complexes anchoring transmembrane receptors to the actin cytoskeleton [70]. SAP102 controls NMDA and AMPA receptor localization during synaptogenesis, and this role is taken over by PSD95 during maturation [71]. NrCAM interaction with SAP102 and/or PSD95 has been suggested to contribute to the remodelling of dendritic spines induced by Sema3F [16]. At present, whether L1 or CHL1 can bind to synaptic MAGUK is not known, but they do not bind to the specific MAGUKs that were shown to interact with NrCAM [68, 69].

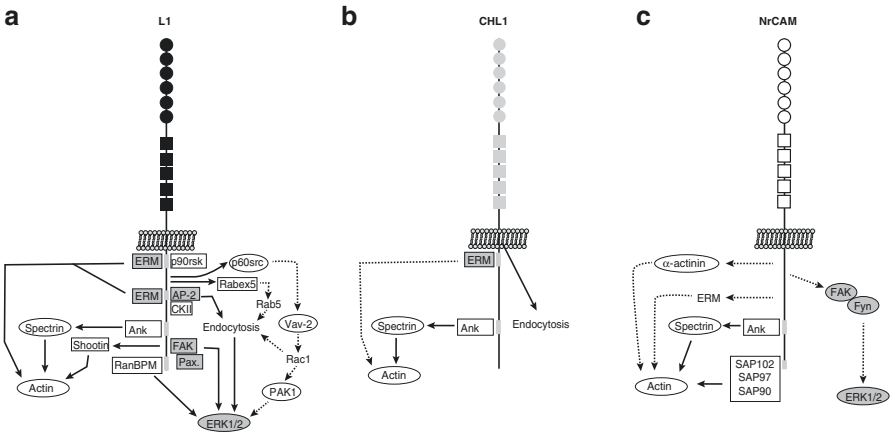


Fig. 4.4 Summary of the interactors and signalling pathways of the L1CAMs shown to interact with NRPs. Direct interactors are schematized with rectangles. Signalling molecules that are not known to interact directly but are activated are represented in ovals. Rectangles and ovals are filled in grey when the molecules were demonstrated to be involved in L1CAM-dependent signalling induced by Sema3s. Plain arrows depict experimentally demonstrated pathways. Dotted arrows were placed when interactions are inferred from the literature. Abbreviations: *ERM* ezrin/radixin/moesin, *Pax.* Paxillin, *FAK* focal adhesion kinase, *CKII* casein kinase II, *Ank* Ankyrin

4.3.1.2 Interactions with Ankyrins

Ankyrins are large cytoplasmic proteins that anchor transmembrane proteins to the actin cytoskeleton via spectrin [72]. $Ankyrin_G$ and $Ankyrin_B$ genes are expressed in the developing nervous system and both can interact with L1CAMs. Ankyrins bind to a well-conserved region of the cytoplasmic domain of L1CAMs [60, 73, 74]. The ankyrin-binding motif contains two conserved tyrosines. Phosphorylation of the tyrosine in the SFIGQY sequence prevents ankyrin recruitment [74, 75] (Fig. 4.5). Phosphorylation and thereby ankyrin binding can be modulated by different factors and is developmentally regulated [75]. Ankyrin-L1CAM interactions are crucial for L1CAMs compartmentalization to the axon initial segment, the paranode of myelinated axons and to some synapses [76, 77]. Whether Ankyrins contribute to L1 function during axon formation is still debated but some data suggest it is likely. $Ankyrin_B$ and L1 knockout mice exhibit similar abnormalities affecting the development of some axon tracts such as the corpus callosum [78]. In addition, expression in knock-in mice of an L1CAM mutant that cannot interact with Ankyrins disturbs the topographic organization of the retinal axons in the colliculus [79]. A possible mechanism would be that Ankyrin regulates the membrane mobility of L1CAMs in developing axons. L1 coupling to the retrograde flow does not rely directly on Ankyrin in growth cones but does in lamellipodia [66]. In addition, in neuronal cell lines, Ankyrin binding seems to favour anchoring of L1 to stationary actin and prevents the coupling of L1 to actin retrograde flow [65]. Ankyrin might be responsible for actin-dependent retrograde mobility of NrCAM as it depends on the last 70 amino acids of the L1 cytoplasmic domain, which includes the ankyrin-binding site [64]. Ankyrin could control membrane mobility and coupling of L1CAMs to the F-actin retrograde flow directly or indirectly in a

Fig. 4.5 Alignment of the cytoplasmic domains of mouse L1, CHL1 and NrCAM. Transmembrane domain is in *light grey*. *Plain lines* signify that the motif or region was demonstrated to mediate interaction. *Dotted lines* are used to highlight sequence conservation of motifs characterized on other L1CAMs but not yet demonstrated to be functional on the considered L1CAMs. When known, the amino acids that define the consensus are highlighted in colour. The interactors are indicated, except for two putative motifs present on the NrCAM sequence. The motif boxed in pink corresponds to an internalization consensus; the sequence boxed in orange is the homologue of the Ezrin-binding sequence of neurofascin. The Ankyrin-binding region is shown with a blue line, and the FIGQY motif that crucially controls Ankyrin/L1CAM interaction is boxed. Asterisk (*) indicates the conserved cysteine that is palmitoylated on L1CAMs

context-dependent manner. In conclusion, it would be interesting to evaluate the role of Ankyrins in Semaphorin-induced responses found to require L1CAMs.

4.3.1.3 Interactions with Ezrin

Ezrin belongs to the 4.1 protein family, which binds to membrane receptors through the N-terminal FERM domain and to F-actin via the C-terminal domain. Ezrin was found to bind L1 on two sites, one juxtamembrane sequence matches the consensus ERM-binding motif found in other CAMs, and a second region encompassing the RSLE sequence [80–83] (Fig. 4.5). Interestingly both regions appear to be required for the binding of Ezrin to the native L1 cytoplasmic tail [82]. Although most of L1CAMs can interact with Ezrin, the interaction does not rely on the same sites. CHL1 cytoplasmic region only contains the juxtamembrane Ezrin-binding domain [84]. Ezrin binds to another motif on neurofascin [83]. Ezrin also co-localizes with NrCAM [60]. However, no direct interaction was found between NrCAM and Ezrin despite the fact that NrCAM cytoplasmic sequence contains all three characterized Ezrin-binding motifs [83] (Fig. 4.5). The recruitment of Ezrin to NrCAM could be mediated by EBP50, a PDZ-containing protein that bridges Ezrin to other transmembrane proteins [85]. Ezrin appears as a key mediator of L1CAMs-mediated responses to Sema3A. First, Sema3A dynamically regulates Ezrin activation. Second, the CHL1 mutant that fails to interact with Ezrin cannot trigger Sema3A-induced collapse response or growth inhibition [84]. Third, inhibition of Ezrin blocks the L1-dependent collapse response to Sema3A and disturbs the axon orientation of cortical neurons induced by Sema3A [86]. L1CAM interaction with Ezrin could regulate T-actin dynamics. Ezrin was shown to couple L1 to F-actin flow suggesting that it could control the

generation of traction force [82]. However, other proteins, such as Shootin1, can also couple L1 to F-actin retrograde flow in the growth cone and regulate L1-dependent outgrowth [67]. In addition, Ezrin binds the F-actin barbed end and could favour F-actin depolymerization [86]. At present, although it is clear that Ezrin is an important mediator of the Sema3A response, whether or not Ezrin function relies on the direct regulation of actin dynamics is not clear and deserves further investigation.

4.3.2 Regulation of Endocytosis by L1CAMs

Endocytosis is a key event, taking place during the response to Semaphorins. Endocytosis is required for growth cone collapse but also during growth cone turning and adaptation to Sema3A [56, 87–91]. Similarly, L1 internalization in the growth cone has been known for a long time [92–94]. Intriguingly after Sema3A treatment, both NRP1 and L1 disappear from the surface of the growth cone and co-localize within early vesicles soon after [19]. Furthermore, L1 endocytosis appears to be necessary because treatments that block L1 internalization also inhibit Sema3A responses [12, 19, 56, 86].

4.3.2.1 Clathrin-Mediated Internalization

The L1 cytoplasmic tail contains a YRSL sequence which binds to the clathrin adaptor AP2 and supports L1 internalization in the growth cone [92, 93] (Fig. 4.5). L1 internalization induced by Sema3A seems to be clathrin dependent. First, L1 co-localizes with clathrin heavy chain after Sema3A treatment. Second, the phosphorylation of the YRSL motif, which prevents AP2 binding to L1, decreases after Sema3A application [19, 95]. Third, the N-terminal domain of Ezrin, which binds to the same YRSL motif, inhibits L1 and NRP1 internalization. This suggests that the transient decrease of the interaction between L1 and Ezrin could facilitate AP2 recruitment and L1 internalization [86] (Fig. 4.6). Clathrin-dependent internalization could regulate different aspects of the growth cone response to Sema3A. First, the clathrin-dependent internalization mediated by L1 could control the growth cone surface. It was estimated that clathrin-mediated endocytosis supports removal of 1.8 % of the growth cone surface per minute and thereby contributes substantially to the membrane removal required for the growth cone to turn [87]. Second, L1-mediated internalization could control the cell surface levels of receptors and/or adhesion molecules. In cancer cells, L1 was shown to facilitate β1 integrin internalization, and this effect requires the AP2-binding sequence on L1 [98]. Third, L1 internalization could control key intracellular events. It is now wildly accepted that the endosomes are endowed with important signalling functions [99]. L1 supports ERK activation only after its internalization [94, 100]. Interestingly, the Sema3A response relies on MAPK [57, 101] suggesting that L1 signalling after internalization could contribute in this way (Fig. 4.6).

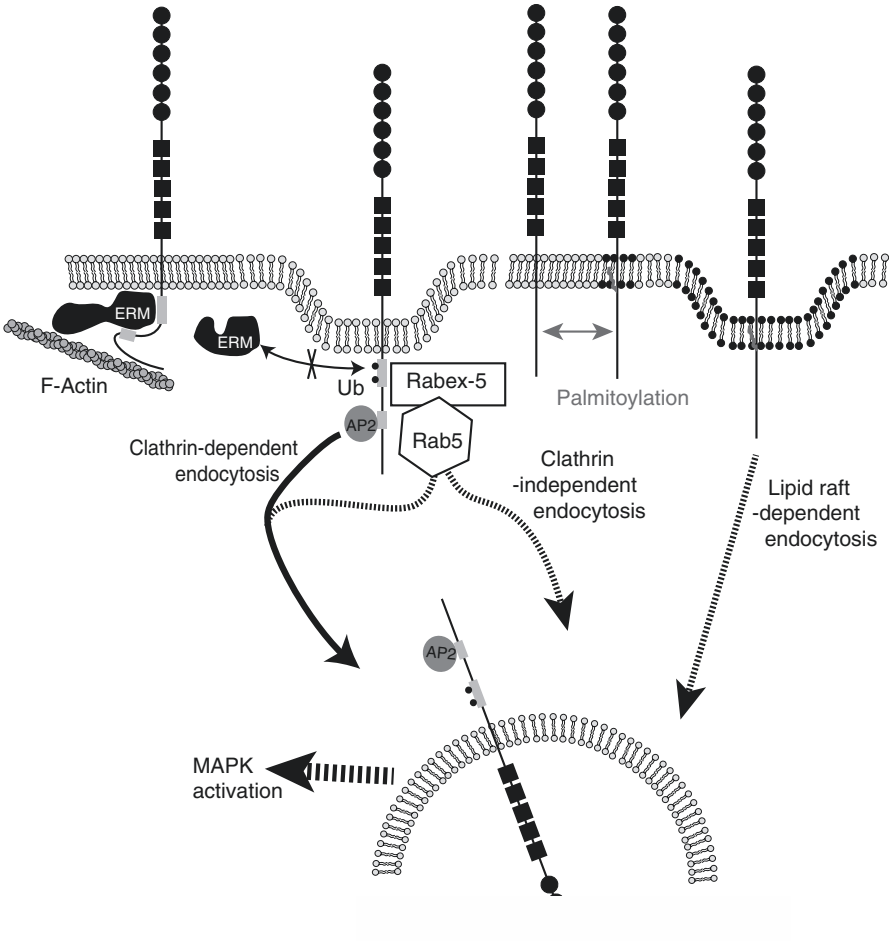


Fig. 4.6 Functional model of L1 internalization. Ezrin binds to juxtamembrane domain of L1 and could compete with AP2 binding. When bound to Ezrin, L1 would be coupled to F-actin (retrograde flow). Ubiquitination of the lysines present on the juxtamembrane domain of L1 could impair Ezrin interaction and as a consequence release the AP2 motif [96]. This would be consistent with the lack of AP2 and active Ezrin in growth cones and the Ezrin inhibitory effect on L1 internalization [86]. AP2 would then bind to L1 and enable clathrin-mediated internalization. In addition, when ubiquitinated, L1 interacts with Rabex-5, which facilitates Rab5-mediated endocytosis and trafficking [97]. L1 internalization would lead to MAPK activation. L1 could also be palmitoylated and partitioned to lipid raft (*in black*) and be internalized via lipid raft-mediated pathways. Abbreviations: *ERM* ezrin/radixin/moesin, *Ub* ubiquitin

4.3.2.2 Clathrin-Independent Endocytosis

It is important to consider that clathrin-independent mechanisms are also required for the growth cone to respond to Semaphorin 3A. Indeed, this response was demonstrated to rely on macropinocytosis and lipid raft-mediated internalization [90, 91, 102, 103]. In some cases, it was even shown that clathrin-dependent

internalization is dispensable [91, 102]. The specificities and the different contributions of clathrin-dependent and clathrin-independent endocytosis to the Sema3A response are not yet known. However, different internalization pathways could be induced, depending on the doses, the responses or the neurons considered. In agreement, the contribution of clathrin-mediated endocytosis to Sema3A internalization was observed to increase in late-born cortical neurons [91]. Although not documented for the Semaphorins, it has been shown that EGF receptor internalization switches from a clathrin-dependent to a lipid raft clathrin-independent pathway when EGF concentration increases [104]. Alternatively, it could also be that different pathways are sequentially induced to trigger different aspects of the response. In support, NRP1 was first found in endosomes derived from the clathrin-dependent route and then sorted into lipid raft-rich endosomes. This sorting depends on the GPI-anchored IgCAM TAG-1, which is crucial for Sema3A response [19].

Whether L1 could mediate clathrin-independent internalization in response to Sema3A is not yet demonstrated. Nevertheless, L1 is still internalized when the YRSL motif is absent or mutated indicating that L1 can be internalized via other pathways [97, 105]. In addition, L1 was recently shown to interact with Rabex-5 and this interaction controls its endocytosis [97]. Rabex-5 is a guanine nucleotide exchange factor (GEF) for the GTPase Rab5. Rab5 regulates biogenesis, transport and fusion of early endosomes [106, 107]. Rab5 is found on both clathrin-coated vesicles and macropinocytosomes [108–110], appears to regulate endosome formation within the growth cone [111] and was recently found to mediate Sema3A-induced collapse [112]. Thus, a Rabex-5/Rab5 could support a clathrin-independent endocytosis of L1 in the growth cone (Figs. 4.4 and 4.6). The L1 transmembrane domain contains the cysteine that is palmitoylated and mediates lipid raft partitioning of neurofascin [113] (Fig. 4.5). Thus, L1 could be enriched in lipid rafts, as other L1CAMs, and be internalized via this entry route (Fig. 4.6). L1 could indirectly trigger Rac1-mediated internalization that is stimulated by Sema3A [90] as L1 activates Vav2, a GEF known to stimulate Rac1 [114] (Fig. 4.4a).

Finally, whether the internalization of other IgCAMs is a key aspect of the response to other Sema3s remain to be tested experimentally. CHL1 lacks the AP2-binding site found on L1 but was found to be internalized via lipid rafts (Fig. 4.4b). CHL1 needs to be palmitoylated for internalization (Fig. 4.5). Interestingly, CHL1 palmitoylation and raft partitioning are dynamically regulated by oligomerization [115]. The sequence that corresponds to the AP2-binding site of L1 is predicted to be a relatively weak binding consensus on NrCAM. However, NrCAM possesses another AP2-binding motif suggesting that NrCAM could be internalized by an AP2/clathrin-dependent mechanism [116] (Fig. 4.5). Alternatively, NrCAM is also found in lipid rafts, and this targeting might also be regulated by clustering as Caveolin accumulates after ligand-induced clustering of NrCAM [64].

Overall, these data thus point to important contribution of L1CAMs in receptor/ligand trafficking occurring during the growth cone response to secreted Semaphorins.

4.3.3 IgCAMs regulate activation of signalling molecules during Sema3A response

L1CAMs are also known to generate various intracellular signalling cascades. Initial studies emphasized the role of FGF receptors in generating signalling downstream of L1 [117]. It is now clear that L1CAM can interact with a variety of non-receptor tyrosine kinases and other signalling molecules, such as caseine kinase II, p60src, Vav2, PAK1 or RanBPM, which mediate axon growth downstream of L1 [59] (Fig. 4.4). Whether they are also activated by L1 during Semaphorin-mediated growth cone responses remain to be established. Nevertheless, Sema3A was shown to trigger L1-dependent signal involving kinases.

4.3.3.1 MAPK Activation via FAK Can Modulate Adhesion Complexes

L1 can mediate ERK1/2 activation after homophilic binding [94, 100], and responses to Sema3A require ERK1/2 activation [57, 101]. Recent evidences show that L1 can also mediate MAPK activation after Sema3A application (Fig. 4.4). First, Sema3A induces ERK1/2 activation in COS7 when L1 is co-expressed with NRP1. Second, L1 mutants that fail to support ERK activation inhibit the response of cortical neurons to Sema3A [57]. The turnover of adhesion points contracted by the growth cone structures is indispensable for growth cone advance, but also for both collapse and growth cone turning [118]. Functional assays revealed that a suboptimal non-collapsing Sema3A dose destabilizes growth cone adherence points. Such disassembly could be visualized with the adhesion complex adaptor Paxillin [119]. Interestingly, inhibitors of MAPK or L1 mutants unable to activate ERK1/2 were found to prevent this Sema3A-induced reduction of Paxillin staining in growth cone [57].

A key component of this signalling mechanism is focal adhesion kinase (FAK). L1 was shown to activate MAPK via FAK recruitment, and mutated FAK forms abrogated the Sema3A-mediated growth cone collapse [57]. The 1146–1176 region of the cytoplasmic domain of L1 was shown to interact with the FERM domain of FAK (Fig. 4.5). This interaction is induced by Sema3A and leads to FAK activation. FAK recruitment to Sema3A receptor appears highly specific to the NRP1/L1 complex. First, the interaction is only detected when L1 is co-expressed with NRP1 but does not require the cytoplasmic domain of NRP1. Second, FAK was not recruited to or activated by Plex/NRP1 receptor complexes. Additional data suggested a model whereby Sema3A triggers adhesion disassembly via a L1-FAK-MAPK leading to release Paxillin^{+} adherent points (Fig. 4.7). In support, Sema3A induces Paxillin phosphorylation, on a tyrosine known to be phosphorylated by FAK. Furthermore, Paxillin interaction with L1 is alleviated after Sema3A addition (Fig. 4.7). In neurons expressing L1 mutants for ERK1/2 activation, disassembly of Paxillin-positive adhesion contacts and collapse response to Sema3A are both abrogated [57].

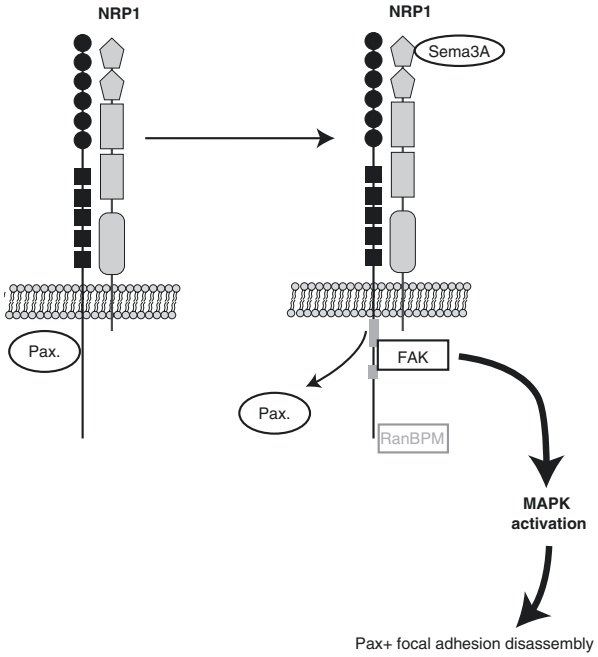


Fig. 4.7 Model of the signalling pathway leading to disassembly of Paxillin-positive adhesion sites during Sema3A-mediated growth cone collapse. In the absence of Sema3A, L1 interacts with Paxillin. In the presence of Sema3A, Paxillin is released from L1, which now can interact with FAK. L1/FAK interaction leads to FAK activation, MAPK activation and disassembly of Paxillin^{+} adhesion complexes. L1-induced activation of MAPK can be FAK independent suggesting that other signalling molecules such as RanBPM that also interacts with L1 (*light grey*) could be involved. Abbreviations: *Pax.* Paxillin, *FAK* focal adhesion kinase

Experimental data also suggested that L1 can activate MAPK via a FAK-independent cascade after Sema3A treatment [57]. Interestingly, the C-terminal region of L1 was shown to interact with RanBPM [120]. RanBPM is a signalling molecule that associates with Sos, a GEF for Ras, and triggers the Ras/MAPK cascade [121]. This interaction was found to trigger ERK activation after L1 cross-linking and to contribute to L1-dependent axon growth [120] (Fig. 4.4). Future work is needed to test if this interaction also supports L1-dependent activation of ERK induced by Sema3A (Fig. 4.7). ERK1/2 phosphorylation is also induced during attractive and repulsive responses to Sema3B [15]. Localization of active FAK differs between attractive and repulsive conditions suggesting that FAK could generate signalling specificity. Indeed, FAK was found to interact with Fyn, a nonreceptor kinase of the Src family, only under attractive conditions (Fig. 4.4c). Moreover, Sema3B induces Fyn activation only in neurons that exhibit an attractive response to Sema3B [15]. Whether and how NrCAM, the L1CAM associated with NRP2 in the Sema3B complex, mediated Sema3B-induced ERK activation, the location of active FAK or Fyn activation is still unknown.

4.3.4 Complementarity, Specificity and Redundancy Between Plexins and IgCAMs

Since PlexinA and IgCAM cytoplasmic domains differ substantially, and both can trigger intracellular signals, different combinations of receptor subunits could mediate distinct specific responses to Sema3s. It is unclear whether NRP/Plex and NRP/lgCAM form distinct and independent holoreceptors or not. In vitro, expression of NRP1/L1 subunits in COS cells triggers Sema3A-induced cell collapse, as does NRP1/PlexinA1 complex [56]. Nevertheless, L1 was found dispensable for some neuronal responses to Semaphorins, whereas PlexinAs appear to be obligatory components. For example, L1 is unnecessary for Sema3A-induced cell death of sensory neurons [122]. In contrast, the Sema3A-induced growth cone responses of the same neurons require L1 [12]. Protein distribution data and biochemical analysis support that Plexins and IgCAMs form common receptor complexes. First, IgCAMs and PlexinAs are often co-expressed in the same neurons [57, 91]. Second in cortical neurons, NRP1 associates with PlexAs and L1, and both PlexA and L1 signalling are required during Sema3A-induced collapse response [57]. Similarly, NrCAM co-immunoprecipitates with NRP2 and PlexinA3 from brain lysate [16]. Nevertheless, the presence of NrCAM in the receptor complex appears not predictive of the attractive or repulsive nature of the Sema3 responses. Indeed, NrCAM is expressed in both anterior and posterior piriform cortical neurons, which exhibit opposing responses to Sema3B [15]. Increasing knowledge of Plexins and L1CAMs signalling give a rather complex picture. Plexins and L1CAMs share some effectors. For example, both L1 and PlexinAs were shown to interact with RanBPM [120, 123]. Both can also activate Rac1. L1 activates Rac1 via the GEF Vav2 [114] while PlexinAs do it by the release of the GEF FARP2 [124]. L1 and Plexin both activate Rab5. L1 interacts with the Rab5 GEF, Rabex-5 [97]. PlexinA1 interacts with the Rab5 effector, Rabatin-5, which associates with Rabex-5 [112, 125]. Thus, Plexin could also mediate internalization, as L1 does. Indeed, PlexinAs and L1 could have redundant or synergistic effects during Rab5 and Rac1-mediated endocytosis after Sema3A treatment. Finally, PlexinAs could regulate the L1 signalling. In support, the activation of CRMP2 by PlexinAs after Sema3A treatment impacts on L1 internalization [126–128]. Indeed, the internalization of L1 mediated by AP2/numb complex has been shown to be inhibited by CRMP2 in axons [66].

Overall, these examples highlight possibilities for cooperation and cross regulation between L1CAMs and PlexinAs that may occur to a greater extent than initially anticipated.

4.4 Regulation of Semaphorin-Mediated Cell Responses by IgCAMs

In addition to their contribution as components of the Sema3 receptor complexes, a function of IgSFCAMs as modulators of the growth cone responses to Semaphorins has emerged from several studies.

4.4.1 Soluble IgSFCAMs switch Semaphorin-mediated repulsion to attraction

In coculture assays, soluble forms of Ig superfamily cell adhesion molecules were found to reverse the response of neuronal growth cones to Sema3s from repulsion to attraction. This property was reported for two IgSFCAM members, L1 and NrCAM, in three different cell types. First, soluble L1 could convert the repulsive effect of Sema3A exerted on cortical neurons into attraction [18]. Second, a similar effect was also observed for DRG neurons [18].Third, soluble NrCAM could convert the repulsion exerted by Sema3B and Sema3F to attraction in a population of neurons establishing the posterior part of the anterior commissure in the brain [15].

The well-characterized human mutations in the L1 gene helped to elucidate the molecular mechanisms underlying the switch. A considerable number of point mutations affecting the L1 gene located at Xq29 are causal of a complex syndrome of neurodevelopmental disorders, referred to as MASA syndrome or X-linked hydrocephalus [129]. This has allowed investigators to map the domains of the L1 protein engaged in the interactions with its binding partners. Similarly, a five-amino-acid sequence of the L1 Ig1 domain was identified as allowing the recruitment of L1 to NRPsl. Indeed, a single mutation, the L120V, reported in a patient and targeting an amino acid within this sequence abrogated L1-NRP1 binding in cis. Interestingly, the switch from repulsion to attraction also requires the same integral amino acid sequence, indicating that soluble L1 might exert the conversion through binding in trans to NRP1. This was further supported by the observation that soluble L1 forms having human mutations that impair their ability to undergo homophilic and heterophilic interactions with other IgSFCAMs did not prevent the switch from repulsion to attraction. Finally, investigation of the signalling pathways downstream of L1 trans binding to NRP1 was conducted using ex vivo assays, which indicated that the switch involves the activation of NO-cGMP pathway [18].

4.4.2 Soluble NrCAM Switches on the Repulsive Response to Semaphorins

More recently, two novel types of crosstalks linking IgCAMs and Semaphorin signalling have been uncovered which reinforces the idea that IgCAMs act as regulators of the growth cone sensitivity to Semaphorin guidance signals. Both were discovered to contribute to the guidance of commissural axons across the midline in the developing spinal cord.

Commissural axon navigation has provided one of the best models to examine how neuronal growth cones vary their sensitivity to guidance cues. It is largely admitted that the midline local environment provides both attractive and repulsive cues to commissural axons. A temporal sequence controls their responsiveness to these cues. First, the growth cones perceive attractive cues which guide them towards the midline, and, second, after crossing, the perception of repulsive cues then instructs the axons to move away from the midline, towards their next destination. Such a sequence implies that the sensitivity to the midline repellents is first

silenced before crossing and then switched on after crossing. Together with Slit proteins, the Semaphorin3B was found to act as a midline repellent in the vertebrate spinal cord [130, 131]. Accordingly, spinal commissural axons are initially insensitive to the cue until they cross the midline [131]. How the sensitivity of commissural axons to midline repellents is controlled has been the topic of intense investigations over years, and many aspects remain still poorly understood. Pioneer studies of midline crossing in the drosophila ventral cord established that this is achieved through regulation of guidance receptors. Before crossing, the presence of the Slit receptor Robos at the cell surface is prevented through sorting to the proteasome by commissureless proteins. In addition trans interactions between Robo2 in midline glial cells and Robo1 proteins, which escaped degradation by comm and could reach the growth cone surface, inhibit Slit repulsion to prevent premature responsiveness of pre-crossing axons [132, 133]. Upon crossing, comm is downregulated, and as a consequence, Robos become available in commissural growth cones to transduce the Slit repulsive signal [134]. While comm is not conserved in vertebrates, an array of mechanisms targeting the guidance receptors has been discovered, testifying that as in the drosophila ventral cord, tight control of receptor distribution and interactions is instrumental to set in time and space the responsiveness to midline repellents.

The sensitivity of spinal commissural axons to Sema3B appeared also controlled through regulation of the Sema3B receptor. Initially at the pre-crossing stage, NRP2 and its co-receptor PlexinA1 are both synthesized by commissural neurons. While NRP2 is available on commissural axons, PlexinA1 is only present at low levels at the surface of the pre-crossing axon shaft. Its cell surface distribution is increased in the crossing and post-crossing axon segments [130, 135]. Combinations of pharmacological, imaging and biochemical approaches revealed that PlexinA1 is synthesized but submitted to a cleavage before crossing by calpain proteases, which prevents its sorting at the cell surface, and that the processing is suppressed by signals released by floor plate cells when commissural growth cones navigate the midline. Unexpectedly, one of the active floor plate cues turned out to be NrCAM. In

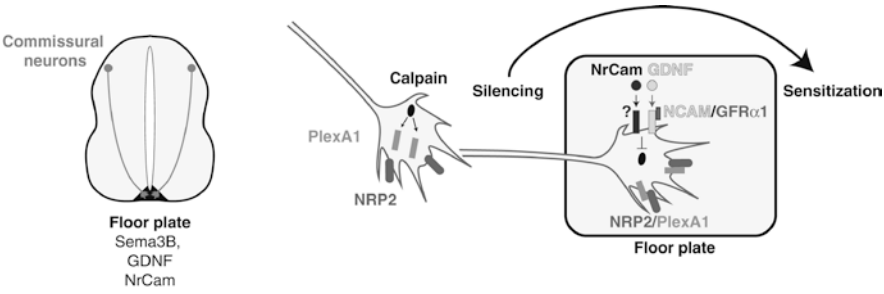


Fig. 4.8 IgCAM/Semaphorin interplays during midline crossing of spinal commissural axons. The sensitivity of commissural axons to repulsive Sema3B is regulated at the midline. Before crossing, PlexinA1 is submitted to a cleavage, which prevents its expression at the cell surface, thus desensitizing commissural growth cones to Sema3B. During crossing, floor plate NrCAM and GDNF acting through NCAM act in synergy to suppress PlexinA1 processing, which becomes available at the cell surface, thus allowing Sema3B to repel commissural growth cones

situ hybridization of embryonic sections showed that the floor plate is the major source of NrCAM transcripts in the spinal cord at these stages, with in addition a moderate expression level in the dorsal spinal cord where commissural neurons reside. Consistently, a soluble NrCAM form, which might result from ectodomain shedding, could be detected in the medium of isolated floor plate tissue cultures (FP^{cm}). In dissociated commissural cultures, FP^{cm} application resulted in a marked increase of PlexinA1 cell surface distribution, and this effect was significantly reduced when the FP^{cm} was produced with tissue from NrCAM null embryos. Thus, in addition to binding in cis to NRP2 and participating in the Sema3B receptor complex, NrCAM also regulates, non-cell autonomously, the surface expression of PlexinA1. Analysis of calpain activity using Tboc, a substrate of calpain whose cleavage produces fluorescence, showed that NrCAM inhibits calpains, consistent with the finding that PlexinA1 is prevented from the cell surface through calpain-mediated processing [130].

Additional investigations of the signals regulating calpain processing during midline crossing led to the discovery of a second cue, the neurotrophic factor GDNF, whose expression in the embryonic E12 spinal cord is strictly restricted to the floor plate [135]. GDNF shares with NrCAM the ability to regulate calpain activity in commissural neurons and to trigger increase of PlexinA1 cell surface levels. Single and double genetic deletion of NrCAM and GDNF revealed the two cues synergized in vivo to regulate PlexinA1 levels and midline crossing. While the commissural receptor of NrCAM remains unknown, GDNF was found to act on calpain activity via NCAM and not RET. Indeed, NCAM but not RET was detected along commissural axons. The GDNF co-receptor GFRαl is also present along commissural axons. NCAM and RET gene deletion in mice confirmed that RET was not necessary for commissural axons while in contrast NCAM loss perturbed their trajectory in the floor plate. Moreover, GDNF loses its ability to inhibit calpain activity in NCAM null commissural neurons [135]. Altogether these findings established that a GDNF/NCAM pathway regulates axon sensitivity to Sema3B by modulating the cell surface availability of PlexinA1, illustrating an additional mode of crosstalks between IgCAMs and the Semaphorin signalling (Fig. 4.8).

Several other biological contexts exist in which similar molecular crosstalk could be implicated. A first context is limb innervation, which share many molecular players with midline crossing. Motoneurons in the ventral spinal cord are organized in columns, each of them having specific pattern of connections. The lateral motor column is additionally subdivided into a medial and a lateral part, which innervate the ventral and the dorsal part of the limb, respectively. Combinations of cues act to guide the trajectory of the different spinal nerves within the limb, including Semaphorins, Ephrins and Netrins [136]. Interestingly, a GDNF source was discovered at the base of the limb, which, by acting through the receptor RET, cooperates with Ephrins to control the dorsal-ventral choice of motor axons, according to their sub-column identity [137]. This GDNF source could in addition regulate the sensitivity of motor axons to the Semaphorins, as it does at the ventral midline for commissural axons. Indeed, PlexinAs and NCAM are expressed by the spinal nerves, and several Semaphorins are secreted in the developing limb [138, 139]. Whether

Sema3B is especially playing some role in motor axon guidance has not been assessed. However, PlexinA1 is shared by several Semaphorins, including Sema3A and Sema3C [140], two Semaphorins whose expression in the limb has already been documented [138, 141]. Moreover, analysis of Sema3A and NRP1 null mutants revealed defects in the timing and fasciculation pattern of the limb nerves [141, 142].

Second, the formation of the enteric nervous system might be another interesting context to explore, although very little is yet known concerning the contribution of Semaphorin signalling to the migration of enteric neural crest cells towards and in the gut, which will give rise to the enteric neuronal population. Indeed, GDNF/RET signalling plays a key attractive role on the enteric neural crest cells in the gut mesenchyme. GDNF mRNA is first present at high levels in the foregut and second later on in the coecum. This dynamic expression thus correlates with the timing of invasion by the enteric neural crest cells. Sema3A is also detected with a pattern that suggests a repulsive effect. Finally while the profile of Plexins remains poorly defined, expression of the IgSFCAMs L1 and NCAM by migrating enteric neural crest cells has already been established [143].

4.4.3 Trans Interactions Between IgSFCAMs and Plexin/Semaphorin Interactions Abrogate Semaphorin Repulsion

Investigations of the mechanisms guiding visual axons at the optic chiasm have provided an additional context to reveal the interplays between IgSFCAMs and the Semaphorin signalling [144]. To ensure binocular vision, retinal ganglion cells establish ipsilateral and contralateral projections. The two classes of axons initially converge towards the ventral midline of the diencephalon, and then diverge at the optic chiasm, with the contralateral axons crossing the midline. As appears to be the case for other systems of projections with ipsilateral and contralateral components, ipsilateral retinal axons are sensitive from the onset to midline repellents preventing them from crossing, while the sensitivity of contralateral axons to these cues is temporary abolished, which enables them to cross the midline. Sema6B is one such midline repellent for retinal ganglion cell axons, including the contralateral ones, which were shown to express both PlexinA1 and NrCAM. Interestingly, midline glial cells and surrounding neurons also produce NrCAM and PlexinA1. *Trans* interactions, with their counterparts on the axons, switch Sema6B-mediated repulsion to attraction, which, as a result, facilitates midline crossing. Open questions for future investigations relate to the exact configuration of these macro-complexes and the downstream signalling they activate to alter the repulsive growth cone response to Sema6B [144].

Interestingly in the chick embryo, Sema6B and PlexinA2 are expressed by commissural neurons. In addition, PlexinA2 is expressed by the floor plate and can undergo trans binding with Sema6B. Data from in ovo manipulations provided a functional model whereby Sema6B acts as a receptor and Sema3B/PlexinA2 trans interaction modulates the sensitivity of the axons to floor plate-derived cues. Although the contribution of NrCAM was not assessed in this molecular context, it

is striking to note that it is expressed by floor plate cells and mediates adhesive cell-cell interactions with commissural axons via axonin [145]. Thus a tempting model would be that NrCAM also contributes to the dynamics of Sema3B/PlexinA3 macro-complexes taking place during the pre-crossing to the post-crossing commissural axon navigation.

Overall these different examples illustrate the complexity and diversity of molecular strategies by which IgCAMs provided by the local environment at specific choice points of axon and cell migration regulate the spectrum of effects exerted by the Semaphorins.

4.5 Insights for Pathologies

The molecular players of the IgCAM/Semaphorin crosstalk have been all implicated in pathological contexts. We report here some of these contexts, which would be particularly interesting for further examination, in the light of the findings obtained from the neurodevelopmental studies. These are neuropsychiatric disorders and cancer.

4.5.1 Neuropsychiatric Disorders

Genetic studies associate NrCAM with several psychiatric and neurological disorders, such as autism and schizophrenia. For example, translational convergent functional genomics (CFG) approaches were undertaken to identify genes involved in schizophrenia, which classified NrCAM, as well as NCAM, in the top list of candidate biomarkers. Whole-exome sequencing (WES) and blood cell transcriptome of male patients with idiopathic autism syndrome disorders identified Sema6B as one of the genes having intronic mutation causing deregulated expression [146].

Deregulations of Sema3A and PlexinA1 transcript levels were reported in the prefrontal cortex of patients with schizophrenia [147]. Perturbations of CRMP2 functions, a known effector of the Semaphorin signalling, including for Sema3B [148] have also been linked to neurological disorders such as Alzheimer and schizophrenia [149]. Finally several years ago, a link was also suggested between GDNF and schizophrenia [150]. More recently, serum levels of GDNF were found to be lower in patients with schizophrenia than in healthy controls [150]. Furthermore, analysis of polymorphisms has revealed significant association of some variants of GFRα, the GDNF co-receptor required with NCAM to trigger Sema3B repulsion, with schizophrenia [151].

4.5.2 Cancer

Cancer is a second context in which IgCAM/Semaphorin crosstalk might play a role. Multiple studies were conducted which testify the strong implication of

Semaphorin signalling in tumorigenesis and cancer cell dissemination (see other book chapters [2]). In glioma, Sema3B deregulations were found with consequences that are debated, as some studies reported that increased Sema3B expression is correlated with poor prognosis [152], and others with good prognosis [153, 154]. This might reflect the versatility of the Semaphorin functions, which vary depending on multiple parameters including the cell types and the environmental contexts. Deregulations of NrCAM have also been associated with cancer, including brain tumours. Early findings established that NrCAM is expressed at high levels in malignant brain tumours and promotes proliferation [155, 156]. A strong literature also supports a link between GDNF and glioma. High transcription levels of GDNF and GFRα were observed in glioma [157, 158]. High-grade glioma cells secrete higher levels of GDNF than low-grade ones [159]. GDNF knockdown in glioma cells injected in the brain of mouse models could reduce tumour growth [160]. Finally, initial report of the human Sema6B gene product indicated its expression in glioblastoma cell lines [161].

Thus, many of the molecules of the IgCAM, Semaphorin, NRP and Plexin families have been found to assemble dynamic macro-complexes, which contribute to key aspects of the development of nerve connections. They are expressed, often in a deregulated way, in cancer. Therefore, it is highly plausible that alterations of the IgCAM/Semaphorin crosstalks contribute to some pathogenic processes, and a better understanding of the modalities and functional properties of these crosstalks could certainly be of great interest for the design of novel therapeutic targets.

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Neuropilin-Dependent Signaling and Neuropilin-Independent Signaling of the Guidance Molecule Sema3E

5

Fanny Mann and Sophie Chauvet

Contents

Abstract

Neuropilins are receptors for all but one secreted (class 3) Semaphorins. The exception is Sema3E that directly binds and activates the Plexin-D1 receptor to induce cell repulsion. In this chapter, we review works that identified an unexpected role of Neuropilin-1 as a gating switch in Sema3E/Plexin-D1 signaling, that converts repulsion into attraction, and discuss the implications of this finding for brain wiring.

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5.1 How and Why the Authors Became Interested in Neuropilins?

The authors study the axon guidance signaling pathways that determine wiring specificity in the mammalian brain. They became interested in neuropilins, paradoxically, when working on an atypical, neuropilin-independent semaphorin ligand: Sema3E. They discovered a novel "gating" function of Neuropilin-1 that converts Sema3E-induced repulsion to attraction. The fine dissection of the mechanism and physiological function of the Neuropilin-1-dependent Sema3E signaling has led to an improved understanding of normal brain development and altered connectivity in schizophrenia.

5.2 Introduction

Neuropilin-1 and Neuropilin-2 are single-pass transmembrane proteins that serve as high-affinity receptors for a subclass of secreted axon guidance ligands, known as class 3 semaphorins (Sema3s). The short cytoplasmic domain of neuropilins, which lacks an identifiable catalytic domain, is not required for transducing semaphorin signaling. Therefore, most Sema3s act through holoreceptor complexes that include a neuropilin as the binding subunit and a plexin (Plexin-A or Plexin-D) as the signal-transducing subunit [41]. One exception to this model is the ligand Sema3E, which directly binds to and activates a plexin receptor, namely, Plexin-D1, without requiring any neuropilin co-receptor [26].

Semaphorin signaling controls a wide range of biological processes, including neural circuit formation, angiogenesis, cardiogenesis, bone homeostasis, and regulation of the immune system. The activation of plexins by Sema3s, either directly (Sema3E) or indirectly via neuropilins (other Sema3s), initiates repulsive signaling cascades that lead to cell detachment and retraction. In certain contexts, however, regulatory mechanisms have been discovered that switch the semaphorin response from repulsion to attraction, further expanding the functional repertoire of these guidance cues. In recent years, attention has been focused on the role of Neuropilin-1 as an unexpected key determinant of the Sema3E repulsion-attraction switch. This role does not meet the classical definition of neuropilin's function in semaphorin signaling, as, in this particular setting, the presence of Neuropilin-1 in the receptor complex does not facilitate ligand binding but instead determines the outcome of receptor signaling.

In this chapter, we will present some of the evidence behind a neuropilin-independent function of Sema3E, and we will then review studies that identified a neuropilin-dependent function of Sema3E in the establishment of a major axonal tract of the mammalian brain, the fornix. Current views on how Sema3E utilizes Neuropilin-1 as part of a multimeric receptor complex to induce an attractive cellular response will be further highlighted. Finally, we will focus on recent work involving alterations in downstream components of this non-canonical semaphorin signaling in an animal model of schizophrenia with congenital absence of the fornix.

5.3 Sema3E Induces Cell Repulsion in a Neuropilin-Independent Manner

The transcript for Sema3E was initially isolated from mouse tumor cells by Christensen et al. [8]. It encodes a secreted protein that contains a ~500 residue N-terminal "Sema" domain, which is the signature feature of the semaphorin protein family, followed by a C2 immunoglobulin-like domain and a C-terminal basic amino acid-rich region [8, 31]. While structurally similar to other Sema3s, Sema3E differs in its ability to activate plexin signaling without requiring any neuropilins for binding. Indeed, Sema3E binds directly to the Plexin-D1 receptor with a dissociation constant (K_D) of 0.13 nM that is not influenced by the presence or absence of neuropilins [6, 26]. In comparison, another semaphorin that signals through Plexin-D1, namely, Sema3C, does not bind the plexin receptor alone but requires Neuropilin-1 as a binding co-receptor, as the Neuropilin-1/Plexin-D1 complex has a greater affinity for Sema3C than does Neuropilin-1 alone [22, 48]. In this regard, Sema3E resembles membrane-bound (class 4–7) semaphorins, many of which require only plexins for signaling.

The reason why transmembrane semaphorins and Sema3E bind to plexins while other Sema3s need neuropilins as "glue" is not yet understood. The percentage of homology at the Sema domains bearing the binding specificity cannot explain these differences. For example, the Sema domain of Sema3E shares higher homology at the amino acid level with Sema3G (53.8 %), which interacts with neuropilins, than with the other Plexin-D1-binding semaphorin, Sema4A (29.3 %) [49]. Binding sites for semaphorin receptors are indeed located in three-dimensional structures of the folded Sema domain [21], and crystallographic studies of the binary Sema3E/Plexin-D1 complex are still awaited to answer this important question.

The binding of Sema3E to Plexin-D1 in vitro induces a rapid retraction and collapse of the cell [26, 36] (Fig. 5.1a). An in vivo function of Sema3E was first described in the mouse, where it controls blood vessel development. In the trunk region of mouse embryos, expression of Sema3E in somites repels the growth of adjacent intersomitic blood vessels (ISVs), which express Plexin-D1. In both *Sema3e*$^{-/-}$ and *Plxnd1*$^{-/-}$ embryos, ISVs ectopically extend throughout the somites, resulting in a loss of their normal segmental organization [26]. In contrast, mice doubly homozygous for a null mutation in *npn-2* and a mutation in *npn-1,* which abolishes semaphorin binding sites (*npn-1sema-*), display normal intersomitic vasculature, thus confirming that Sema3E can function in a neuropilin-independent manner to repel endothelial cells [26]. Since then, a role of Sema3E has been identified in the developing nervous system, where it contributes to the development of several Plexin-D1-expressing axon tracts, many of which lack significant neuropilin expression. These include the striatonigral and corticofugal fibers and specific sensory-motor projections [6, 9, 14, 19, 34]. Most neuronal defects observed in *Sema3e* and *Plxnd1* mutant mice are consistent with the repulsive activity of this ligand-receptor pair, as revealed by precocious axonal outgrowth, defasciculation, misguidance, or ectopic synapse formation.

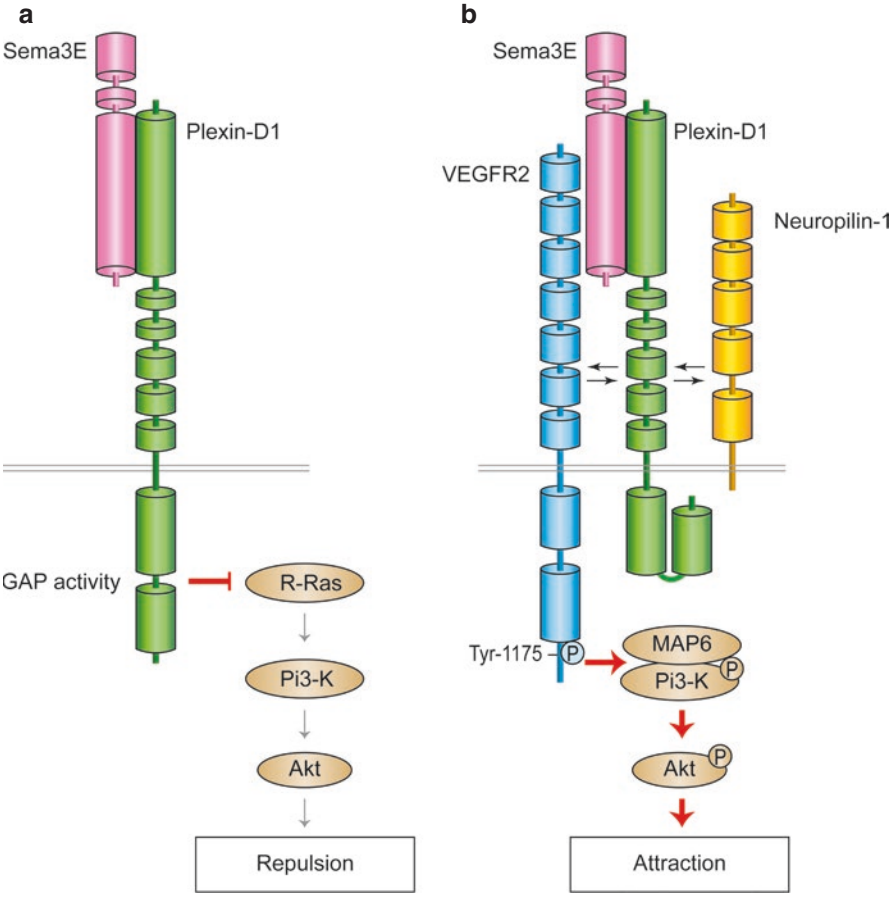


Fig. 5.1 Molecular models for axonal growth cone responses to Sema3E. Sema3E repels the growth of neuronal axons that express Plexin-D1 alone (**a**). In contrast, axons of neurons expressing Plexin-D1, Neuropilin-1, and VEGFR2 show an attractive response to Sema3E (**b**). In both cases, Plexin-D1 is the ligand-binding subunit for Sema3E. However, whereas Plexin-D1 is responsible for initiating cellular signal transduction inducing axon repulsion (**a**), VEGFR2 serves as the signal-transducing subunit during axonal attraction (**b**). Gating of Sema3E signaling from repulsion to attraction is achieved by Neuropilin-1, which inhibits the Plexin-D1-mediated repulsive signal and promotes the VEGFR2-mediated attractive signal (**b**). MAP6 controls the activation of the PI3K/Akt signaling pathway through recruitment of the PI3K to the activated trimeric receptor complex of Sema3E (**b**)

5.4 Does Sema3E Interact with Neuropilins?

Is Plexin-D1 the unique receptor for Sema3E? Binding experiments with alkaline phosphatase (AP)-tagged Sema3E protein and sections of mouse embryo brains revealed intense binding in several axonal tracts that is completely lost in *Plxnd1*$^{-/-}$ embryos. However, AP-Sema3E binding also occurs, albeit at a lower

level, on fibers of the corpus callosum in both wild-type mice and *Plxnd1*$^{-/-}$ mutants (F. Mann and S. Chauvet, unpublished observations). This suggests the existence of a second, lower-affinity receptor for Sema3E that has yet to be identified. The classical Sema3 binding partner Neuropilin-1 is expressed in the developing corpus callosum and could be a potential candidate [25]. Indeed, early binding studies using chick semaphorins suggested that Sema3E could bind Neuropilin-1 with a similar affinity to the prototypical Sema3A protein [16]. In contrast, binding experiments using mammalian proteins have revealed that Sema3E is unable to bind Neuropilin-1 or Neuropilin-2 [6, 26]. It is unclear whether these divergent results may reflect the fact that, through vertebrate evolution, mammalian Sema3E and Neuropilin-1 have lost the ability to interact, similar to what has been described for other evolutionarily conserved families of axon guidance molecules [50]. Alternatively, the binding differences may be due to differential processing of Sema3E precursor proteins between studies rather than to the species of origin of the proteins. Indeed, Sema3E is expressed as a proprotein of 87 kDa that forms dimers and is susceptible to proteolytic cleavage catalyzed by furin proprotein convertases, yielding a smaller fragment of approximately 61 kDa [7]. Proteolytic cleavage of other Sema3s by furin is known to modulate their activity and ability to bind to Neuropilin-1 [29, 45, 48]. Whether the full (87 kDa) and furin-processed short fragments (61 kDa) of Sema3E differ in their binding affinity for Neuropilin-1, as well as whether this may have any physiological implication, awaits further investigation.

5.5 Neuropilin-1, a Functional Gating Switch in Sema3E/Plexin-D1 Signaling: From Repulsion to Attraction

As mentioned above, Sema3E serves as a repulsive axon guidance cue for multiple classes of projection neurons expressing Plexin-D1. There is, however, one notable exception to this: axons of pyramidal neurons of the subicular cortex, a subregion of the hippocampal formation, are attracted by Sema3E [6]. Similar to repulsion, attraction is also dependent upon Sema3E binding to Plexin-D1 on growing axons [6]. Therefore, what determines the outcome of Sema3E signaling? A large part of the answer has emerged from the observation that projection neurons in the subicular and piriform cortex, two areas that differ in their guidance responses to Sema3E, express different kinds of neuropilin receptors: Neuropilin-1 and Neuropilin-2 are both expressed in subicular neurons, whereas neurons of the piriform cortex express only Neuropilin-2. Although both neuropilins can physically interact with Plexin-D1 through their extracellular domains, functional studies demonstrated that Neuropilin-1, but not Neuropilin-2, was required to mediate the attractive effect of Sema3E, which is consistent with its cell-type-specific expression [6]. Moreover, knocking down Neuropilin-1 expression by siRNA completely reversed the polarity of the response of subicular neurons, which were then repelled by Sema3E, as were piriform neurons that naturally lack Neuropilin-1. Conversely, ectopic expression of Neuropilin-1, or the addition of a soluble form of the ectodomain of Neuropilin-1,

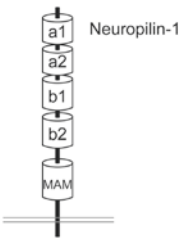
converted the repulsive response of piriform neurons to Sema3E into attraction [6] (Figs. 5.1b and 5.2). These data provided the first evidence for a "gating" function of Neuropilin-1 in semaphorin-dependent axon guidance that differs significantly from other roles reported for neuropilins, whose interactions with co-receptors contribute to initiating a signal but do not switch the sign of the response, as occurs with Plexin-D1.

Which domain of Neuropilin-1 mediates this "gating" function? The extracellular portion of neuropilins comprises five distinct domains: two N-terminal CUB domains (named a1 and a2) and two coagulation factor V/VIII homology domains (named b1 and b2), which are required for the binding of Sema3 and vascular endothelial cell growth factors (VEGFs), and a MAM domain (meprin/A5/mu, named c domain), which has been implicated in homodimerization. Functional dissection of the Neuropilin-1 ectodomain involved the MAM domain, which is itself sufficient to mimic the effect of Neuropilin-1 on Sema3E signal by converting repulsion into attraction [4]. Moreover, this study confirmed that the gating function of Neuropilin-1 is independent of the ligand-binding domains [4] (Fig. 5.2). Recently, another study revealed that Neuropilin-1 guides vascular development independently of its ability to interact with Sema3 or VEGF ligands, instead through its capacity to function as a VEGFR2 co-receptor to enhance VEGFR2 activation [20, 25]. Therefore, the modulation of co-receptors may be a much more general mechanism by which neuropilins contribute to cell signaling than previously assumed.

5.6 Neuropilin-1 Acts with VEGFR2 to Modulate Sema3E/Plexin-D1 Signal

The mechanisms by which engagement of plexin-neuropilin receptor complexes by Sema3s initiates repulsive signaling cascades have begun to be elucidated and have been reviewed elsewhere [33]. Particularly intriguing, then, is the question of how the Plexin-D1-Neuropilin-1 complex initiates an opposing, attractive signaling cascade in response to Sema3E. In general, as mentioned above, Sema3E/Plexin-D1 signaling is

a



b

	Gating activity
Neuropilin-1	YES
Neuropilin-1^{Sema-}	YES
Neuropilin-1Δa2	YES
Neuropilin-1Δb1	YES
Neuropilin-1Δb2	YES
Neuropilin-1ΔMAM	NO
Soluble ectodomain of Neuropilin-1	YES
Soluble MAM domain of Neuropilin-1	YES

Fig. 5.2 The gating activity of Neuropilin-1 on Sema3E signal is mediated by its MAM domain. (**a**) Schematic representation of the different extracellular domains of Neuropilin-1. (**b**) Different mutant forms of Neuropilin-1 were overexpressed in neurons of the piriform cortex, or bath-applied, to test their ability to switch Sema3E signaling from repulsion to attraction. The MAM domain of Neuropilin-1 is both necessary and sufficient to exert its gating function

repulsive and involves the GTPase Activating Protein (GAP) domain in the plexin cytoplasmic region for triggering intracellular cascades [43]. When complexed with Neuropilin-1, however, the cytoplasmic region and GAP activity of Plexin-D1 appeared dispensable for attractive guidance signals [4], indicating that Plexin-D1, although required for ligand binding, does not mediate attractive signaling. Thus, signal transduction must be mediated by a third component of the Plexin-D1/Neuropilin-1 heteromeric receptor complex. A pharmacological screen for effectors of Sema3E-induced attraction identified the PI3K/Akt pathway, an important signaling cascade that can be activated by receptor tyrosine kinases (RTKs), and led to the later identification of the RTK VEGFR2 as the signaling subunit of the Sema3E receptor [4].

Attention was drawn to VEGFR2 as a neuronal mediator of guidance signaling after important similarities have begun to emerge between the mechanisms used to pattern the vascular and the nervous systems. Several members of the "canonical" families of axon guidance molecules have been shown to regulate angiogenesis, with Sema3E being one of them. Conversely, the possibility that a master regulator of endothelial cell function, VEGFR2, also contributes, either alone or with the Neuropilin-1 co-receptor, to nervous system development has been a subject of intensive investigation. For example, VEGFR2 has been revealed as crucial in mediating the attractive guidance of spinal commissural neurons to VEGF through downstream activation of Src family kinases [35]. In that context, VEGFR2 operates independently of the co-receptor Neuropilin-1, which is not expressed in spinal commissural neurons [35]. In another system, VEGF acts through Neuropilin-1 independently of VEGFR2 [15]. Conversely, neurons of the subicular cortex co-express VEGFR2 and Neuropilin-1, but their axons are unresponsive to VEGF ligands [4]. Instead, loss of VEGFR2 in subicular neurons completely blocked the attractive response to Sema3E [4]. Systematic dissection of the underlying mechanisms has revealed that VEGFR2 forms a trimeric complex with Neuropilin-1 and Plexin-D1 to which Sema3E binds, activating the PI3K/Akt pathway through the phosphorylation of Tyr1175 in VEGFR2 [4] (Fig. 5.1b).

Neuropilin-1 was found to enhance Sema3E-induced phosphorylation of VEGFR2 and its subsequent signaling, as had been reported for VEGF signaling in endothelial cells; however, this is not its sole function. Indeed, Neuropilin-1 and VEGFR2 do not have symmetric roles in subicular neurons: while the loss of Neuropilin-1 function switches an attractive response to Sema3E into repulsion, the loss of VEGFR2 function blocks the response to Sema3E but does not convert it to repulsion. This was explained by the fact that, in the latter case, the presence of Neuropilin-1 in the remaining receptor complex prevents initiation of repulsive signaling by keeping the Plexin-D1 receptor in an inactive state [4]. The exact mechanism by which Neuropilin-1 operates is currently unknown. Interaction with Neuropilin-1 is thought to induce a conformational change in the intracellular domain of plexins that allows specific proteins, such as the FERM domain-containing GEF protein FARP2 (FERM, RhoGEF, and pleckstrin domain protein 2, also known as FIR), to interact with plexins [42]. It has been shown that the binding of the ligand Sema3A to Neuropilin-1 induces the dissociation of FARP2 from the plexin receptor, resulting in the activation of the Rac GEF activity of FARP2. Subsequent activation of Rac is essential for Rnd1 recruitment to plexin and activation of downstream signaling events, such as

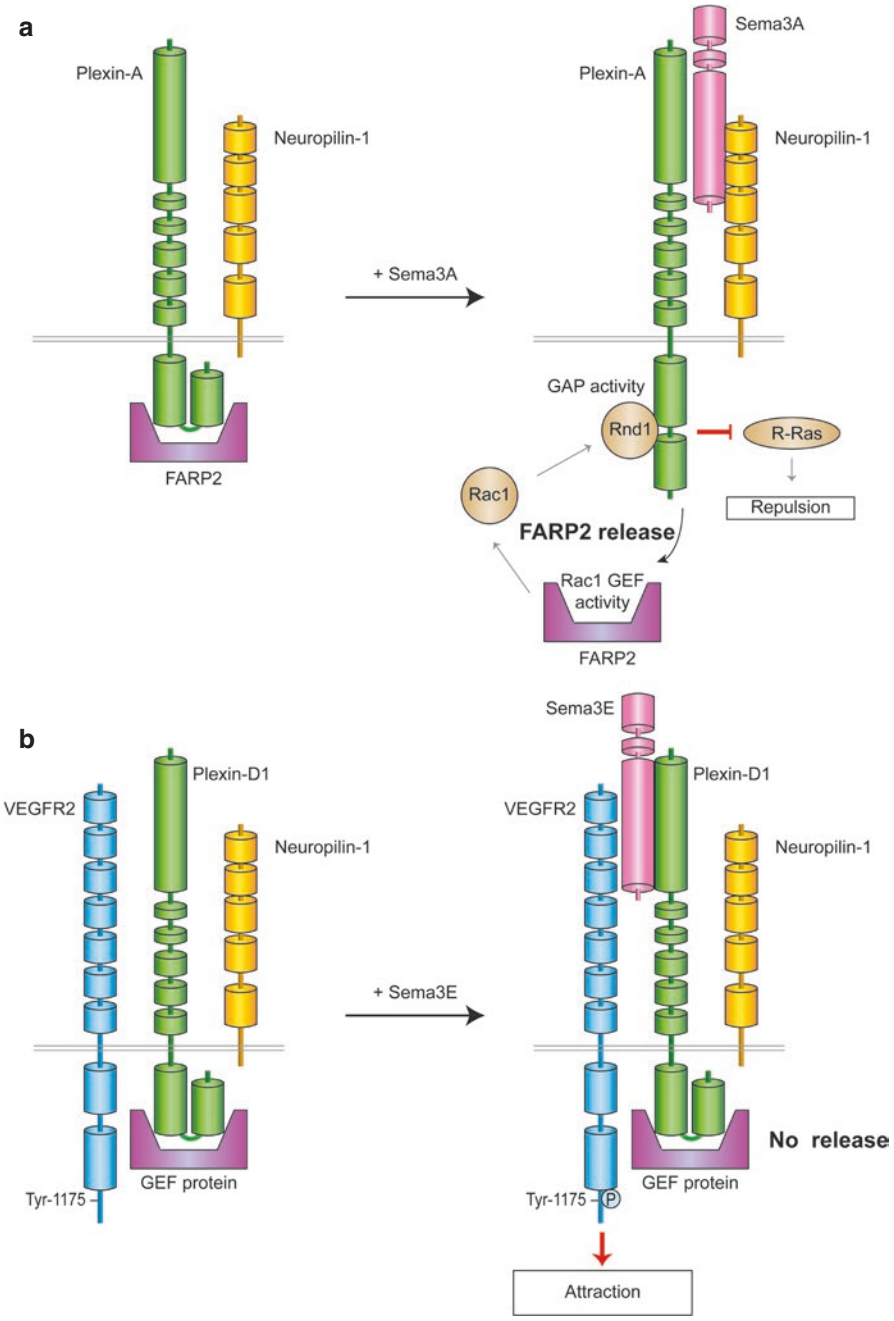
activation of the intrinsic GAP activity of plexin [42] (Fig. 5.3a). However, it could be envisioned that, in the particular case of Sema3E, the direct binding of the ligand to the plexin subunit rather than to Neuropilin-1 may fail to cause the release of FARP2 (or another essential GEF protein) from Plexin-D1 and therefore fail to activate downstream Plexin-D1-mediated repulsive signaling (Fig. 5.3b). In conclusion, the "gating" function of Neuropilin-1 in Sema3E signaling relies on the ability of Neuropilin-1 to both promote VEGFR2-mediated attractive signaling and inhibit Plexin-D1-mediated repulsive signaling.

To date, pyramidal neurons of the subicular cortex are the only identified population of neurons in the mammalian brain that exhibits an attractive response to Sema3E. In zebra fish embryos, a role for Sema3E as a permissive factor or attractant has been proposed to facilitate retinal axon growth across the midline at the optic chiasm [10]. Genetic data suggest that a Neuropilin-1-dependent mechanism mediates this effect [10]. Outside the nervous system, a chemoattractive activity of Sema3E has also been reported on Plexin-D1-positive macrophages with a pro-inflammatory (M1) phenotype [39]. Consistent with results in neurons, disruption of Neuropilin-1 or inhibition of the VEGFR2/PI3K/Akt pathway abrogated Sema3E-induced migration of macrophages [39]. These data further support the general concept that Sema3E acts as a repellent in a neuropilin-independent manner but as an attractant in a neuropilin-dependent manner. However, the conditions for the occurrence of an attractive response to Sema3E are likely to be more stringent than the simple coexpression of Neuropilin-1, Plexin-D1, and VEGFR2. For example, while endothelial cells express all three components of the attractive trimeric receptor for Sema3E, they often show a repulsive response to Sema3E both in vivo and in vitro, with the only endothelial cell line reported so far to be attracted by Sema3E being the SV40-transformed mouse endothelial cells (SVEC-40 cells) [7]. It is therefore likely that additional mechanisms that control the local assembly of cell surface receptors into multimeric complexes determine the outcome of Sema3E signaling.

5.7 Altered Neuropilin-Dependent Sema3E Signaling in a Model of Schizophrenia

The gating mechanism by which Neuropilin-1 modulates Sema3E signaling in subicular neurons has important implications for the development of a major axonal pathway of the forebrain and for the establishment of normal behaviors in adult

Fig. 5.3 Possible mechanism for the repression of Plexin-D1 signaling by Neuropilin-1. (**a**) In the presence of Neuropilin-1, FARP2 interacts with the intracellular domain of Plexin-A. Sema3A mediates dissociation of FARP2 from Plexin-A. Subsequent activation of Rac by GEF activity of released FARP2 allows the binding of Rnd1 to Plexin-A and the downregulation of R-Ras by GAP activity of Plexin-A. (**b**) As with Plexin-A, Neuropilin-1 may regulate the interaction between Plexin-D1 and FARP2 (or another GEF protein). The direct binding of Sema3E to Plexin-D1, rather than to Neuropilin-1, may fail to cause the release of FARP2 (or another GEF) from Plexin-D1, which would then remain inactive


a
Plexin-A
Neuropilin-1
FARP2
+ Sema3A
Sema3A
Plexin-A
Neuropilin-1
GAP activity
Rnd1
R-Ras
Repulsion
Rac1
FARP2 release
Rac1 GEF activity
FARP2
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Plexin-D1
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Tyr-1175
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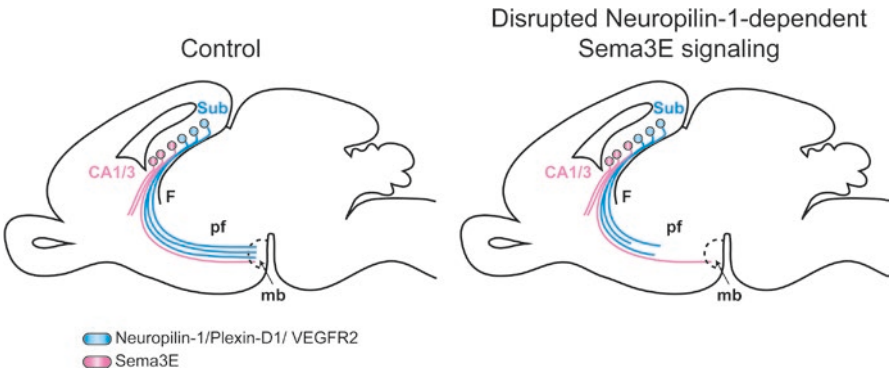

Fig. 5.4 Neuropilin-dependent Sema3E signaling is required for proper development of the fornix fiber tract. Schematic representation of the neuropilin-dependent function of Sema3E in the prenatal development of the postcommissural fornix. Subicular axons (*blue*) express Plexin-D1, Neuropilin-1, and VEGFR2. Sema3E is expressed by pyramidal neurons of the hippocampal CA1/3 fields (*pink*) and therefore is likely to be present along the full length of CA1/3 efferent projections adjacent to subicular axons. In the absence of neuropilin-dependent Sema3E signaling, the development of subicular projections is severely delayed, and only a few fibers reach their target in the caudal hypothalamus. *CA1/3* cornus ammonis 1 and 3, *F* fornix, *pf* postcommissural fornix, *Sub* subiculum, *mb* mammillary bodies

mice [4, 6]. Pyramidal neurons of the subicular cortex, which express the tripartite receptor complex Plexin-D1/Neuropilin-1/VEGFR2, extend long-distance axonal projections through the fornix and the postcommissural part of the fornix (which turn and extend behind the anterior commissure) to eventually reach the mammillary bodies of the hypothalamus (Fig. 5.4). Expression of *Sema3e* mRNA has been detected in pyramidal cells of the developing cornus ammonis (CA)1 and CA3 fields of the hippocampus, just adjacent to the subiculum [6]. Because CA1/CA3 projections grow alongside subicular axons in the initial portion of the fornix tract, they have been proposed to provide a local source of chemoattractive Sema3E signal that guides subicular axons out of the hippocampus and along the fornix [6] (Fig. 5.4). Consistent with this idea, in mouse embryos with disrupted Neuropilin-1-dependent Sema3E signaling, although subicular neurons are still present, their projections were severely reduced and failed to reach their target in the hypothalamus [4, 6]. This leads to a complete disappearance of the postcommissural fornix that was seen in embryos lacking the Sema3E ligand [6] or components of the receptor [4, 6] (Fig. 5.4). Interestingly, fornix abnormalities persist into adulthood.

From a functional perspective, the fornix is part of a larger neuronal network, called the Papez circuit, which is involved in learning and memory, emotion, and social behavior. The behavioral consequences resulting from the disruption of the Neuropilin-1-dependent Sema3E signaling have been explored in *Sema3e*$^{-/-}$ mice, which are viable. The mutant mice showed a reduced anxiety level in a test based on rodents' aversion to open space, as well as defective working memory in a spatial memory task [6]. These behavioral deficits are consistent with impairment of the Papez circuit.

A resulting question, therefore, is whether alterations in neuropilin-dependent Sema3E signaling could underlie abnormal brain wiring in neurodevelopmental and psychiatric disorders accompanied with emotional and memory defects. In possible support of this idea is the recent finding of an alteration of the neuropilin-dependent Sema3E signaling pathway in a mouse model of schizophrenia, the *Map6* (microtubule-associated protein 6) knockout mice [2, 12]. Prominently expressed in neurons, MAP6 (also known as STOP, for Stable-Tubule-Only-Polypeptide) binds to cytoskeletal microtubules and provides resistance to depolymerizing conditions, such as cold temperature [5], but can also have other non-microtubular localizations and functions [3, 23, 24]. *Map6*$^{-/-}$ mice are fully viable but exhibit learning and memory deficits and severe behavioral abnormalities in adulthood, including disorganized activity and social withdrawal [2, 18, 46]. These behavioral alterations are reminiscent of several schizophrenia-like symptoms and can be alleviated by treatment with neuroleptics, the antipsychotic agents principally used in schizophrenia [2, 11].

To search for the neural substrate that may underlie these behavioral and cognitive alterations, anatomical brain connectivity of adult *Map6*$^{-/-}$ mutants was assessed using diffusion tensor imaging (DTI) tractography [12]. Of the major axon tracts of the forebrain, all but one were present in *Map6*$^{-/-}$ mice, although reductions in size and integrity were reported in specific fiber tracts, including the forebrain commissures (anterior commissure and corpus callosum), in comparison to wild-type animals. In contrast, a specific absence of the postcommissural fornix was consistently observed in the mutant brains. This defect was already apparent during embryonic development, indicating a neurodevelopmental problem [12].

The finding that *Map6* was required for the proper development of the postcommissural fornix has led to the investigation of a possible role of MAP6 in axonal responses to Sema3E. Results revealed that MAP6 specifically regulates the neuropilin-dependent function of Sema3E. Indeed, neurons from the subicular cortex of *Map6*$^{-/-}$ mouse embryos failed to respond to the attractive activity of Sema3E in vitro, despite the normal expression of the trimeric Plexin-D1/Neuropilin-1/VEGFR2 receptor complex [12]. In contrast, neurons from *Map6*$^{-/-}$ piriform cortex maintained a normal repulsive, neuropilin-independent response to Sema3E [12]. From a mechanistic perspective, MAP6 was found to directly interact with the Sema3E trimeric receptor complex and could be immunoprecipitated from subicular neurons together with Neuropilin-1 [12]. The loss of MAP6 expression had no apparent effect on the phosphorylation of VEGFR2 on Tyr1175 that is induced by Sema3E binding, indicating that MAP6 likely acts downstream of the receptor activation. Interestingly, the role of MAP6 in Sema3E signaling was found to be independent of its microtubule-binding activity, instead requiring stretches of proline residues contained in the N-terminal domain of the protein [12]. This proline-rich domain (PRD) was found to interact with a broad range of Src homology 3 (SH3) domain-containing proteins, including the p85 regulatory subunit of PI3K [12]. These results support a model in which MAP6 contributes to the Neuropilin-1-dependent function of Sema3E by controlling the activation of the PI3K/Akt signaling pathway through the recruitment of PI3K to the activated receptor complex [12] (Fig. 5.1b).

Studies investigating the brains of schizophrenic patients have reported decreased fornix volume and integrity at illness onset [13, 17, 27, 28]. Early fornix alterations have been correlated with greater severity of psychotic symptoms [1] and greater episodic memory impairments, which is a consistent symptom of schizophrenia [30, 32, 40]. Thus, atypical fornix connectivity might account for the behavioral and cognitive impairments in patients with schizophrenia. However, the causative mechanisms are not yet defined. Because genetic association and expression studies in human brains have suggested a possible pathological role for MAP6 in schizophrenia development [38], the contribution of MAP6, and potentially of neuropilin-dependent Sema3E signaling, to the processes that result in schizophrenia merits further attention.

Conclusion

Neuropilins have pleiotropic and essential functions in embryogenesis and adult life by regulating a variety of signaling pathways, involving multiple growth factors and semaphorins. Dysregulation of neuropilin function has been linked to neurological disorders including autism and schizophrenia [37, 44, 47]. The above reviewed studies illustrate how dissecting the fine molecular details of neuropilin functions, either as initiators or modulators of downstream signaling, may help reveal specific neuronal phenotypes that contribute to disease mechanisms and clinical symptoms.

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Part II

Neuropilins as Regulators of Developmental and Immune Processes

The Role of the Neuropilins in Developmental Angiogenesis

6

James T. Brash, Anastasia Lampropoulou, and Christiana Ruhrberg

Contents

Abstract

The process of angiogenesis, defined as the sprouting of new blood vessels from existing ones, is a key process in the development of the cardiovascular system and is tightly regulated by a plethora of signalling pathways. The transmembrane receptor neuropilin 1 (NRP1) is expressed in blood vascular endothelial cells to regulate angiogenesis by binding to several different ligands and receptors. Here, we provide an overview of the various functions of NRP1 in angiogenesis during both embryonic and postnatal blood vascular development with particular reference to studies that have defined these functions on the cellular and molecular level. We additionally discuss briefly possible roles for the NRP1 homolog NRP2 in vascular development and the requirements for NRP1 and NRP2 in lymphatic vascular development.

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Abbreviations

ECM	Extracellular matrix
HSPG	Heparan sulphate proteoglycan
VEGF	Vascular endothelial growth factor A

6.1 Introduction

The cardiovascular system is the first organ system to develop during embryogenesis in vertebrates. Angioblasts initially give rise to a honeycomb-shaped blood vessel network in the yolk sac and form the paired dorsal aorta in the embryo proper in a process termed vasculogenesis. These blood vessels subsequently expand by angiogenesis into a vast network capable of sustaining tissue metabolism throughout the body (reviewed by [53]). Under physiological conditions, angiogenesis occurs both during embryonic and perinatal development, whilst adult endothelium is usually quiescent and becomes proliferative again only in specific circumstances, for example, in the cycling uterus, during pregnancy or during wound healing and in other pathological conditions (reviewed in [14, 35]). Angiogenic vessel sprouts are composed of filopodia-studded endothelial tip cells, which lead the migrating sprouts, and endothelial stalk cells that proliferate and form the lumen (e.g. [26]). In addition to expanding the vasculature by infiltrating host tissues, the vessel sprouts have to fuse to each other to add new perfused circuits to the expanding plexus. The transmembrane protein neuropilin (NRP) 1 is expressed in the vascular endothelium to modulate responses to extracellular stimuli that promote angiogenesis. Here, we will discuss the role of NRP1 in developmental angiogenesis, including its interaction with several distinct ligands and other receptors that regulate endothelial cell behaviour. We will also briefly review the contribution of the second neuropilin, NRP2, in vascular development, including lymphatic vascular development.

6.2 NRP1 Is Essential for Developmental Angiogenesis

The first evidence implicating NRP1 in cardiovascular development was generated through the analysis of genetically engineered mice that overexpress this protein [42]. These mice are embryonic lethal, with excessive growth of leaky blood vessels [42]. Subsequently, *Nrp1*-null mouse embryos were generated and shown to also be embryonic lethal [41]. These mice have impaired vascularisation of neural tissues, including the brain and spinal cord, as well as defective remodelling of the great vessels of the heart, including the pharyngeal arch arteries and the outflow tract [41]. They also have abnormal vascular development within the yolk sac, which is essential for the oxygenation of the mammalian embryo and also gives rise to the first blood cells [37, 41].

NRP1's specific functions in promoting sprouting angiogenesis have been extensively studied in the mouse embryo hindbrain, which is vascularised early in

development to support tissue growth (reviewed by [54]). Hindbrain vascularisation begins on around embryonic day (E) 9.5 in the mouse, when neural progenitor-derived vascular endothelial growth factor (VEGF) stimulates vessel sprouting from the perineural vascular plexus towards the ventricular zone [8, 33, 55]. From E10.5 onwards, these sprouts grow as radial vessels before turning at near right angles when they approach the subventricular zone (SVZ) and then anastomosing with neighbouring sprouts to form the subventricular vascular plexus (SVP). Sprout anastomosis is aided by tissue macrophages, a process that is readily observed in flat-mounted E11.5 hindbrain tissue [18]. Accordingly, an extensive intraneural vascular network beneath the hindbrain ventricular zone is established by E12.5 [18, 55]. The development of the hindbrain early in embryogenesis makes it a useful organ model to study developmental angiogenesis in mice with prenatal embryonic lethality such as *Nrp1*-null mice, as long as they survive past E10.5 [21].

Analysis of the mouse embryo hindbrain showed that heterozygous *Nrp1*-null mutant mice have delayed SVP formation, whereas *Nrp1*-null mice fail to establish this vascular plexus altogether [20, 23, 27]. Specifically, the vessels of *Nrp1*-null mice sprout towards the SVZ normally, but then fail to branch or anastomose with neighbouring vessels [20, 27]. Accordingly, radial vessels terminate in tuft-like structures in *Nrp1*-null mutant hindbrains (Fig. 6.1a, b). Although non-endothelial cell types, including neural progenitors and macrophages, also express NRP1 and contribute to hindbrain vascularisation by secreting VEGF or promoting anastomosis, respectively, the NRP1 requirement for angiogenesis is exclusive to the endothelium [20]. This was demonstrated through the comparison of SVP development in mice lacking endothelial-, macrophage- or neural progenitor-expressed NRP1. These mice were generated with Cre/LoxP technology by combining floxed conditional *Nrp1*-null alleles with the *Tie2-Cre*, *Csf1r-iCre* or *Nes-Cre* transgenes that recombine predominantly in the endothelial/macrophage, macrophage or neural lineage, respectively [20]. Thus, only mice lacking endothelial NRP1 phenocopied the hindbrain vascular defects observed in the *Nrp1*-null mice.

Even though NRP1 is normally expressed in both tip and stalk cells of vessel sprouts (Fig. 6.1a), it has a particularly important role in promoting filopodia formation and therefore tip cell function [20, 23]. Thus, NRP1-deficient hindbrains lack morphologically identifiable tip cells despite the expression of tip cell genes [20] (Fig. 6.1a, b). Moreover, the analysis of hindbrains from mice with mosaic targeting of NRP1 in endothelial cells showed that cells retaining NRP1 preferentially adopted the tip rather than stalk position in vessel sprouts and enabled the sprouting of vessels in which stalk cells lacked NRP1 [20] (Fig. 6.1c). Notably, the number of recombination-resistant, NRP1-retaining tip cells in mosaically targeted endothelium correlated with the extent of vascular branching, supporting the idea that NRP1 function is essential for tip cell-mediated vessel sprouting [20].

The endothelial function of NRP1 has also been studied during retinal angiogenesis in the mouse. This tissue is vascularised after birth, when endothelial sprouts headed by filopodia-studded tip cells migrate towards astrocyte-localised VEGF in the retinal periphery and tip cells fuse to each other in a process that depends on tissue macrophages [18] and extracellular matrix (ECM) [23, 26, 49, 55], as

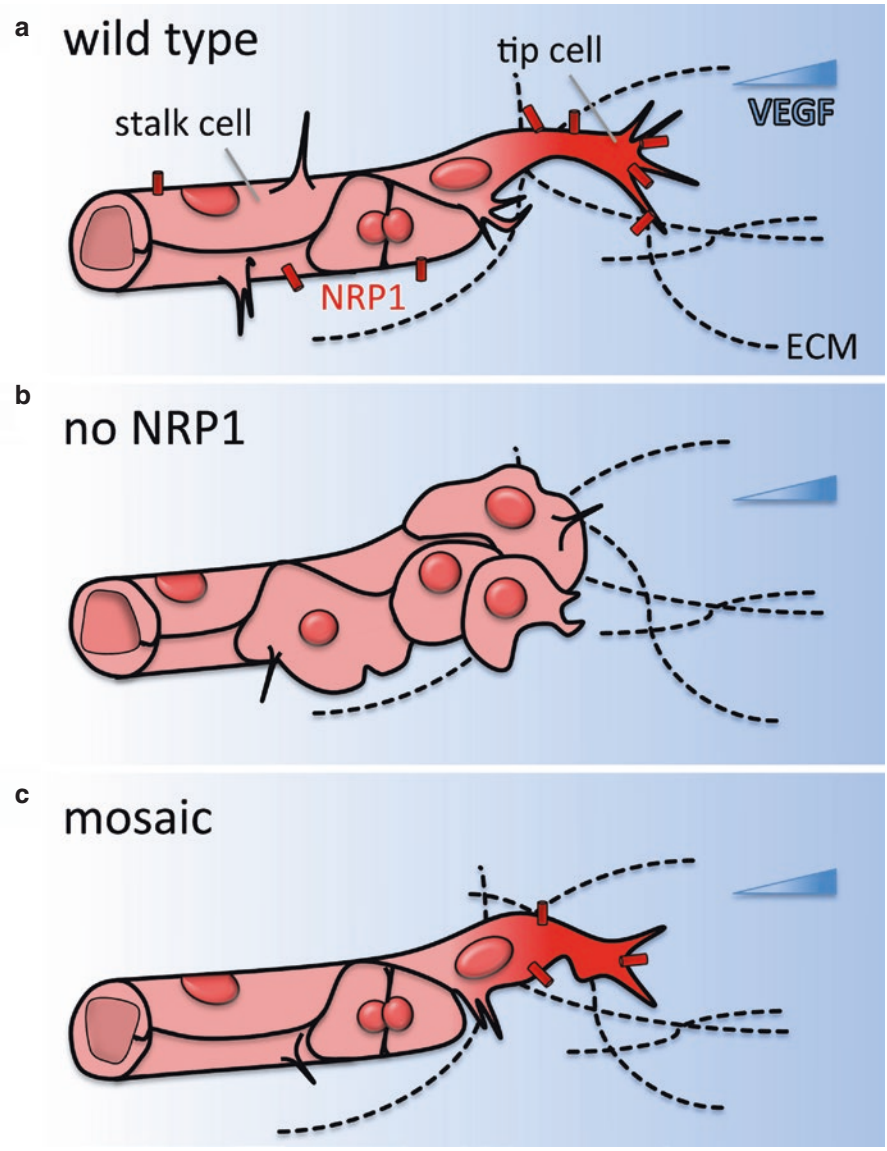

Fig. 6.1 Essential role for NRP1 in angiogenesis. (**a**) NRP1 is expressed in endothelial tip and stalk cells of vessels that extend filopodia and migrate in response to VEGF gradients and ECM, in particular in neural tissues such as the brain and retina. (**b**) Lack of endothelial NRP1 impairs filopodia formation and vessel sprouting, leading to the formation of blind-ended vascular tufts in neural tissues. (**c**) Mosaic targeting of NRP1 in endothelial cells showed that cells retaining NRP1 adopt a tip rather than a stalk position in vessel sprouts and can rescue vessel sprouting in response to VEGF and ECM

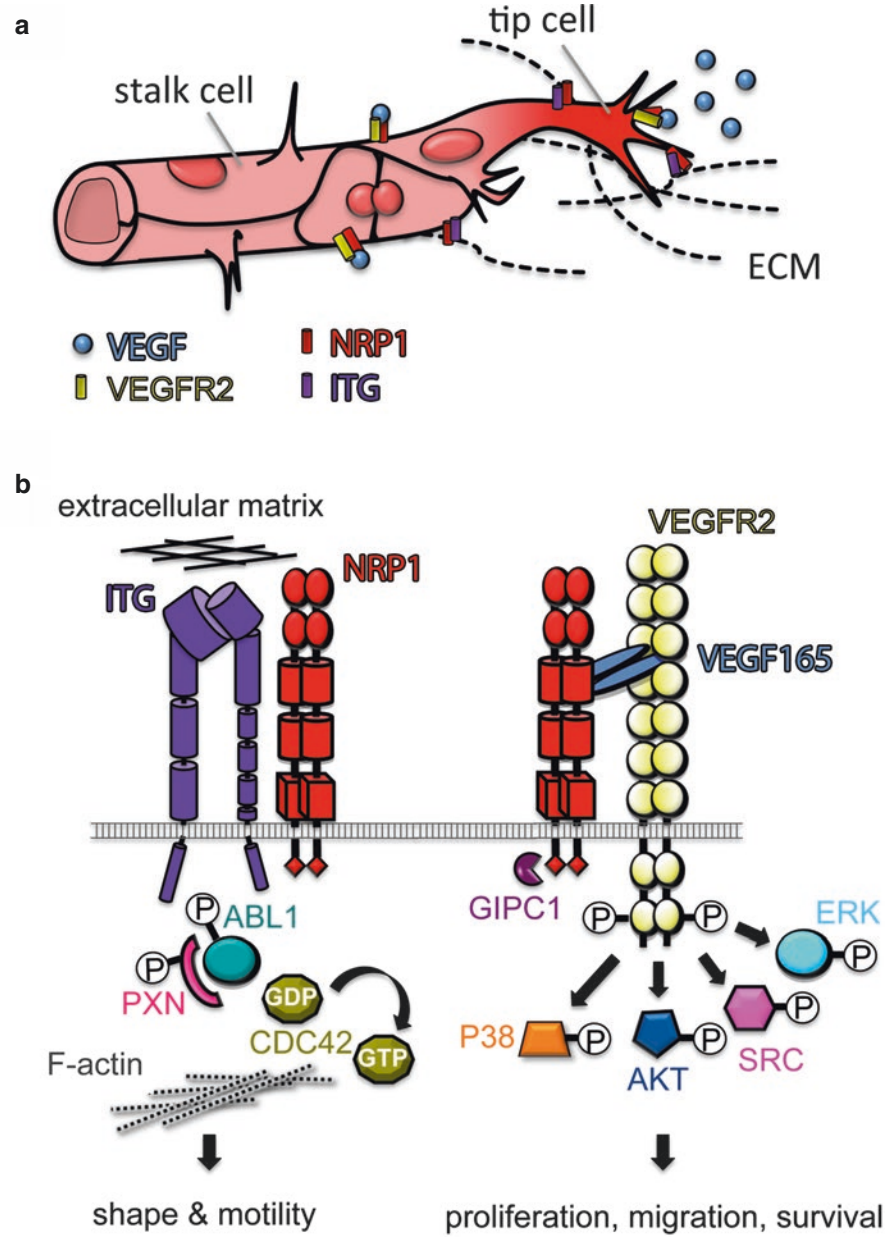

Fig. 6.2 NRP1 promotes angiogenesis through roles in VEGF and ECM signalling. (**a**) In endothelial cells, NRP1 forms complexes with VEGFR2 to enable a VEGF response, which is known to promote endothelial cell migration and proliferation. In parallel, NRP1 interacts with integrins (ITG) and enables migration on integrin ligands in the ECM of neural tissues such as the brain and retina. (**b**) NRP1-binding VEGF induces the formation of a VEGFR2/NRP1 complex that promotes downstream signalling through ERK and p38, and possibly AKT, for cell proliferation, migration and survival. VEGF binding to VEGFR2 also promotes the activation of SRC familiy kinases, but it is not yet known whether this response relies on NRP1. Independently of its role as a VEGFR2 co-receptor, NRP1 activates ABL1 and CDC42 to modulate actin remodelling and promote paxillin phosphorylation and filopodia formation for endothelial cell migration

reviewed in [54]. As *Nrp1*-null mice are embryonic lethal, postnatal studies of NRP1 function in the vasculature require temporally inducible deletion. This has been achieved by breeding mice carrying floxed *Nrp1*-null alleles to mice carrying a tamoxifen-inducible *Cre* transgene under the control of the endothelial *Pdgfb* promoter. Upon NRP1 depletion through tamoxifen treatment between perinatal day 2 (P2) and P5, the retina develops vasculature with decreased network density and fewer vascular branch points [23, 50]. Similar to earlier observations in the hindbrain [20], the analysis of retinal angiogenesis showed that NRP1-expressing cells in mosaically targeted vasculature adopted the tip cell position [3].

Vascular stability is also compromised in developing mice lacking endothelial NRP1 [34]. For example, mouse embryos containing floxed *Nrp1* alleles and a Cre transgene under the control of the endothelial-specific *Alk1* promotor have extensive oedema and haemorrhage by E16.5 [34]. These defects are particularly evident in the ganglionic eminence and thalamus [34].

6.3 NRP1 Ligands in Developmental Angiogenesis

As a transmembrane protein on the endothelial cell surface, NRP1 interacts with several ligands or their receptors to mediate their signal transduction (Fig. 6.2a) (reviewed in [51]). Amongst the most studied of these ligands are a member of the VEGF family termed VEGF165 and the class 3 semaphorins SEMA3A and SEMA3C.

In mammals, the VEGF family consists of five secreted glycoproteins with a signature cysteine knot motif. The prototypical member of this family is VEGF-A, hereafter referred to as VEGF. VEGF is a potent proangiogenic factor that acts variably as an endothelial chemoattractant, mitogen and survival factor (e.g. [2, 15]). The VEGF gene is comprised of eight exons that are alternatively spliced to produce three main isoforms in humans, referred to as VEGF121, VEGF165 and VEGF189 to indicate the number of amino acids that comprise the mature protein [53]. VEGF189 contains the protein domains encoded by all 8 exons, whilst VEGF165 lacks the exon 6-encoded domain and VEGF121 lacks both the exon 6- and exon 7-encoded domains. The mouse isoforms are one amino residue shorter and are therefore termed VEGF120, VEGF164 and VEGF188.

The functions of the exon 6- and 7-encoded domains are twofold. Firstly, they facilitate VEGF binding to heparin in vitro and, accordingly, are thought to promote the interaction between VEGF and heparan sulphate proteoglycans (HSPGs) on the cell surface or in the ECM in vivo [53]. In agreement, VEGF189 displays the strongest and VEGF121 the weakest ECM affinity, and VEGF165 exhibits intermediate ECM affinity [53]. Through their differential strength of retention in the ECM, the VEGF isoforms establish chemotactic gradients that provide powerful migration cues and are detected by endothelial filopodia [55]. Moreover, heparin stabilises VEGF165 and by preventing its loss of activity through oxidation in vitro [28]. Even though the enzyme HS6ST2, which is required for the sulphation of HSPGs, interacts with VEGF during zebrafish vascular development [13], direct proof that

VEGF binding to HSPGs is important for angiogenesis is still lacking. The second function of the alternatively spliced exon 7-encoded domain is to work in conjunction with the exon 8 domain to mediate binding of VEGF to the b1b2 domain of NRP1 [47]. Thus, the VEGF165 and VEGF189 isoforms have a high affinity for NRP1 and, in this fashion, are able to evoke responses of different amplitude and quality compared to VEGF121, which lacks the exon 7-encoded domain and therefore has a poor affinity for NRP1 (reviewed in [51]). These responses are discussed in detail below.

The class 3 semaphorins SEMA3A, SEMA3C and SEMA3E have been implicated in vascular development. SEMA3E binds to plexin D1 (PLXND1) and affects blood vessel formation independently of the NRPs [32], although there is evidence that NRP1 modulates SEMA3E signalling through PLXND1 during axon guidance [12]. SEMA3C instead signals through NRP1/PLXND1 complexes to induce cardiac outflow tract septation during embryogenesis in a mechanism that involves neural crest cell-induced endothelial-to-mesenchymal transition [48]. Neither of these processes is reviewed in detail here.

Studies in chick and fish have suggested that SEMA3A signalling through NRP1 regulates vascular development [1, 57, 61]. Thus, exogenous SEMA3A inhibits VEGF-induced angiogenesis in the chick chorioallantoic membrane by inhibiting the VEGF-induced activation of the focal adhesion kinase (FAK) and the cellular homolog of the ROUS sarcoma kinase (SRC), two intracellular proteins that promote angiogenesis via cytoskeletal remodelling [1]. Moreover, morpholino-induced knockdown of the *sema3a1* or *sema3a2* homologs in fish inhibits intersomitic vessel sprouting. However, mice lacking SEMA3A or all SEMA3 signalling through NRP1 or both NRP1 and NRP2 have normal developmental angiogenesis [7, 32, 63]. Moreover, mice lacking both SEMA3A and VEGF164 showed vascular defects similar to mice lacking VEGF164 only [63]. Accordingly, different conclusions regarding the role of semaphorin signalling have been reached depending on the species studied, with no obvious role for SEMA3A signalling in developmental angiogenesis in mice. In contrast, SEMA3A plays an important role in the murine lymphatic system by promoting lymphatic valve development by signalling through NRP1 and PLXNA1 [7, 39].

SEMA3A is also important for pathological angiogenesis in the mouse [11, 38, 44]. This has been shown in a model of oxygen-induced retinopathy (OIR), in which neonatal mice are exposed to hyperoxia and then returned to normoxia; this process causes vascular regression and inflammation in the retina, culminating in abnormal vessel growth (reviewed in [54]). Thus, SEMA3A is secreted from retinal neurons in response to inflammation to repel new vessel growth away from avascular retinal areas towards the vitreous, and SEMA3A inhibition promotes beneficial retinal revascularisation [38]. SEMA3A expression also inhibits tumour angiogenesis by inducing endothelial cell apoptosis and normalises tumour vasculature, with possible benefits to anti-tumour drug delivery, by promoting pericyte coverage of tumour vessels [11, 44]. Thus, SEMA3A recruits NRP1-expressing monocytes, which then secrete growth factors such as transforming growth factor beta (TGFβ) and platelet-derived growth factor beta (PDGFβ) to attract pericytes [10, 68].

Intravitreal delivery of SEMA3C also impairs vascular development in the postnatal mouse retina by reducing the number of tip cells and inhibits pathological neovascularisation in the OIR model [65]. However, it is not known whether these effects are mediated by NRP1 or NRP2; moreover, it has not yet been examined whether endogenous SEMA3C contributes to retinal vascularisation.

6.4 VEGF Signalling Through NRP1 in Vascular Development

As NRP1 was first identified as a co-receptor for the VEGF receptor tyrosine kinase VEGFR2 in endothelial cells upon VEGF165 stimulation [58], the exclusive explanation for the angiogenic defects of *Nrp1*-null mice was initially thought to be disrupted VEGF signalling (reviewed in [51]). Several lines of evidence were obtained that support this hypothesis, including in vitro studies of porcine aortic endothelial cells, which endogenously lack VEGFR2 and NRP1, but can be transfected with expression plasmids for one or both of these receptors to establish their relative contribution to VEGF signalling [58]. In these cells, VEGF-stimulated, VEGFR2-mediated activation of ERK1/2 and p38MAPK signalling and chemotaxis was enhanced by NRP1 [6, 58]. In cultured embryoid bodies, VEGF also requires NRP1 to activate p38 MAPK kinase, whose inhibition attenuates angiogenesis [40]. In agreement with a role for VEGF binding to NRP1 to regulate chemotaxis in vivo, mice with a mutation that abrogates VEGF binding to NRP1 have reduced radial outgrowth of vessels in the perinatal mouse retina [22, 25]. In addition, these mice have impaired arteriovenous specification and vascular smooth muscle coverage of retinal arteries [22, 25].

In contrast to the result obtained for retinal angiogenesis, the role of NRP1 in promoting VEGF signalling appears to be of little significance for embryonic angiogenesis. Thus, unlike mice lacking endothelial NRP1 altogether, mice defective in VEGF binding to NRP1 have no obvious defects in brain vascularisation [22, 25], even on a *Nrp2*-null background (A. Plein and C. Ruhrberg, unpublished observations). The milder vascular phenotype of mice lacking VEGF binding to NRP1 suggests that NRP1 also has VEGF-independent functions in vascular development.

6.5 NRP1 as a Regulator of TGFβ Signalling

The cytokines TGFβ1, TGFβ2 and TGFβ3 regulate migration, proliferation and apoptosis in various cell types [17]. Studies in T cells suggest that the latent and activated forms of TGFβ1 bind to NRP1 at the site that also binds VEGF165, and that soluble NRP1 activates latent TGFβ1 [29]. In breast cancer cells, NRP1 interacts with the TGFβ receptors TGFβR1 and TGFβR2 to help activate their effectors SMAD2 and SMAD3 [30]. In contrast, two recent studies suggested that NRP1 dampens endothelial TGFβ signalling via both the SMAD2/3 and the SMAD1/5/8 pathways [3, 34]. In particular, it was demonstrated that NRP1-mediated

suppression of TGFβ signalling promotes the tip cell phenotype and therefore blood vessel growth during retinal angiogenesis [3]. Moreover, DLL4 in endothelial tip cells activates notch signalling in neighbouring endothelial cells to downregulate NRP1 and thereby induces excessive TGFβ signalling and stalk cell behaviour [3]. Endothelial NRP1 loss also increases TGFβ signalling via SMAD2/3 in the mouse embryo hindbrain [34]. Interestingly, in vitro and genetic mouse studies suggest that neuroepithelial β8 integrin interacts with endothelial NRP1 in trans and reduces endothelial SMAD2/3 activation [34]. It is not known whether these opposing roles can be explained by a direct integrin-NRP1 interaction, their competition for binding to latent TGFβ and/or additional roles for NRP1 in regulating TGFβR receptor activation.

6.6 NRP1 Promotes VEGFR2-Independent Angiogenesis in Response to Integrin Ligands

Several studies have shown that the integrin subunits β1 and β3 interact with NRP1 in tumour and endothelial cells [24, 52, 62]. However, the function of integrin-NRP1 interactions in angiogenesis is complex. On the one hand, tissue culture models measuring endothelial wound healing or aortic ring sprouting as well as in vivo models of tumour angiogenesis showed that αvβ3 integrin can negatively regulate angiogenesis that has been stimulated through the VEGF-NRP1 axis [52]. On the other hand, in vitro studies in human umbilical artery endothelial cell showed that NRP1 organises fibronectin assembly by promoting intracellular trafficking and recycling of activated α5β1 integrin in a mechanism that relies on the NRP1 cytoplasmic domain [62]. This mechanism has been proposed to promote angiogenesis by enhancing endothelial cell migration [62]. However, mice lacking the NRP1 cytoplasmic domain have normal angiogenesis [19, 43]. Therefore, the in vivo importance of the NRP1 cytoplasmic domain for integrin recycling and fibronectin remodelling in the vasculature remains to be identified; in particular it would be interesting to determine whether NRP1 regulation of α5β1 contributes to pathways previously shown to be dependent on the NRP1 cytoplasmic domain, including arteriogenesis and arteriovenous patterning [19, 43]. Interestingly, a role for NRP1 in fibronectin fibril assembly has also been observed in cancer cells, in which NRP1 recruits the cellular homolog of the Abelson leukaemia oncogene ABL1, an intracellular tyrosine kinase, in a NRP1 cytoplasmic domain-dependent fashion [66]. Two prior studies suggested that loss of NRP1 also impairs endothelial cell adhesion to low concentrations of fibronectin [45, 62]. However, this role for NRP1 in cell adhesion was not observed when endothelial cells encountered concentrations of fibronectin used for routine tissue culture [50].

Studies in human dermal microvascular endothelial cells and primary mouse lung endothelial cells showed that NRP1 forms a complex with ABL1 and recruits the ABL1 substrate paxillin in response to fibronectin, leading to paxillin phosphorylation and therefore focal adhesion remodelling for endothelial cell migration (Fig. 6.2b) [50]. Even though the NRP1 cytoplasmic domain mediates ABL

recruitment in cancer cells [66], it is dispensable for angiogenesis [19]. As at least two integrin subunits, β1 and β2, associate with ABL1 to promote cell adhesion and migration [5, 16], the NRP1 cytoplasmic domain and integrins may have partially redundant roles in recruiting ABL1 to focal adhesion.

Studies in bone marrow-derived macrophages demonstrated that the association of integrins with ABL1 and SRC family kinases promotes cell migration by regulating the activity of CDC42 and RAC1 [5]. We have recently shown that NRP1 interacts with ABL1 in endothelial cells to activate CDC42 in response to fibronectin stimulation, which then facilitates actin remodelling (Fig. 6.2b) [23]. Demonstrating the importance of this pathway in vivo, the pharmacological inhibition of either ABL1 or CDC42 during postnatal development reduces the number of vascular branch points and tip cells, similar to loss of endothelial NRP1 [23, 50]. Moreover, the endothelial deletion of CDC42 impairs filopodia formation and vascular sprouting in postnatal mouse retina [4].

Interestingly, the retina of endothelial NRP1 knockout mice showed defective vascular extension in addition to the lateral branching and tip cell defect seen in mice lacking ABL1 and CDC42 activation [22, 23, 25]. This defect was similar to that seen in the retina of NRP1 mutants lacking the VEGF binding site, even though the latter do not have defects in the number of lateral branch points or tip cells [22, 23, 25]. These data suggest that NRP1 has a dual role in postnatal angiogenesis by controlling ECM-induced ABL activation and VEGF-mediated VEGFR2 signalling (Fig. 6.2b) [23, 50].

6.7 NRP2 Contributes to Vascular Development

In contrast to *Nrp1*-null mice, mice lacking NRP2 do not have obvious developmental angiogenesis defects, although a vascular role for the receptor has been described in the OIR model [56, 67]. Nevertheless, NRP2 appears to partially compensate for NRP1 in developmental angiogenesis, because mice with homozygous null alleles for either *Nrp1* or *Nrp2* that additionally carry a heterozygous null mutation in the other gene have defective yolk sac vascularisation [60]. Moreover, loss of semaphorin signalling through both NRP1 and NRP2, but not loss of NRP2 or loss of semaphorin signalling through NRP1 alone, prevents remodelling of the cardiac outflow tract into the aortic and pulmonary arterial trunks [31, 48]. Despite their partially redundant roles in the blood vasculature, both neuropilins have distinct roles in lymphatic vascular development, with *Nrp1*-null mice having defective lymphatic valves and *Nrp2*-null mice lacking properly formed lymphatic vasculature [7, 46, 67]. Whereas the lymphatic defects of *Nrp1*-null mice have been attributed to defective SEMA3A signalling [7, 46], the lymphatic defects of *Nrp2*-null mice are thought to reflect a role for NRP2 in promoting signalling of the VEGF homolog VEGF-C through the VEGFR2 homolog VEGFR3 [64, 67]. Exogenous administration of the NRP2 ligand SEMA3F also inhibits the growth of retinal and choroidal

endothelial cells in explant culture [9]. However, there are presently no reports that mice lacking SEMA3F have developmental vascular abnormalities or reduced ocular vascular pathology.

6.8 Conclusions and Future Directions

NRP1 is now well recognised as an indispensable signalling receptor in multiple processes important for cardiovascular development, spanning the period from yolk sac angiogenesis in the early embryo to angiogenesis after birth. NRP1's diverse roles in cardiovascular development can be attributed to its involvement in a repertoire of vascular processes and its interactions with a range of ligands and receptors. Nevertheless, our understanding of NRP1's precise molecular functions in angiogenesis is still evolving. We have learnt in recent years that one of the two best-known ligands of NRP1, SEMA3A, is not required for developmental angiogenesis. Besides, signalling through the VEGF-NRP1 signalling axis, although once thought to be the primary function of NRP1 in blood vessels, was lately recognised to be only one of several pathways by which NRP1 promotes vascular development. Accordingly, NRP1 has additional roles in promoting ECM-stimulated angiogenesis and in regulating TGFβ signalling. Moreover, it is likely that other, currently unidentified NRP1 pathways play prominent roles in developmental angiogenesis, because the NRP1 ligands relevant for yolk sac and brain angiogenesis have not yet been defined. A potential candidate is galectin 1, which interacts with NRP1 and stimulates VEGFR2 signalling and endothelial migration in vitro in a NRP1-dependent manner [36]. The proangiogenic hepatocyte growth factor (HGF) also binds NRP1 in vitro and stimulates angiogenesis in a mouse matrigel model in a mechanism that can be inhibited by antibodies to NRP1 [59]. However, the relevance of these ligands for NRP1 signalling in developmental angiogenesis remains to be determined. Thus, NRP1's versatility in regulating several different signal pathways continues to add complexity to experimental work aiming to understand how NRP1 modulates endothelial functions in different contexts, such as embryonic versus postnatal developmental angiogenesis or pathological neovascularisation. Moving forward, it will be important to distinguish NRP1's roles in these diverse angiogenic settings and to determine how they may diverge from NRP1's roles in other vascular processes such as arteriogenesis, vascular remodelling and ligand-induced vascular hyperpermeability. It will also be important to understand what the endogenous roles of NRP2 and its ligands are in vascular development of the eye and how it may be able to compensate for NRP1 in yolk sac vascularisation.

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Neuropilins in Lymphatic Development and Function

7

Jinah Han, Georgia Zarkada, and Anne Eichmann

Contents

Abstract

The lymphatic vascular system is important to maintain extracellular fluid homeostasis and crosstalk with the immune system. Neuropilin 1 and 2 (Nrp1, Nrp2) receptors have emerged as important regulators of lymphangiogenesis. This review summarizes recent findings regarding the roles of Nrp1 and 2 in lymphatic development.

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7.1 The Lymphatic Vascular System

The lymphatic system is a network of tubes that drains interstitial fluid from tissues and returns it to the circulatory system. It is present in vertebrates and mammals and performs multiple critical functions, including maintenance of tissue fluid homeostasis, trafficking of antigen presenting cells, and absorption and transportation of dietary lipids from the intestine. It consists of lymphatic capillaries, precollecting and collecting lymphatic vessels. The lymphatic capillaries are blind-ended vessels that are specialized in fluid and cell uptake. They are highly permeable due to a discontinuous basement membrane, loose intercellular junctions, and anchoring filaments that attach to the extracellular matrix and pull junctions apart when tissue fluid accumulates. The precollecting and collecting vessels are covered by pericytes/smooth muscle cells and possess contractile function required to transport lymph. In addition, luminal valves in the collecting lymphatic vessels prevent lymph backflow [6] (Fig. 7.1).

The development of the lymphatic vasculature involves lymphatic endothelial cell (LEC) specification, sprouting and migration, as well as maturation, remodeling, and formation of valves. In the mouse, the lymphatic system starts to develop after the establishment of the blood circulation. LECs differentiate from blood

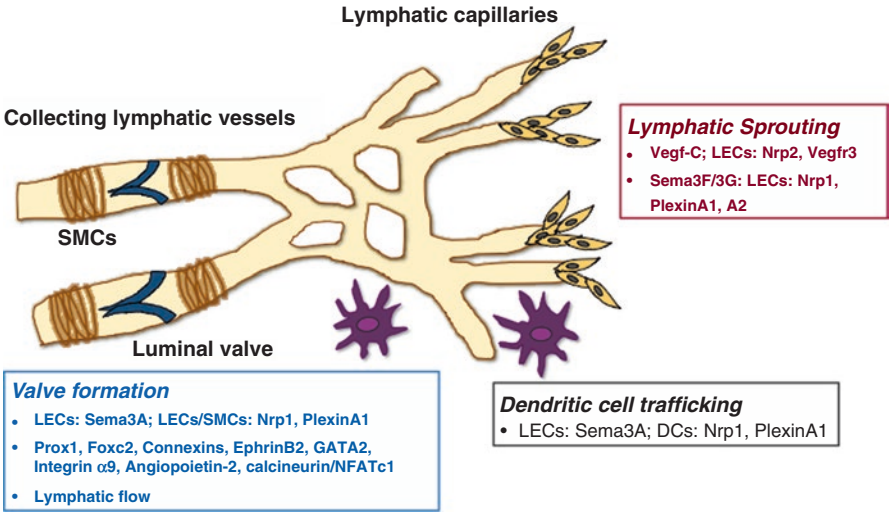

Fig. 7.1 Neuropilins in lymphatic vasculature development. The lymphatic vasculature consists of sprouting capillaries and larger collecting lymphatic vessels. Collecting lymphatic vessels are covered by smooth muscle cells (*SMCs*) and have luminal valves. Antigen-presenting dendritic cells (*DCs*) enter the lymphatic vascular system through afferent lymphatic vessels. Neuropilins, their co-receptors, and their ligands are involved in various aspects of lymphatic system development and function, as described in detail in the text.

endothelial cells (BECs) in the cardinal vein (CV) at embryonic day (E) 9.0. At that time the SRY-related HMG domain transcription factor Sox18 is expressed in BECs lining the dorsolateral area of the CV [27]. The activation of SOX18 is positively regulated by ERK activation and negatively by AKT, which phosphorylates and inactivates RAF1, leading to ERK shutdown [20]. Once activated, Sox18 cooperates with the orphan nuclear transcription factor Coup-TFII and induces the expression of another transcription factor Prox1 as early as E9.5 [88].

Prox1 is required for LEC specification, as shown in Prox1 mutants that lack all LECs [99]. Mechanistically, the targets of Prox1 that specify LEC identity include the Vegf-C receptor Vegfr3 [88]. Once Prox1 expression is activated, Prox1-positive LEC precursors bud from the dorsal side of the CV in a polarized manner and form the primitive lymph sacs, which can be found along the anteroposterior axis of the mouse embryo. The migration of LECs is chiefly orchestrated by Vegf-C/Vegfr3 signaling [44]. Vegf-C signaling via the receptor tyrosine kinase Vegfr3 is critical for LEC survival, proliferation and migration, via PKC-dependent activation of ERK and PI3/Akt phosphorylation [57].

The lymphatic system is largely derived from veins in mice [77] and in zebrafish [50, 103]. In mouse embryos, a second source of LECs distinct from the cardinal vein (CV), which is located at the lower edge of the superficial venous plexus, has been identified [35]. Likewise, in zebrafish some LECs arise from mesoderm-derived angioblasts residing within the ventral wall of the cardinal vein [63]. In addition some lymphatic vessels in the murine lumbar and dorsal skin as well as some of the mesenteric lymphatics develop from non-venous cells through a process defined as lymphvasculogenesis [59, 89]. A similar mechanism has been described for cardiac lymphatics, with some of them originating from hemogenic endothelium [49]. Finally, in birds and amphibians separate mesenchymal lymphangioblasts contribute to the formation of the lymphatic vascular system [67, 79, 100].

Following the formation of the primitive lymphatic capillary plexus, the peripheral lymphatic vessels form by sprouting lymphangiogenesis and mature into capillaries, precollecting and collecting vessels. During lymphatic vessel maturation, the collecting lymphatic vessels deposit basement membrane, form valves and recruit smooth muscle cells [102]. The lymphatic vessels continue to grow, remodel, and mature during postnatal development [19, 66, 91]. Interestingly, lymphatic vessels rely on Vegfr3 signaling only for the first two postnatal weeks in mice and become relatively resistant to Vegfr3 blockade thereafter [45, 106].

Fluid flow has gathered attention as an important factor in lymphatic development [97]. In vitro systems demonstrated that lymphatic endothelial cells respond to flow changes [14] and synergistically work with growth factors to stimulate lymphangiogenesis [36]. In vivo evidence also supports flow-regulated growth and remodeling of the lymphatic vasculature. It has been reported that lymphatic flow directs lymphangiogenesis by guiding cell migration [12, 32]. During embryonic stages, increased interstitial fluid pressure is accompanied by the stretching of LECs, which leads to increased Vegfr3 signaling, β1 integrin activation, and LEC proliferation that results in lymphangiogenesis [73]. Flow is also involved in valve

formation and collecting lymphatic vessel maturation [78, 92], and a recent study suggests a novel role of Vegfr3 as a fluid flow sensor regulating vascular remodeling both in blood and lymphatic vasculature [7].

7.2 Nrp1 and 2

Nrp 1 and 2 are single-pass transmembrane glycoproteins. They consist of a large extracellular domain that includes two CUB homology domains, two coagulation factor V/VIII homology domains and a MAM domain, and a short cytoplasmic tail with no catalytic function, which however presents a PDZ binding site [8, 28]. Nrp1 and Nrp2 are about 100 kDa in size, and their amino acid sequences are 44 % identical. Originally identified as receptors for secreted class III semaphorins (Sema 3) that mediate repulsive signals during axonal growth [75], Nrps are also expressed in endothelial cells and have emerged as major regulators of vascular morphogenesis. Nrp1 is mainly expressed in arterial endothelium, while Nrp2 is expressed preferentially in veins and lymphatic vessels [37, 38, 60, 105]. In keeping with their distinct expression patterns, Nrps play largely non-overlapping roles in the vasculature, with Nrp1 regulating arteriogenesis and angiogenesis and Nrp2 regulating lymphangiogenesis. However, important exceptions exist: Nrp1 is required in lymphatic vasculature for valve formation, while Nrp2 has as yet incompletely understood functions in blood endothelial cells.

Ligands of Nrp1 and 2 include Sema3 and Vegf family members, which are proteins with distinct structure and function that bind to different sites of Nrp1. Disruption of Nrp-Sema3 or Nrp-Vegf interactions results in defective neuronal and vascular development, respectively. Thus, Nrps appear to play selective roles in the neural and vascular systems through specific interaction with different ligands [33, 62]. Again, important exceptions to this rule exist: Vegf binding to Nrp regulates guidance events in the nervous system [22, 80, 82], while Sema3 binding to Nrps regulates specific aspects of vascular development as described in the chapters below (Fig. 7.2).

7.3 Nrp2 in Lymphatic Development

Analysis of Nrp2 expression in the vasculature by immunostaining and in situ hybridization revealed labeling of veins and lymphatic vessels [37]. At E10, mouse embryos displayed Nrp2 expression in the cardinal vein but not in the dorsal aorta. After E13, the expression of Nrp2 in the cardinal vein decreased, while expression levels increased in the lymphatics that are formed close to the cardinal vein. The lymphatic specific expression of Nrp2 remains high throughout development and in the adult and co-localizes with lymphatic EC markers, such as Vegfr3 and podoplanin.

As suggested by this expression pattern, genetic ablation of Nrp2 during development results in abnormal formation of lymphatic vessels, demonstrating an

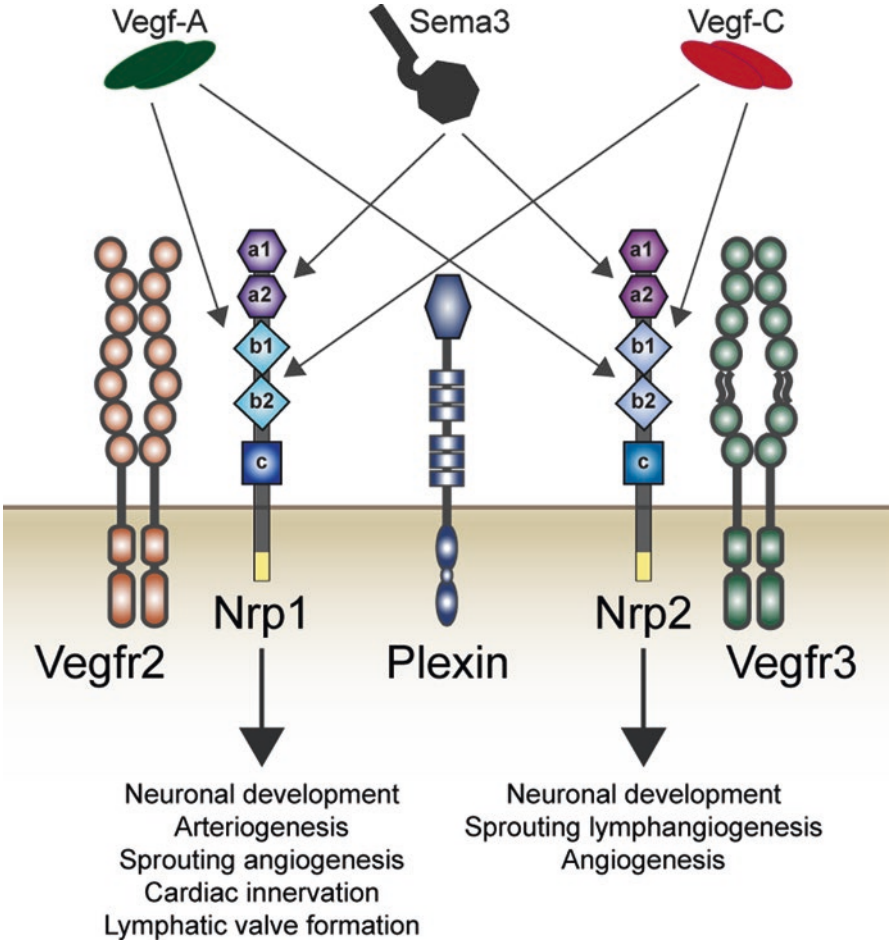

Fig. 7.2 A schematic summary of Nrp signaling in endothelial cells. Nrp1 and Nrp2 consist of two CUB domain repeats (a1/a2), two FV/VIII domain repeats (b1/b2), a MAM domain (c), a transmembrane, and a relatively short cytoplasmic region (40–43 amino acids). Semaphorins bind to the a1 and a2 domains, while interactions with Vegfs rely mostly on the b1 domain.

important role of Nrp2 in lymphangiogenesis [101, 105]. Several groups independently generated homozygous Nrp2 deletions and reported increased rates of neonatal mortality [17, 30, 96, 105]. During embryonic stages (~E18), homozygous mutants are obtained at approximate Mendelian frequency, but surviving Nrp2−/− mice ratio is lower than the expected frequency. Some juvenile/adult Nrp2−/− mice survive, but these are smaller than their littermates and reported as very poor breeders or infertile [17, 30, 96, 105].

Nrp2−/− embryos exhibit defects in guidance and patterning of neural crest cells and abnormal projections of sympathetic and sensory axons [17, 30, 81, 96]. In the vasculature, PECAM-1-positive blood vessels and capillaries are intact in

Nrp2−/− embryos at E13 and E15. However, sprouting of lymphatic capillaries is strongly reduced, leading to enlargement of jugular lymph sacs and fewer, enlarged, and abnormally patterned peripheral lymphatic vessels in the skin, heart, diaphragm, lung, and intestine of Nrp2−/− mice. BrdU incorporation studies revealed a reduction in the number of proliferating LECs in the skin, suggesting that Nrp2 is required for LEC proliferation. Interestingly the morphological defects of lymphatic vessels in the Nrp2−/− mice are not accompanied by functional defects such as edema. Thus, Nrp2 regulates sprouting lymphangiogenesis.

Pharmacological treatment with antibodies blocking VEGF-C binding to Nrp2 (anti-Nrp2^{B}) [15] confirmed VEGF-C dependence of Nrp2 function in lymphatic vessels [101]. In vivo modulation of Nrp2 signaling using anti-Nrp2^{B} results in selective disruption of lymphatic sprouting in various tissues. For example, anti-Nrp2^{B} treatment significantly affects the development of tail dermal lymphatic network, resulting in a less complex and immature hexagonal ring pattern. In the intestine, where lymphatic sprouting occurs during early postnatal stages (between P0 and P2), anti-Nrp2^{B} treatment results in decreased numbers of sprouting lacteal, but no difference in the lacteal length. Treatment of anti-Nrp2^{B} after postnatal day 3 does not affect the established lymphatic structure, further supporting the role of Nrp2 in lymphatic endothelial cell sprouting, rather than lymphatic vessel extension or maintenance. Expectedly, the expression level of Nrp2 appears to be higher in tip cells of new sprouts, and inhibition of Nrp2 greatly reduces sprout formation and alters lymphatic tip cell behavior in vitro and in vivo. In vitro bead sprouting demonstrated that treatment with anti-Nrp2B reduced the number of lymphatic sprouts induced by VEGF-C treatment. Interestingly, live imaging of in vitro sprouting revealed that anti-Nrp2B does not affect sprout initiation, but it induces sprout stalling and retraction which result in reduced number of sprouts. This observation may explain the reduction in the number of sprouts in vitro and reduced lymphatic sprouting and altered tip cell morphology in vivo [101].

Although Nrp2 has been known to act as a co-receptor for Vegfr3 in LECs, the molecular interaction between Nrp2 and other angiogenic molecules is largely unknown. Genetic interactions between Nrp2 and Vegfr3, but not between Nrp2 and Vegfr2, have been identified in double heterozygous mice [101]. Neither Nrp2± nor Vegfr3± mice show defects in lymphatic vessel morphology, but double heterozygous animals show impaired branching, enlarged lymphatic vessels and sprouting defects, which are similar to the defects observed in Nrp2−/− and anti-Nrp2^{B}-treated animals. Furthermore, the lymphatic defect in Nrp2−/−; Vegfr3± mice is severely aggravated compared to Nrp2−/− mice, indicating the genetic interaction between Nrp2 and Vegfr3. On the other hand, Nrp2 and Vegfr2 double heterozygous mice do not show lymphatic defects. Thus, while Nrp2 is able to augment VEGF-C-induced activation of both VEGFR2 and VEGFR3 in vitro [15], Vegfr2 appears to have a limited role in developing lymphatics in vivo [106].

Compared to Vegfr3 or Prox-1, which are master regulators of lymphatic development, the role of Nrp2 appears to be restricted to sprout formation. As such, Nrp2 modulates lymphatic endothelial tip cell extension and prevents tip cell stalling and

retraction, but does not play a significant role in lymphatic endothelial cell specification at early stages of lymphatic development. This suggests that Nrp2 might act downstream of initial LEC specification governed by Vegfr3 or Prox-1. Chicken ovalbumin upstream promoter transcription factor II (COUP-TFII) is another critical molecule for lymphatic development and lymphatic endothelial cell specification [55]. Knockdown of COUP-TFII results in decreased expression of Nrp2 and impaired lymphatic sprouting. This suggests that COUP-TFII is acting upstream of Nrp2 in order to control lymphangiogenesis. Recently, BRG1 has been reported as an upstream regulator of COUP-TFII in venous EC specification, and it remains to be determined if BRG1 also regulates COUP-TFII in the context of lymphatic development [18].

In addition to positively modulating lymphatic sprouting in response to Vegf-C, Nrp2 also responds to Sema3F and Sema3G, which negatively modulate LEC growth and sprouting during dermal lymphatic network formation [95]. Sema3F and Sema3G show differential expression in epidermis and arteries, respectively, and genetic deletion of Sema3F and Sema3G induces lymphatic hyperplasia in vivo. In vitro, treatment of Sema3F and Sema3G inhibits Vegf-C-induced LEC sprouting in an Nrp2-dependent manner. Hence, Nrp2 may have dual function in lymphatic vasculature by interacting with different ligands, Sema3s and Vegf-C [95]. PlexinA family members, another receptor complex for Sema3s, are also expressed in lymphatic vasculature, and mutant mice deficient in PlexinA1 and A2 display similar phenotype to Sema3F and G mutants, indicating that repulsive Sema3 signaling via Nrp2 involves PlexinA co-receptors that serve to fine-tune dermal lymphatic patterning.

7.4 Nrp2 in the Blood Vasculature

Global Nrp2 mutants are viable and display normal cardiovascular development, and the gross morphology of their veins and other blood vessels appears indistinguishable from wild-type embryos [105]. Adult Nrp2−/− mice have normal retinal vessels, yet Nrp2 expression is upregulated in blood vessels during pathological retina neovascularization. Global Nrp2 knockout mice developed less neovascularization compared to the controls in a model of oxygen-induced retinopathy, as well as in a model of transgenic retinal Vegf overexpression driven by the rhodopsin promoter. This data suggest that Nrp2 plays a role in blood vessel growth in hypoxia- and Vegf-driven angiogenesis, which cannot be substituted by Nrp1 signaling [83]. These effects could be partially attributed to the ability of Nrp2 to enhance Vegfr2 activity in response to specific isoforms of Vegf (Vegf $_{165}$ and Vegf $_{121}$) and HGF [84, 90]. Furthermore, compound Nrp1 and Nrp2 mutant mice show more severe vascularization defects and exhibit earlier embryonic lethality (E 8.5) compared to Nrp1 mutants [94]. This suggests a possible functional redundancy between Nrp1 and Nrp2 and signifies some distinct roles for Nrp2 in blood vessel morphogenesis. However, the mechanisms through which Nrp2 drives angiogenesis are poorly understood.

7.5 Sema3A-Nrp1 in Lymphangiogenesis

The Nrp1 ligand Sema3A was initially thought to compete with Vegf for binding to the same site, thereby antagonizing Vegf signals [86]. Crystal structure has shown that both ligands bind to distinct sites [2], and functional assays revealed that Sema3A does not inhibit VEGF-induced phosphorylation of VEGFR2 although it inhibits VEGF-induced activation of ERK1/2 [34]. Mouse genetic experiments have shown that neither Sema3A mutants nor mice lacking the Sema3A binding sites in Nrp1 display obvious angiogenesis defects [24, 33]. Cardiovascular defects appear restricted to the heart, where Sema3A signaling regulates innervation, and Sema3A mutants develop lethal cardiac arrhythmia [39]. Sema3 is also known to inhibit Vegf-induced angiogenesis and induce vascular permeability with or without Vegf under normal condition as well as pathologic condition such as diabetic retinopathy [1, 16]. In addition, Sema3A signaling is involved in lymphatic crosstalk with the immune system and in lymphatic valve formation [8].

Sema3A is expressed in adult lymphatic vessels and Sema3A signals regulate the entry of dendritic cells (DCs) into lymphatics via interaction with PlexinA1-Nrp1 receptor complexes expressed in DCs [93]. Sema3A expression on lymphatic vessels suggested a potential role for Sema3A in lymphatic vessel development, and indeed, analysis of lymphatic vessels of Sema3A−/− neonatal mice revealed that Sema3A selectively regulates lymphatic valve formation but not sprouting or assembly of lymphatic vessels. Lymphatic valve formation is initiated at late embryonic stages by specification of valve-forming cells that express high level of Prox1 and Foxc2 [72, 78]. Upregulation of the transcription factors Prox1 and Foxc2 initiates and regulates LEC migration and orientation in order to develop mature valve leaflets. Connexins [43], ephrinB2 [58], GATA2 [47], integrin α9 [9], angiopoietin-2 [19], and calcineurin/NFATc1 [51] are known to be involved in lymphatic valve formation. Interestingly a recent study verified fluid shear force as another important factor formation of lymphatic valves [92].

We and others found that Sema3A strongly binds to valve-forming areas of mesenteric lymphatic vessels expressing Nrp1 and PlexinA1 [13, 42]. In the mesenteric lymphatic vessels of homozygous Sema3A mutant mice (Sema3A−/−), Foxc2 and Integrin α9 expressing valves are still formed but are abnormally smaller, indicating that the absence of Sema3A causes this morphogenesis defect. Sema3A−/− mice also exhibited abnormal smooth muscle cell coverage in the valve regions. Further evidence for the role of Sema3a-Nrp1 signaling in lymphatic valve formation in vivo was provided by analysis of mice carrying a mutated form of Nrp1, which lacks the Sema3A binding site ($Nrp1^{sema3a-/-}$), and by using a blocking antibody that inhibits Sema3a binding to Nrp1 [69]. In both cases, inhibition of Nrp1-Sema3A interactions resulted in smaller valves and abnormal smooth muscle cell coverage as seen in Sema3a−/− mice. Absence of Sema3a-Nrp1 signaling does not affect other aspects of lymphatic morphogenesis, such as lymphatic sprouting or collecting lymphatic vessel development. Interestingly, Nrp2 is not expressed in lymphatic valves, and Nrp2 mutants show normal lymphatic valve formation, suggesting a selective role for Nrp1 in lymphatic valve development.

7.6 Nrp1 in Arteriogenesis and Angiogenesis

Nrp1 has been originally identified as a co-receptor for Vegfr2, enhancing its activity in the presence of $VEGF_{165}$ [87, 98]. Nrp1-deficient mice as well as endothelial-specific Nrp1 mutants die at mid to late gestation due to severe cardiovascular defects including impaired heart development, vessel enlargement, and defective vessel branching and sprouting [29, 41, 46]. These defects are due to important roles of Nrp1 in arteriogenesis as well as angiogenesis [85]. Interestingly, Nrp1 uses different signaling mechanisms to mediate both processes.

Notably, available evidence indicates that defective arteriogenesis in Nrp1 mutants is due to abnormal Vegf signaling. Nrp1 signaling is critical for arterial differentiation [61] and mice expressing a modified Nrp1 receptor unable to bind Vegf or lacking the Nrp1 cytoplasmic domain both display impaired arterial morphogenesis [23, 25, 53]. Biochemical and genetic experiments have shown that Nrp1 and Vegfr2 form complexes in response to Vegf and that complex formation depends on binding of the adaptor protein synectin to the PDZ domain of Nrp1. The PDZ-dependent interaction between Nrp1 and Vegfr2 promotes trafficking of endocytosed Vegfr2 from Rab5+ to EAA1+ endosomes and prevents PTPN1 (PTP1b)-mediated dephosphorylation of Vegfr2 at Y(1175), the site involved in activating ERK signaling. Genetic deletion of the Nrp1 cytoplasmic domain (Nrp1cyto), or of synectin, both lead to highly similar arteriogenesis defects that can be rescued by restoring ERK activation via endothelial Ptpn1 deletion [52–54]. These data support a model whereby Nrp1 signals regulate arteriogenesis by promoting Vegfr2-dependent ERK activation.

Interestingly, despite prominent arteriogenesis defects, mice lacking the Nrp1cytoplasmic domain or synectin-deficient mice do not show any angiogenesis defects, suggesting that Nrp1 triggers angiogenesis through a different mechanism. Mice lacking the Vegf binding domain in Nrp1 do not exhibit the severe cardiovascular defects observed in the endothelial specific and the full Nrp1 knockouts [25], suggesting that Nrp1 mediates important pro-angiogenic functions through VEGF-independent mechanisms. One possible mechanism involves the activation of the non-receptor tyrosine kinase ABL1 by Nrp1 in response to fibronectin/integrin signals; this pathway has been shown to control EC motility in vitro and in vivo [74]. In addition, Nrp1 modulates the Tgfβ/BMP9 signaling axis and regulates angiogenic responses independently of Vegf/Vegfr2. Inducible endothelial-specific genetic deletion of Nrp1 in a subset of retinal cells revealed that Nrp1 deficient cells are severely deficient in their ability to attain the capillary tip position [3]. Nrp1 in tip cells was found to restrict Smad2/3 activation downstream of Alk1/Alk5. Accordingly, combined deletion of Alk1 or Alk5 rescued tip cell formation in Nrp1-deficient cells. Tip cells also upregulate expression of the Notch ligand Dll4, which activates Notch signaling in neighboring endothelial cells. Notch activity suppresses Nrp1 expression, thus allowing activation of the angiosuppressive Smad2/Smad3 signaling and adoption of stalk cell behavior [3]. These data reveal a novel, Vegf-independent Nrp1 mechanism of action that accounts for its role in tip cell formation and sprouting angiogenesis. Exploiting the distinct Nrp1 signaling modes

regulating angiogenesis and arteriogenesis might be used clinically to selectively enhance arteriogenesis in patients with coronary or peripheral artery disease.

It is not known if a similar mechanism of action of Nrp takes place also in LECs. The expression of Nrp1 on LECs in hardly detectable in vivo; yet knockdown of Nrp1 in human dermal lymphatic endothelial cells significantly reduced VEGF-C induced signaling in vitro [21]. The role of Notch signaling in LECs also remains controversial. In vitro, LECs respond to VEGF/VEGF-C stimulation in a similar manner as blood endothelial cells; LECs expressing higher amounts of Dll4 adopt the tip cell position, and Notch activation restricts lymphatic cell sprouting downstream of VEGF/VEGF-C activation [107]. In mice, conditional deletion of Notch1 in LECs resulted in LEC overproliferation and lymphatic vessel overgrowth, as well as enhanced sprouting and filopodia formation [26]. On the other hand, Notch inhibition by blocking antibodies restricted lymphatic vessel growth in postnatal mice [65] and Dll4/Notch activation has been shown to maintain filopodia formation in intestinal lacteals [10]. LECs express components of the Tgfβ pathway, such as Acvr2b, Bmpr2, Alk1, and endoglin, and respond to BMP9/10 stimulation by upregulating the expression of Alk1 target genes, including Smad6 and Id1 [64]. With respect to the effects of Tgfβ/BMP9 signaling on LECs, most of the literature supports a suppressive role. In vitro, TGFβ signaling inhibits lymphatic cell proliferation and migration [68]. In vivo, Tgfβ activation restricts LEC sprouting and lymphatic vascular plexus formation in the mouse skin during development, and mice lacking either Tgfβr1 or Tgfβr2 show edema and a hyperplastic skin lymphatic network in the skin [40]. Finally, postnatal inhibition of Alk1 signaling using an inducible global Cre (RosaCreERT2) results in enlarged and dilated lymphatic vessels [104]. Whether Nrp1/2 could suppress Tgfβ/BMP9 signaling in order to elicit stalk behavior in LECs during sprouting lymphangiogenesis independently of Vegf-C/Vegfr3 remains to be discovered.

7.7 Conclusions and Perspectives

Research described above provides firm genetic evidence for Nrp signaling in vascular development and some understanding of the cellular context as well as ligand and co-receptor requirement. Further use of cell-type-specific and inducible receptor and ligand deletions will provide a more complete picture and allow defining roles of Nrps and ligands in pathological vascular development including tumor angiogenesis [11, 34]. In addition, in vitro studies will clarify signaling events downstream of Nrp1 and 2 signaling in endothelial cells.

In addition to Vegf and Sema3 family members, Nrps affect other growth factor signaling systems as well, including Tgfβ, Pdgf, and EGF signaling [31, 71, 76]. Elucidation of mechanisms regulating this crosstalk and its biological significance remain important challenges in the field. Additional exciting questions are potential roles of Nrps in fluid flow-dependent lymphangiogenesis, as suggested by Nrp1 function in lymphatic valve formation and Nrp2 function as a co-receptor for Vegfr3. Finally, Nrps may be important in the lymphatic vessels of tissues previously

thought to be devoid of lymphatics, including the eye and the central nervous system [4, 5, 48, 56, 70].

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Functions of Neuropilins in Wiring the Nervous System and Their Role in Neurological Disorders

8

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Contents

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Abstract

The proper wiring of the nervous system depends on an orderly series of events, beginning in embryonic development with neuronal migration, axon and dendrite development, and guidance events and continuing in postnatal development with synaptogenesis, pruning of axonal projections, and synapse refinement. In the nervous system, neuropilins function mainly with the class 3 secreted semaphorins (Sema3s) to mediate a majority of these developmental processes. Neuropilins bind to Sema3s as obligatory cell surface co-receptors and form a complex with the type A plexin family members, as well as with cell adhesion molecules and other modulatory co-receptors, to activate intracellular signaling networks that, in most cases, influence cytoskeletal dynamics and neuronal morphology. Changes to neuronal morphology are known to regulate neural connectivity and activity. In this chapter we will focus on recent discoveries of neuropilin functions, mediated by Sema3 signaling, to regulate wiring of the nervous system. In addition, we will highlight some of the emerging roles neuropilins play in neurodevelopmental and neuropsychiatric disorders.

8.1 Introduction

In the nervous system, neuropilins were originally identified by Takagi et al. [100] using monoclonal antibodies, which he called neuronal recognition molecules, with homologies to complement components and coagulation factors. Later work demonstrated that neuropilins are expressed prominently on olfactory axon subclasses in the chick developing nervous system, and that they can function as cell adhesion molecules with lamina-specific expression patterns in the chick optic tectum [37, 89, 101, 118]. Since the late 1990s, when two independent groups confirmed neuropilins to be receptors for class 3 secreted semaphorins, their role as axon guidance receptors in the nervous system has been the predominant focus [38, 47, 60]. More recent studies have elucidated the diverse roles of neuropilins in the nervous system beyond axon guidance, from cell migration and differentiation, to dendritic morphogenesis and synaptic pruning, to cell death and neurodegeneration, of which this is not an exhaustive list. In this chapter we will give a brief overview of the numerous roles for neuropilins in distinct neural development processes and the current understanding of how neuropilins function in the nervous system. We will also discuss in more detail some of these roles in the context of semaphorin-neuropilin/plexin signaling and their relevance to neurodevelopmental and neuropsychiatric disorders.

8.2 Role of Neuropilins in Neural Development

8.2.1 Neuropilins as Co-receptors for Class 3 Secreted Semaphorin Signaling in Axon Guidance

In vertebrates, two neuropilins (Nrps) have been identified, Nrp1 and Nrp2, which are type I transmembrane proteins that harbor a short cytoplasmic tail [36]. While most members of the semaphorin family bind directly to a plexin (Plxn) receptor

[113], members of the class 3 secreted semaphorins (except for Sema3E) require binding to a neuropilin receptor [18, 47, 60, 102]. Evidence for Nrp1/PlxnA1 as the physiological receptor complex for Sema3A-induced axon guidance events first came from Sema3A-dependent cellular morphology changes in Nrp1/PlxnA1 expressing, but not in Nrp1 only expressing, COS-7 cells [103]. Later, sensory neuron explant cultures demonstrated that the cytoplasmic tail of type A plexins is required for Sema3-Nrp-mediated repulsion of axons [86], suggesting that plexins are the signal-transducing receptors. In addition, it was demonstrated that the Nrp1/PlxnA1 complex has a higher affinity for binding Sema3A than does Nrp1 alone [103]. Subsequently, studies in mutant mouse lines of Sema3s, Nrps, and PlxnAs confirmed that Nrps/PlxnAs are functional complexes in vivo [41, 47, 57, 60, 107].

Interestingly, it has been demonstrated that different Nrp/PlxnA receptor complexes are used by similar neuronal cell types for Sema3-mediated axon guidance events. In embryonic chicken dorsal root ganglion (DRG) neurons, Sema3A-mediated Nrp1/PlxnA1 receptor signaling leads to the recruitment of the FERM domain-containing guanine nucleotide exchange factor (GEF) Farp2 to PlxnA1, which is required for Sema3A-mediated axon repulsion and growth cone collapse in vitro [109]. In mouse DRG neurons, Sema3A-dependent growth cone collapse is mediated through Nrp1/PlxnA4, which can also recruit Farp2 for downstream signaling [72]. That different Nrp1/PlxnA complexes are employed by different species to activate a similar downstream signaling cascade for axon guidance events suggests a mechanism for how these receptors evolved.

8.2.2 Neuropilins Are Involved in Sema3-Mediated Repulsion and Attraction Events

Nrps forming specific complexes with other cell surface receptors, such as cell adhesion molecules (CAMs) has given different neurons a strategy to diversify their Sema3 signaling, which in turn leads to distinct neuronal functions. For example, Nrps interacting with different CAMs has allowed them to switch from Sema3-mediated axonal repulsion to attraction. Here, we will only highlight the functions of Nrps and their interaction with CAMs in regard to nervous system development, while a more detailed review of Nrps' interaction with CAMs and its biological significance will be discussed in another chapter in this book.

Neuropilin 1 can associate with L1 CAM to form a receptor complex [14], in addition to type A plexins (PlxnAs), in dissociated cortical neurons from neonatal mice. This recruitment of L1 CAM to Nrp1 following Sema3A stimulation induced focal adhesion kinase and mitogen-activated protein kinase (FAK and MAPK, respectively) activation, leading to the removal of paxillin-positive adhesion points in axonal growth cones and to their collapse [6]. Interestingly, cross talk between Sema3A-Nrp1/L1 and L1-L1 signaling was shown to switch the repulsive effects of Sema3A on DRG axons to attraction [14]. In contrast, Nrp2, forming a complex with NrCAM to activate the FAK/Src signaling cascade, distinguishes the bifunctional, attractive versus repulsive, axon guidance activity of Sema3B in sorting axons of the anterior commissure [31]. Interestingly, axons of mesodiencephalic dopamine neurons (mdDA), originating from the substantia nigra and ventral tegmental area, require Sema3F-Nrp2 signaling for axonal repulsion and fasciculation

as they project along the medial forebrain bundle during early embryonic development (Fig. 8.1). However, as they arrive at the border of the developing white matter and cortical plate of the presumptive medial prefrontal cortex, Sema3F-Nrp2-mediated attraction is necessary to orient these mdDA axons dorsally toward the pial surface for proper target innervation (Fig. 8.1) [58]. Whether this switch from repulsion to attraction is from an intrinsic mechanism of Nrp2 signaling or mediated by cross talk/association with a CAM is unclear.

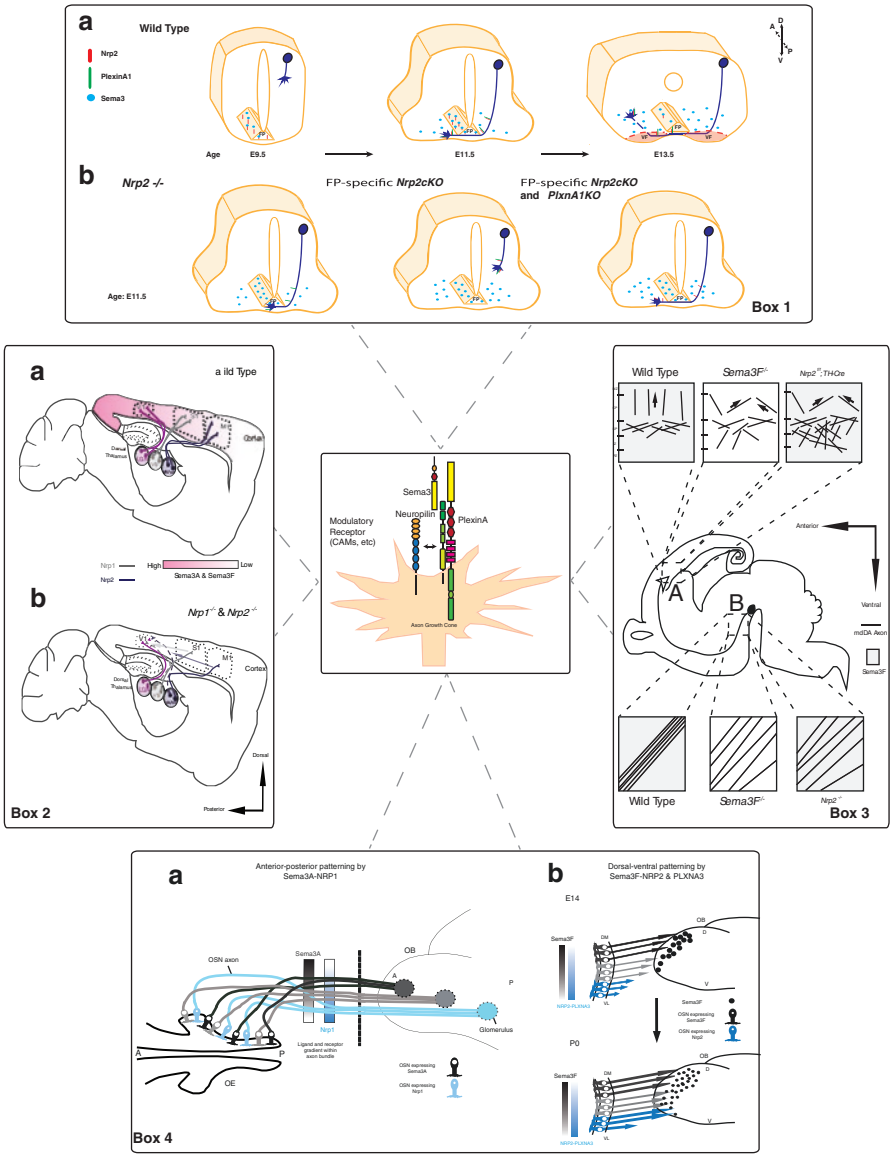

Fig. 8.1 Neuropilin-dependent axonal guidance events in the developing mammalian nervous system. *Middle box*: Schematic illustration of a growth cone expressing neuropilins (Nrps) forming receptor complexes with other receptors such as plexinAs (PlxnAs) and modulatory receptors, including cell adhesion molecules (CAMs). The interactions between the Sema3 ligands and distinct receptor complexes provide the ability for Sema3-Nrps signaling to diversify in mediating a diverse range of axonal guidance events. The individual boxes illustrate a few examples of the different early developmental processes controlled by Sem3A-Nrps in the nervous system. *Box 1*: Axon guidance of commissural neurons in the developing spinal cord: (**a**) schematic diagrams of WT E9.5–E13.5 spinal cords. At E9.5 (*left*), specific expression of Nrp2 in floor plate (*FP*) along with Sema3B (*red sticks* and *light blue dots*) is observed, along with Nrp2-/PlxnA1-positive dorsal commissural neuron (CN) axons beginning to project ventrally toward the midline (*dark blue*). At E11.5 (*middle*), Nrp2 is highly expressed in the floor plate, sequestering Sema3B, which attenuates the repulsion effects experienced by dorsal Nrp2-/PlxnA1-positive commissural axons, thus allowing them to approach and enter the midline. By age E13.5 (*right*), FP-derived Nrp2 is greatly downregulated, enabling Sema3B repulsion of Nrp2-/PlxnA1-positive midline crossing and contralateral axons to leave and project away from the FP to be guided along the longitudinal axis by other guidance cues toward rostral brain targets. (**b**) A schematic illustrating the effects of the global loss of Nrp2 (*Nrp2*$^{-/-}$, *left*) at E11.5 when the majority of commissural axons are crossing the midline. Specific loss of Nrp2 in the FP (FP-specific *Nrp2* cKO, *middle*) results in premature repulsion of ipsilateral (pre-crossing) Nrp2-/PlxnA1-positive axons in the ventral spinal cord. Inhibition of PlxnA1 signaling in FP-specific *Nrp2* cKO (*right*) enables Nrp2-/PlxnA1-positive axons to regain ability to cross the midline. *Box 2*: Sema3-Nrps-dependent guidance of thalamocortical axon projections. (**a**) Schematic showing Sema3A and Sema3F, high-posterior and low-anterior, gradient expression in the developing cortex. In *dark blue*, axons from the motor thalamic nuclei (*VA/VL*), expressing Nrp2, project to targets in the motor cortex (*M1*) and are repelled by Sema3F. In light gray, axons from the ventral basal thalamic nuclei (*VB*) expressing Nrp1 project to targets in the somatosensory cortex (*S1*) and are repelled by Sema3A. (**b**) In *Nrp2*$^{-/-}$ and *Nrp1*$^{-/-}$ animals, axons from VA/VL and VB, respectively, are mistargeted posteriorly to the visual cortex (*V1*, *dashed line*). *LGN* lateral cingulate nuclei. *Box 3*: Sema3F-Nrp2 signaling mediates bifunctional axon guidance events of mesodiencephalic dopaminergic (mdDA) neurons rostral projection to their medial prefrontal cortex (mPFC) targets. (**a**) Schematic showing the mdDA axon projections to the developing cortical plate in the mPFC of WT, *Sema3F*$^{-/-}$, and *Npn2f/f;TH-Cre* mice. In WT mice, terminal axon projections are attracted dorsally toward the pial surface. In *Sema3F*$^{-/-}$ mice, the density of mdDA axon innervation in the superficial layers of the mPFC is reduced, and axons ectopically innervate deeper cortical areas, displaying random orientation. In *Npn2f/f;TH-Cre* mice, similar to *Sema3F*$^{-/-}$, mdDA axons ectopically innervate deeper cortical areas, and axons within the cortical plate display a random orientation. (**b**) Schematic showing the proximal projections of developing mdDA axons in the mesodiencephalon of WT, *Sema3F*$^{-/-}$, and *Npn2*$^{-/-}$ mice. In WT mice, mdDA axons are tightly fasciculated into bundles due to Sema3F-Nrp2 surround repulsion. In both *Sema3F*$^{-/-}$ and *Npn2*$^{-/-}$ mice, mdDA axons are defasciculated. *Box 4*: Olfactory sensory neuron (*OSN*) axons employ distinct Sema3s-Nrps axonal guidance mechanisms to innervate specific olfactory bulb (OB) targets. (**a**) Subsets of OSNs in the olfactory epithelium (*OE*) project their axons to OB targets along the anterior-posterior axis by expressing complementary gradients of Sema3A and Nrp1, which enable the organization of axonal positions within the bundle prior to arriving at their targets. (**b**) OSN axons originating from the dorsal-medial (*DM*) and ventral-lateral (*VL*) OE project to the OB in a sequential manner. Early-arriving DM axons arriving first to the dorsal region of the OB express and deposit Sema3F at the target, thus establishing a dorsal-high and ventral-low gradient to repel later-arriving VL axons, which express Nrp2/PlxnA3, from dorsal regions, and restrain them to the ventral OB (*Box 4* schematic illustrations adopted from Pasterkamp [81])

While Sema3E also displays bifunctional activities in the developing mammalian brain, it employs a different receptor binding mechanism in guiding descending axonal projections in the CNS. In this case, the PlxnD1 receptor expressed by descending cortical and striatal axons interacts with Sema3E, as previously shown in the vascular system [45], to mediate repulsion of corticofugal and striatonigral axons [15]. However, the presence of Nrp1 and PlxnD1 on subiculo-mammillary axons projecting in the fimbria and fornix is thought to convert the repulsive effects of Sema3E-PlxnD1 to attractive or growth-promoting effects, as shown by the fact that fewer axons reach their targets in the hypothalamus in Sema3E knockout mice, and functional blocking antibodies specific for Nrp1 completely inhibited the axonal growth-promoting effects of Sema3E on subicular neurons. In all other examples, Nrp is required to be the obligatory binding partner for a Sema3; however, it does not appear that Sema3E binds Nrp1, even though Nrp1 and PlxnD1 *cis* interaction is necessary for the switch from repulsion to attraction. The mechanisms underlying these opposite responses are likely to be more stringent than simple co-expression of Nrp1 with PlxnD1 in the same subset of axons and most likely involve different downstream signaling cascades. It will be important for future studies to elucidate the underlying intracellular signaling mechanism of Sema3E-PlxnD1/Nrp1, as currently it is not known.

The theme for Nrp interactions with CAMs can also be observed in Sema3s-mediated guidance of thalamocortical axon projections during embryonic development. The close homolog of L1 (CHL1) was demonstrated to associate with Nrp1 through a sequence in the CHL1 Ig1 domain, which is required for Sema3A-induced growth cone collapse of thalamic neurons [28, 115]. In addition, Nrp1 and CHL1 are co-localized on thalamic axons, and both *Nrp1Sema-* and *CHL1*$^{-/-}$ mutant embryos exhibit defects in somatosensory thalamic axons. In contrast, the interaction between Nrp2 and NrCAM is necessary for Sema3F-dependent guidance of another subpopulation of thalamocortical axons, and genetic deletion of Nrp2 or NrCAM misdirected motor and somatosensory thalamic axons to the primary visual cortex (Fig. 8.1) [115]. NrCAM, L1, and CHL1 are expressed in different but overlapping patterns in developing thalamic axons. Thus, differential expression of CAMs in subpopulations of thalamic axons, coupled with the ability to interact with different Nrps, which mediate Sema3A or Sema3F responses, suggests that Nrp/CAM interactions may serve to increase the specificity of repellent guidance responses in distinct thalamocortical axons. Collectively, the interaction of Nrps with other signaling receptors to form specific Nrp/Plxn receptor complexes or Nrp with other transmembrane molecule complexes has given neurons a strategy to diversify their semaphorin signaling, leading to different cellular functions.

8.2.3 Novel Mechanisms of Neuropilins in Axon Guidance and Target Recognition

Canonical axon guidance mechanisms usually dictate that there is an expression of attractive and repulsive cues in the neuronal environment, which are detected by

receptors or receptor complexes (e.g., Nrp/Plxn) on growing axons/growth cones to either steer toward or away from the source of the cue by changes in the axon/growth cone cytoskeleton network [59, 81, 110]. For example, Nrp2 expressed by vomeronasal axons projecting to the accessory olfactory bulb is required for fasciculation of the nerve and proper zonal targeting of subsets of vomeronasal sensory axons in response to the repulsive ligand Sema3F expressed along the axon pathway and the target cells [20, 21]. In addition, Sema3A-Nrp1 and Sema3F-Nrp2 signaling was demonstrated to mediate spinal cord motor axon guidance at the plexus region and for the proper patterning of subsets of motor and sensory axons innervating the developing mouse limb [51, 52].

It is well established that many guidance cues are key players in topographic map formation in different developing neural systems [13, 29, 32, 52, 65, 88]. The traditional and well-studied mechanism of topographic map formation depends on opposing graded expression of axon guidance cues (usually located at the final or intermediate target) and their receptors on axons/growth cones [90, 98]. Nrp2 was demonstrated to express in an increasing nasal-temporal gradient in retinal ganglion cells (RGCs), whereas the ligand, Sema3F, was found in a graded low-rostral to high-caudal expression in the superior colliculus. Functional RGCs' growth cone collapse assays demonstrated that Sema3F induces growth cone collapse of temporal, but not nasal, RGCs expressing high levels of Nrp2 [19]. In the olfactory system, Sema3A was demonstrated to express in the ventral olfactory bulb (OB) and is required to guide Nrp1-positive odorant receptor neuron (OSN) axons to their glomeruli located lateral and medial in the OB [84, 91, 92, 108]. However, recent findings demonstrated that axonal sorting within the projection pathway before reaching the target is an important mechanism contributing to guidance cue-mediated topographic map formation in vivo. It was shown that levels of Nrp1 in OSN axon terminals, the glomeruli, correlated with the level of cAMP signals [53] and that Nrp1 levels determine the glomerular positioning in the anterior-posterior axis of the OB (Fig. 8.1) [54]. In addition, these researchers found that axons expressing high versus low levels of Nrp1 are segregated within the OSN axon bundle that projects to the dorsal zone of the OB, and either specific deletion or ectopic expression of Nrp1 in these axons shifts their positions within the bundle. Furthermore, Sem3A is expressed not only in the target but also in OSNs, in complementary levels to Nrp1 expression, such that Sema3A-positive OSN axons were found in the area within the bundle with low levels of Nrp1-positive axons. This finding suggests that axons with high levels of Nrp1 are repelled by Sema3A-positive axons, and Sema3A-Nrp1 signaling in OSNs regulate pre-target axon sorting.

In contrast to this pre-target axon sorting mechanism that establishes the topographic map of OSN projections in the anterior-posterior axis, OSN axons projecting along the dorsal-ventral axis are already pre-segregated in separate bundles in the olfactory epithelium. It was shown that Nrp2 and its repulsive ligand, Sema3F, are expressed in complementary gradients by OSN axons that target the dorsal and ventral areas of the OB (Fig. 8.1) [105]. Using an enhanced expression of EYFP-fused Sema3F mouse reporter line, Takeuchi and colleagues demonstrated that the source of the repulsive ligand was not coming from the target cells but rather being

deposited by early-arriving OSN axons projecting to the anterodorsal OB, complementary to the late-arriving Nrp2-positive axons that project to the ventral region of the OB [105]. Thus, the sequential arrival of OSN axons in combination with the graded secretion of Sema3F by early-arriving OSN axons, which is complementary to the late-arriving Nrp2-positive axons, is another variation in the mechanism by which Nrps function to specify the topographic map of the OB.

It was first demonstrated by Zou et al. [124] and later confirmed by others [17, 111] that Sema3s expressed by the ventral midline (VM) floor plate cells and/or within the spinal cord are involved in repelling subsets of Nrp2-positive dorsal commissural axons (CAs) to leave the floor plate and move onto their contralateral trajectory. In addition, in vitro dorsal spinal cord explant outgrowth assays and Nrp2-*null* knockout animals displayed no guidance defects in pre-crossing (ipsilateral) axons, suggesting that Nrp2-positive pre-crossing axons are not responsive to Sema3-mediated repulsion. However, contrasting new data from our group [48] and those previously published by Nawabi et al. [76] point to novel and more complicated mechanisms of how Sema3-Nrp2/PlxnA1 signaling regulates the specific ipsilateral and contralateral segments of commissural axon guidance. Nawabi and colleagues showed that there is a dramatic difference in the expression levels of the signal-transducing PlxnA1 receptor in CAs, very low in ipsilateral and high in contralateral segments of the axon, and calpain proteolytic processing of the PlxnA1 is suggested to be the mechanism underlying this difference in receptor expression patterns. On the contrary, results from our group demonstrated prominent expression of PlxnA1 on both ipsilateral and contralateral segments of Nrp2-positive dorsal CAs, and in vitro dorsal spinal cord explants (containing only ipsilateral axons) are, indeed, inhibited by Sema3B in a dosage-dependent manner [48]. Furthermore, using mouse genetics we demonstrated the repulsive effects of Sema3B on Nrp2-/PlxnA1-positive ipsilateral axons which is greatly attenuated by a novel source of Nrp2 expressed by the floor plate (Fig. 8.1). This suggests that floor plate-derived Nrp2 serves as a molecular sink to bind Sema3B, and this autocrine effect is reminiscent of the autocrine effects of Sema3A-Nrp1 in medial motor column (MMC) axons, which decreased the sensitivity of MMC axon to environmental Sema3A repulsion [74]. Just as silencing Sema3A expression in motor neurons unmasked sensitivity to Sema3A repulsion for MMC axon projection [74], specific deletion of floor plate-derived Nrp2 disrupted and reduced the number of Nrp2-positive CA projections into the ventral ipsilateral spinal cord [48]. Furthermore, this guidance phenotype can be rescued by inhibiting PlxnA1 endogenous receptor signaling in vivo, evidence to support the existence of functional Nrp2/PlxnA1 receptor complexes on ipsilateral CAs.

While there are inconsistencies between our study and Nawabi et al. [76], particularly the differences in the levels of PlxnA1 on ipsilateral segments of CAs, there might be indeed an upregulation of Nrp2/PlxnA1 signaling on contralateral axons, as we have observed an increased sensitivity to Sema3B inhibition on spinal cord explants growing contralateral axons. Perhaps, the more intriguing question, which neither of these studies addressed, is why CNs express guidance receptors on ipsilateral axons prematurely when extra effort is required by the system to suppress their detection of the inhibitory ligands. Future experiments to elucidate the

spatiotemporal expression and trafficking mechanisms of neuropilin and plexin receptors would be valuable to understand this observation.

8.2.4 Regulatory Mechanisms of Neuropilin Expression

As alluded to in the previous sections, the spatiotemporal regulation of Nrps and their Sema3 ligands is critical for the proper control of the magnitude and duration of Sema3-Nrps signaling in neural developmental processes ranging from cell migration, to axon guidance, to synaptic plasticity. At the transcriptional level, *Nrp1* expression is repressed by the neuron restrictive silencer factor NRSF/REST and its corepressor CoREST in both non-neuronal and neuronal cells in controlling cell migration and axon guidance, respectively [5, 62]. *Nrp2* is also transcriptionally repressed by members of the homeobox gene family, Dlx1/Dlx2 and Nkx2-1, in basal forebrain interneuron progenitors, thus allowing them to be insensitive to the Sema3 ligands as they migrate along their route to the cortex during embryonic development [63, 78]. However, the regulation of *Nrp1* in retinal axon growth cones is not as straightforward, as the repression of Nrp1 by CoREST must be alleviated by miR-124, downregulating CoREST. The dynamic control of *Nrp1* expression thus enables retinal growth cones to be sensitive to Sema3A in a spatiotemporal manner that allows the axons to find their appropriate targets [5]. Interestingly, both *Nrp1* and *Nrp2* genes are direct downstream targets of the transcription factor COUP-TFII, which positively regulates their expression for the proper migration of progenitors from the caudal ganglionic eminence [106]. In addition, the dynamic expression of COUP-TFII/*Nrp2* was demonstrated to be a molecular switch for the proper migration of preoptic area GABAergic neurons to the cortex and amygdala [56, 106].

At the posttranscriptional level, a recent study demonstrated that Nrp1 protein levels in zebrafish retinal ganglion cell (RGC) axons are negatively regulated by the RNA-binding protein Hermes and Hermes-depleted RGCs have increased Nrp1 expression in vivo, which resulted in RGC growth cones and axons with premature sensitivity to Sema3A [50]. Nrps also can be the direct targets of microRNAs (miRNAs) in both neuronal and non-neuronal cells [4, 23, 64]. Specifically, the 3′-UTR of Nrp2 contains possible binding sites for over 25 miRNAs, but only miR-188 is associated with synaptic plasticity, which has been shown to be upregulated following in vitro LTP induction in the rat hippocampus [64]. Conversely, the level of Nrp2 is decreased following LTP. Overexpression of Nrp2 decreased the dendritic spine density and reduced the frequency of the miniature EPSCs of rat primary hippocampal neurons, but overexpression of miR-188 is capable of rescuing both phenotypes. These in vitro results are consistent with previous in vivo findings of Nrp2, and its ligand Sema3F, being in the appropriate spatiotemporal location in the postnatal and adult hippocampus to act as a negative regulator of spine morphogenesis [112], and suggest that repression of Nrp2 expression by miR-188 may be a mechanism for hippocampal neurons to maintain basal excitatory synaptic transmission.

Other mechanisms controlling Nrp expression include G-protein-coupled cAMP-dependent signaling pathways in retinal and olfactory sensory neurons, which

positivity regulate Nrp1 levels to increase the sensitivity of sensory axons to Sema3 ligands and consequently facilitate pathfinding [26, 53]. Interestingly, spinal commissural axon growth cone sensitivity to Sema3 repulsion at the midline is also regulated by Shh binding to its receptor Ptch1, which leads to disinhibition of Smo signaling to regulate cAMP levels in commissural neurons [80]. Whether this mechanism has a direct effect on the levels of Nrp2 on commissural axons remains to be determined. Conversely, recent studies in both the spinal cord and brain have begun to investigate the subcellular trafficking of Nrp receptors. Specifically, the transient axonal glycoprotein 1 (TAG1 or CNTN2) was shown to regulate the distinct endocytic pathway of Nrp1 and its co-receptor L1 CAM following Sema3A treatment of mouse sensory neurons [24]. Furthermore, Sema3A treatment of layer 2/3 colossal axon growth cones in vitro can activate the Rab5 early endosome pathway to mediate the trafficking of Nrp1/PlxnA1 complex, where PlxnA1 can directly associate with the Rab5 effector Rabaptin-5 [116]. Moreover, in utero electroporation of Rab5 rescued the positioning of cortical axons within the corpus callosum in Nrp1-deficient mouse brains. Interestingly, in non-neuronal cells, Nrp1 can form a complex with another Rab GTPase family member, Rab7 in the late endosome targeted to the lysosome for degradation; this process was shown to be accentuated by the liver kinase B1 (LKB1) in cultured lung cells [79]. Therefore, Rab small GTPase-mediated subcellular trafficking of Nrp appears to be a common mechanism employed by both neuronal and non-neuronal cells in regulating the cell surface expression of these receptors.

8.2.5 Functions of Neuropilins in Dendritic Morphogenesis and Synapse Elimination

While Nrps are the obligate binding partners for Sema3s in the nervous system, they are also known to bind to other ligands from structurally distinct families, including the vascular endothelial growth factors (VEGF) [44], which are discussed in other chapters in this book. In particular, the distinct functions of Nrp1 were difficult to analyze in *Nrp1*-null animals due to its involvement in the cardiovascular system, which requires both Sema3-Nrp1 and VEGF-Nrp1 signaling, leading to early embryonic lethality [44]. Therefore, the generation of the *Nrp1* flox conditional knockout and a *Nrp1Sema-* knockin mouse line that expresses a mutated Sema3-binding site variant of Nrp1 was among the first studies to demonstrate Sema3-Nrp1 signaling in mediating axon pathfinding of several populations of neurons in the CNS and PNS and the requirement of Nrp1 for proper cortical layer 5 neuron basal dendrite development in vivo [44]. Since then, there is accumulating in vitro and in vivo evidence demonstrating Nrps function in dendritic morphogenesis and synapse elimination by mediating Sema3 signaling [61, 81, 122]. It appears that Sema3A-Nrp1 signaling can promote both the amount of dendrite arborization and spines in cortical neurons [33, 75, 77, 83, 93, 119]. Interestingly, Sema3A-Nrp1/PlxnA4 signaling mediates basal dendrite growth and branching in mouse cortical neurons in vitro and in vivo (Tran et al., 2009; Mlechkovich et al., 2014), Sema3A-Nrp1/PlxnA1 signaling promotes dendrite complexity in mature rat hippocampal neurons in vitro [16]. Sema3F-Nrp2 signaling was demonstrated to restrain spine morphogenesis and excitatory synaptic

transmission [27, 87, 112]. In mice deficient in Nrp2 or its Sema3F ligand and PlxnA3 holoreceptor partner, layer 5 cortical pyramidal neurons and hippocampal granule neurons exhibit supernumerary spines on their dendrites, and electrophysiological recordings displayed altered excitatory synaptic transmissions in both types of neurons (Fig. 8.2) [112]. The neural cell adhesion molecule NrCAM was shown to interact with the Nrp2/PlxnA3 complex through an Nrp2-binding sequence and contributes to Sema3F-Nrp2/PlxnA3 signaling in controlling excess spine elimination in cortical neurons [27].

Interestingly, Sema3F-Nrp2/PlxnA3 signaling is also responsible for the pruning of the mouse hippocampal infrapyramidal tract (IPT), which involves the elimination of both presynaptic (axonal terminals from the IPT) and postsynaptic (spines on CA3 pyramidal dendrites) components (Fig. 8.2) [2, 66, 67]. It was demonstrated that this synaptic elimination process requires Sema3F signaling with Nrp2/PlxnA3 and the recruitment of the RacGAP β2-chimaerin binding to the cytoplasmic domain of Nrp2, leading to inactivation of Rac1 activity [85]. While it is not known whether cell adhesion molecules are involved in this synaptic pruning process, it was demonstrated that β2-chimaerin signaling is not required for restraining spine morphogenesis or repulsive axon guidance. Collectively, these works demonstrate

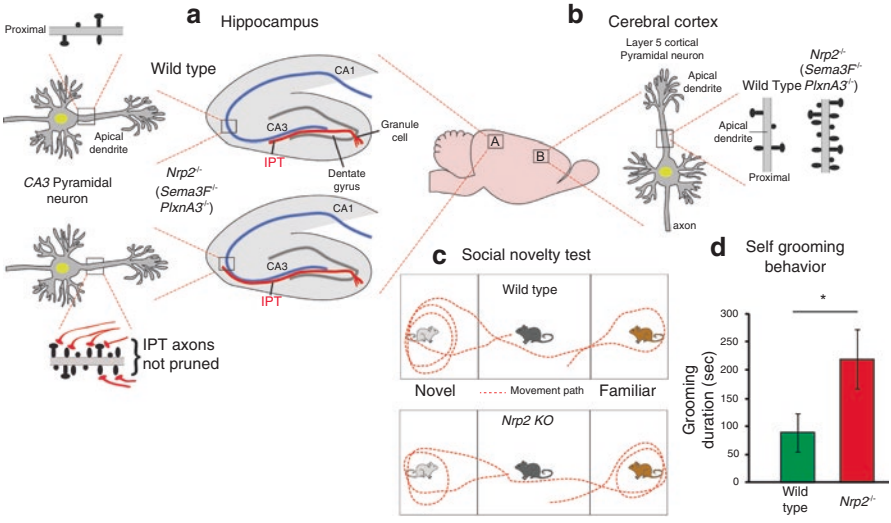

Fig. 8.2 Neuropilin 2 signaling contributes to dendritic morphogenesis, axon pruning, and behavior in the postnatal animal. (**a**) In the mouse hippocampus, *Nrp2*-null mice ($Nrp2^{-/-}$) show a hyperextended infrapyramidal tract (*IPT*, shown in *red*), with synaptic terminals contacting CA3 dendritic fields. Similar axon pruning defects are observed in $Sema3F^{-/-}$ and $PlxnA3^{-/-}$ knockout mice, suggesting that Sema3F-Nrp2/PlxnA3 signaling is required for the pruning of IPT axons. (**b**) Loss of neuropilin 2 increases spine density in proximal apical dendrites of pyramidal neurons in layer 5 of the cerebral cortex leading to increase excitatory synaptic transmission. (**c**) Adult $Nrp2^{-/-}$ animals display impairments in a test of social novelty. Movement analysis shows $Nrp2^{-/-}$ mice equally investigate novel and familiar conspecifics, as opposed to a preference for novelty in wild-type mice. (**d**) Adult $Nrp2^{-/-}$ mice engage in excessive self-grooming behavior compared to wild-type mice (* = significant at $P < 0.05$, error bars = ± 1 SEM; Adopted from Shiflett et al. [95])

the engagement of differential Sema3-Nrp/PlxnA intracellular networks to regulate the distinct functions of Nrps in neuronal wiring.

8.3 Role of Neuropilins in Neuropsychiatric and Neurodevelopmental Disorders

It is now recognized that a number of psychiatric diseases, including schizophrenia and autism spectrum disorder (ASD) arise, in part, from compromised nervous system development [8, 40]. The diverse role for Nrps in brain development has spurred a great deal of interest in understanding the relationship between Sema-Nrp signaling and psychiatric disorders. As will be described in the following sections, ample evidence links polymorphisms in the genes coding for Nrps and their plexin and semaphorin signaling partners, to increased risk for schizophrenia, autism, and other comorbidities (see Table 8.1). Additionally, animals harboring mutations to Nrp genes are beginning to identify brain and behavioral anomalies that are similar to features observed in humans with psychiatric and neurological disorders. Specifically highlighted in this chapter are examples of behaviors in animals that model psychiatric disease symptoms. Finally, changes in expression of neuropilin, semaphorin, and plexin genes have been observed in postmortem analysis of brain tissue in patients with these diseases.

8.3.1 Role of Neuropilins in Neurological and Behavioral Processes in Animal Models

Nrp2 knockout ($Nrp2^{-/-}$) mice are viable into adulthood and show generally intact neurological function, with one notable exception: adult $nrp2^{-/-}$ mice are more prone to develop seizures. Gant et al. observed greater spontaneous seizure activity in $Nrp2^{-/-}$ mice derived from an FVB/NJ background compared to $Nrp2^{+/-}$ and wild-type control strains [39]. Chemoconvulsant-induced seizures were also more pronounced in these mice compared to control animals [39]. Loss of the Nrp2 ligand Sema3F similarly increases seizure-related activity: *Sema3F*-null mice displayed greater epileptiform activity as measured with electroencephalogram (EEG) and were more likely to show postures and movements associated with mild seizure activity during exploratory behavior [87]. Likewise, Yang et al observed downregulation in Sema3F expression in FVB/NJ mice, which are more susceptible to seizures [119]. A similar reduction in expression of Sema3C and Sema3A in the hippocampus was observed after chemoconvulsant-induced seizures in rats [3, 49].

Epilepsy is comorbid with ASD and other neurodevelopmental disorders [9], suggesting they share a common developmental origin. The mRNA encoding Sema3F is a target of the fragile X mental retardation protein (FMRP) and is found in a decreased amount in polysomes from fragile X syndrome patients' cells, suggesting that Sema3F is downregulated in these patients [25]. Mutant mice lacking *Fmr1*, the gene that encodes for FMRP, demonstrate similar defects in the infrapyramidal tract (IPT) to those observed in Sema3F, Nrp2, and PlxnA3 knockout animals, suggesting that regulation of Sema3F by FMRP is important for the correct wiring of the

Table 8.1 Neuropilins in neurological and psychiatric disorders

The receptor (neuropilins)				
	Study type			
Disorder	**Subjects**	**Methods**	**Findings**	**Reference**
Autism spectrum disorder (ASD)	Human	Genome-wide association study in ancestral Han Chinese population	Association of two SNPs of *NRP2* gene with ASD	Wu et al. [117]
	Mice	Cell counts	Decreased number of interneurons in *Nrp2*$^{-/-}$ mice: implications for autism	Gant et al. [39]
	Mice	Behavior	Learning impairments and repetitive behavior in *Nrp2*$^{-/-}$ mice match some of the core features of ASD	Shiflett et al. [95]
Major depressive disorder (MDD)	Human	mRNA expression	Increased mRNA expression in prefrontal cortex in MDD patients	Goswami et al. [43]
Epilepsy and epileptic seizure	Rats and mice	mRNA expression	Decreased mRNA expression of Sema3C, Sema3F, and Nrp2 in CA1 and CA3 fields of the hippocampus	Barnes et al.[3], Yang et al. [120]
	Rats	Protein expression	Upregulation of Nrp2 protein expression in the dentate gyrus and entorhinal cortex	Shimakawa et al. [95]
	Mice	Behavior	Increased seizures in *Nrp2*-deficient mice: implications for epilepsy	Gant et al. [39], Sahay et al. [87]
The ligand (semaphorins)				
	Study type			
Disorder	**Subjects**	**Description**	**Findings**	**Reference**
Anxiety and fear	Mice	Behavior	*Sema3F*$^{-/-}$mice show increased anxiety- and fear-related behaviors and enhanced fear memory.	Matsuda et al. [70]
Schizophrenia	Humans	Protein expression	Sema3A upregulation of protein expression in the cerebellum and prefrontal cortex	Eastwood et al. [30], Gilabert-Juan et al. [42]

hippocampus [55]. Interestingly, 10–20 % of individuals with the fragile X syndrome also develop epilepsy [7]; for review see Yaron and Zheng [120].

In tests of learning and memory, loss of *Nrp2* had deleterious effects on behavior. We found striking impairments in adult *Nrp2*$^{-/-}$ mice on tests of hippocampal-dependent learning and memory, such as the novel object recognition test [94]. This test consists of a learning phase in which mice are exposed to, and are allowed to investigate, two identical objects. After a delay, one of the objects is replaced with a new object with a different texture, color, and shape. In control animals, this "novel" object attracted greater investigatory behavior (sniffing, orienting, etc.) compared to the familiar object. Interestingly, *Nrp2*$^{-/-}$ mice showed no preference for the novel object and instead investigated both the novel and familiar object equally.

We observed a similar impairment in *Nrp2*$^{-/-}$ mice in tests of social novelty (Fig. 8.2). In this test, the test mouse is presented with the opportunity to investigate another mouse that is confined to one compartment or to investigate a novel object. Our control and *Nrp2*$^{-/-}$ mice preferred to investigate the confined mouse over the object. In other words, they displayed a typical preference for social over nonsocial stimuli. We then replaced the object with a second confined mouse. The test mouse now has a choice of investigating a familiar or a novel conspecific mouse. Unlike control mice that prefer to investigate the novel mouse, *Nrp2*$^{-/-}$ mice spent equal time investigating the novel and familiar mice.

In both tests described above, an expression of preference for novelty relies on a multitude of cognitive and motivational processes. The animal must acquire and retain an episodic-like memory of the sensory features of the objects or mouse it encounters during the learning phase. This information must then be retrieved during the test phase to guide its investigatory behavior. Mice must also be sufficiently motivated by novelty to express investigatory behavior in the presence of these stimuli. Performance of *Nrp2*$^{-/-}$ mice in both tests may therefore reflect impaired acquisition or retention of long-lasting episodic-like memory or an impairment in motivational control by novel stimuli. We believe the deficit in *Nrp2*$^{-/-}$ mice is likely an impairment in memory and less likely reflects an impairment in motivation. In both tests, we found *Nrp2*$^{-/-}$ mice investigated stimulus objects during both phases, suggesting an intact interest in these animals in exploring novel stimuli. Indeed, there was no difference between strains in the amount of investigatory activity; rather, *Nrp2*$^{-/-}$ mice simply could not differentiate between novel and familiar stimuli.

In addition to episodic learning deficits, we found impairments in motor learning and stereotyped repetitive motor behavior in *Nrp2*$^{-/-}$ mice. On tests of the accelerating rotarod, control *Nrp2*$^{+/-}$ mice improved their performance across training trials, as shown by the mouse maintaining its position on top of the rotating spindle for a longer duration as the spindle's rotational speed accelerated. In contrast, *Nrp2*$^{-/-}$ mice showed little improvement across trials. This suggests that *Nrp2*$^{-/-}$ mice were impaired in motor skill learning. We also found in observations in their home cage that *Nrp2*$^{-/-}$ mice made longer and more frequent grooming bouts (Fig. 8.2). This suggests that loss of *Nrp2* alters stereotypic motor behavior. Similar increases in grooming behavior have been observed in two autism-related mouse models, the BTBR mouse [96], and mice treated prenatally with valproic acid [71].

As with the neuropilins, only a handful of studies have examined behavior in semaphorin and plexin knockout animals. Recently, the behavior of mice with a deletion of the *Sema3F* gene has been characterized [70]. *Sema3F*$^{-/-}$ mice show an increase in anxiety-like behaviors: they spent less time in the center of the arena during an open-field test and reduced their activity in an elevated plus maze. Similar anxiety-related behaviors were observed in a light/dark transition test. *Sema3F*$^{-/-}$ mice also showed increased freezing to a shock-associated context after tone-shock pairings. Taken together these data suggest loss of Sema3F signaling increases anxiety-related behaviors.

Sema3F is the ligand for Nrp2, and one might expect that loss of either signaling unit would have similar effects on behavior. However, in contrast to the results obtained from *Nrp2*-null mice, no deficits in motor behavior as measured with the accelerating rotarod were observed in *Sema3F*$^{-/-}$ mice [70]. Nor were any deficits in spatial reference memory observed in the Barnes Maze. One possible explanation for these divergent results is that Nrp2 is a receptor for multiple semaphorins including Sema3B, Sema3C, and Sema3G, as well as Sema3F. As Matsuda et al. [70] note, the behavioral effects of loss of *Nrp2* may reflect loss of all Sema3 function.

8.3.2 Relevance to Neuropsychiatric Diseases

Findings from animal studies support the connection between Nrp signaling and psychiatric diseases. Both *Nrp2*- and Sema3F-null mice show greater likelihood of developing seizures, which is often comorbid with ASD [9]. The observed increase in self-grooming behavior in *Nrp2* mice is often interpreted as an expression of excessive stereotypic motor behavior and in that way resembles the repetitive motor behavior that forms a core feature of ASD. Indeed, enhanced self-grooming in mice is often used as evidence supporting a particular strain as a model of ASD because it provides some degree of face validity [71, 95, 96]. Repetitive motor behavior is one of three core ASD features, which also includes communication and social impairments. These behaviors have not been fully assessed in *Nrp2* mice; thus it is premature to claim this animal is a model of ASD. Although *Nrp2* mice show no preference for social novelty, this deficit likely reflects an impairment in episodic memory. Indeed, both *Nrp2*- and *Sema6A*-null mice show similar learning impairments, suggesting that these signaling systems are critical for establishing and/or maintaining proper function in the hippocampus.

Additional results from *Nrp2* and *Sema* knockouts suggest this signaling system may have relevance to other psychiatric disorders. Sema3F-null mice showed increased anxiety-related behaviors across a range of tasks, suggesting a possible link between *Sema3F* and mood disorders. Going forward, the behavioral response of Nrp-deficient mice to pharmacological treatments developed for psychiatric diseases will provide valuable evidence to assess the validity of these mice as disease models. Likewise, interventions based on disrupting or reversing the effects of disrupted Nrp signaling may be a source of new treatments for psychiatric diseases.

8.3.3 Neuropilin-Related Genes Associated with Neuropsychiatric Diseases in Humans

A number of studies have linked members of the semaphorin signaling family to psychiatric disorders. One study examined the relationship between polymorphisms in the NRP2 gene that codes for Neuropilin-2 and incidence of ASD in a Chinese ancestral population [116]. They found a significant association between NRP2 and ASD, with two single-nucleotide polymorphisms (SNPs) located in the NRP2 gene that was significantly associated with ASD status. In addition to Nrps, studies have identified a link between the *PLXNA2* gene and susceptibility to schizophrenia and comorbid psychiatric disorders. *PLXNA2* resides in 1q32, which linkage studies have identified as a candidate locus for schizophrenia [10, 46]. A genome-wide association study of schizophrenia patients in a European-American population identified a number of SNPs in the *PXLNA2* gene that are associated with schizophrenia status [68]. Although this finding has not been fully replicated in Asian ancestral populations [11, 34, 103], a meta-analysis combining datasets from Mah et al. and Fujii et al. supports the association of *PLXNA2* with susceptibility to schizophrenia [1]. Further, based on the SNPs identified by Mah et al. Wray et al. [113] identified *PLXNA2* variants that strongly associated with diagnostic measures of anxiety and depression [113].

In addition to the neuropilins and plexins, genes coding semaphorins have been associated with psychiatric disorders. Fujii et al. examined four SNPs of the *SEMA3D* gene in a Japanese population and found a significant association of one SNP with schizophrenia status, as well as a significant association of *SEMA3D* with schizophrenia in a haplotype analysis [35]. Taken together, these findings suggest that variations in genes encoding members of the semaphorin signaling family may increase susceptibility to psychiatric disorders with a neurodevelopmental origin, such as ASD and schizophrenia. As with other susceptibility genes, variations in semaphorin-coding genes may also confer some cognitive advantages that are evolutionarily adaptive, but may also increase liability for certain neurodevelopmental diseases [22].

8.3.4 Changes in Gene Expression in Patients with Psychiatric Illnesses

Further evidence for neuropilins and their signaling partners' involvement in neuropsychiatric disorders comes from postmortem analysis of brain tissue. Studies examining samples taken from prefrontal cortex have generally shown increased expression of genes coding for Nrps, semaphorins, and plexins. For example, in individuals diagnosed with major depressive disorder, mRNA analysis of prefrontal cortex samples showed increased expression of the *NRP1* gene compared to matched controls [43]. Similarly, using microarray technology, it was found that expression of the genes *SEMA3A*, along with *PLEXNB1*, *SEMA4D*, and *SEMA6C*, was increased in prefrontal cortical samples taken from patients diagnosed with

schizophrenia [42]. In contrast to these results, a reduction in the expression of *PLXNA4* was observed in the anterior cingulate and motor cortex in patients with ASD [98]. In addition to the prefrontal cortex, differences have been observed in the cerebellum in adult individuals with schizophrenia. Eastwood et al. used immunohistochemistry and ELISAs to show increased presence of Sema3A protein in the neuropil in the inner molecular layer of the cerebellum compared to control subjects [30].

These studies suggest that increased expression of semaphorins in the adult brain may have a role in the pathophysiology of schizophrenia and depression. It is not entirely clear, however, what role semaphorins play in these diseases. One possibility is that the increased presence of semaphorins is a consequence of some underlying pathology and not necessarily a cause of the disease itself. For example, it has been shown that NMDA hypofunction in rats will increase expression of semaphorin-related proteins [122]. Alterations in glutamatergic signaling feature prominently in contemporary theories of schizophrenia pathophysiology [73]. Therefore, the increase in semaphorin signaling in adult brain tissue may be a consequence of perturbed synaptic or other neurophysiological functions and is not necessarily itself responsible for disease pathophysiology.

8.4 Summary

Over the past two decades, many diverse functions of Nrps in nervous system development have been identified. While we have discussed some examples and highlighted the novel mechanisms of how Nrp expression is regulated, the disparate functions of Nrps are best understood within the context of class 3 semaphorin signaling in general. That is, the interaction of Nrps with plexin signaling transducing receptors to form specific Nrp/Plxn complexes or Nrps with other transmembrane molecule complexes has given neurons a strategy to diversify their semaphorin signaling, leading to different cellular functions. Since the downstream signaling cascades of Sema3-Nrp usually involve a plexin signal transducing receptor or other modulatory co-receptor, there are only a few immediate downstream interactors identified for Nrps [12, 85]. Nevertheless, the intriguing question of how Nrps choose to directly interact with a downstream effector (for example, β2-chimaerin) or a plexin to form a receptor complex to mediate distinct semaphorin signaling and function remain to be answered. Furthermore, how do Nrps choose between forming a complex with plexins or CAMs when both are often present in the same neuronal population? Does the Sema3 ligand specificity solely dictate these interactions? Future crystal structural work, in combination with in vivo domain functional analysis, should provide invaluable insights into ligand-receptor and receptor complex binding and activation of distinct Sema3-Nrp signaling.

Transcriptional and translational regulation of Nrps adds yet more functional diversity and another layer of mechanistic complexity. Nevertheless, the mechanisms identified to dynamically control the spatiotemporal expression of Nrps are still in the infancy stage, and much remains to be understood. It is likely that there

are other yet to be identified transcription factors that can either positively or negatively control Nrp gene expression based on cell type specificity and function. It is well established that the proper wiring of many neural circuits requires neural activity. Therefore, the idea that Nrp expression can be indirectly regulated by neuronal activity via a microRNA that responds to synaptic plasticity is intriguing, especially in the context of Nrp function in dendritic morphogenesis and synapse elimination during postnatal development. Moreover, accumulating evidence suggests altered expression and function of Nrps and their semaphorin ligands in neurological disorders and injury [69, 82, 120]. A future challenge that may provide important clinical applications is to identify specific transcriptional and translational regulators associated with changes in Nrp expression in neurological diseases.

Given the diverse roles of Nrps in nervous system development, it is not surprising that their disruption in animal models has significant effects on behavior. In mice, loss of Nrp function increases repetitive motor behavior and induces learning impairments. In humans, Nrp gene variants are related to increased ASD incidence. More broadly, semaphorin and plexin gene variants in humans are associated with a number of neuropsychiatric disorders. An important future research direction is to understand how the various developmental processes in the brain mediated by Nrps may be responsible for altered cognitive/mental functions. Such an endeavor will require temporally and spatially specific manipulations of Nrp expression in the brain combined with physiological and behavioral measurements. Ultimately, these multidisciplinary approaches may lead to a better understanding of the underpinnings of developmental neurological disorders and neuropsychiatric disease mechanisms.

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The Roles of Neuropilins in the Immune System

9

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Abstract

Although the neuropilins (Nrps), neuropilin-1 (Nrp1) and its homologue neuropilin-2 (Nrp2), were originally identified as key molecules involved in the development of neurons, it has recently become clear that they also play important roles in the immune system. The function of Nrp1 has been well characterized, mainly in T cells, including regulatory T (T_{reg}) cells, as well as in dendritic cells (DCs), whereas the immunological function of Nrp2 is less clearly understood. In this chapter, we discuss the identification of Nrp1 and its roles in immune systems.

9.1 Introduction

Neuropilin-1 and -2 (Nrp1 and Nrp2) are highly conserved transmembrane proteins originally identified as neuronal guidance molecules that act during neural development and axonal outgrowth [1]. Subsequent work revealed that Nrps function as neuronal receptors for semaphorins such as Sema3A [2, 3]. Today, we know that Nrps have short cytoplasmic domains that mediate signals through interacting co-receptors such as A-type plexins [4, 5]. In the immune system, the function of Nrp1 has been characterized mainly in T cells, especially regulatory T (T_{reg}) cells, as well as in dendritic cells (DCs). By contrast, the function of Nrp2, which is structurally similar to Nrp1, is not well determined. In this chapter, we describe current knowledge regarding the role of Nrp1 in the immune system.

9.2 Neuropilin in Lymphocytes

9.2.1 Neuropilin-1 in Regulatory T Cells

Regulatory T (T_{reg}) cells are a subpopulation of T cells defined by expression of CD4, CD25, and forkhead box P3 (FOXP3). T_{reg} cells suppress induction and proliferation of effector T cells, thereby helping to maintain tolerance to self-antigens and prevent autoimmune diseases [6]. T_{reg} cells consist of two major types, "natural" T_{reg} (nT_{reg}) and "induced/inducible" T_{reg} (iT_{reg}) cells. nT_{reg} cells, which are positively selected in the thymus, have a relatively high avidity for self-antigens. The signal to develop T_{reg} cells comes from interactions between T cell receptor (TCR), expressed on T_{reg} cells, and major histocompatibility complex (MHC) class II molecules displaying self-peptide, expressed on the thymic stroma [7]. By contrast, iT_{reg} cells develop from conventional $CD4^+$ T cells circulating in peripheral tissues.

A recent report showed that Nrp1 expression distinguishes thymus-derived nT_{reg} cells from peripherally derived iTreg cells [8, 9]. Microarray analysis comparing gene expression between thymus-derived nT_{reg} cells and peripherally derived iT_{reg} cells revealed that Nrp1 is expressed at high levels by thymus-derived nT_{reg} cells but not by mucosa-generated peripherally derived iT_{reg} cells. iT_{reg} cells derived from

highly inflammatory environments in animal models of inflammatory disorders, such as the spinal cords of mice with spontaneous autoimmune encephalomyelitis (EAE) or the lungs of mice with chronic asthma, express Nrp1 at high levels, whereas iT_{reg} cells in secondary lymphoid organs express only low levels of Nrp1. These results indicate that distinct types of infiltrating T_{reg} cells expressing Nrp1 are involved in the chronic phase of inflammatory diseases [8]. A contemporaneous study using TCR-transgenic mice with a defined self-antigen specificity showed that Nrp1 is expressed at high levels in thymus-derived nT_{reg} cells, whereas iT_{reg} cells generated in vitro or in vivo lack Nrp1 expression, indicating that Nrp1 distinguishes the nT_{reg} and iT_{reg} subsets [9]. Thus, these studies indicate that Nrp1 represents an excellent candidate marker for distinguishing the two types of T_{reg} cell subset in mouse models. However, in contrast to murine T_{reg} cells, human $Foxp3^+$ T_{reg} cells do not specifically express Nrp1 [10]. The authors of that study carried out several experiments but were unable to demonstrate clear Nrp1 expression in human $CD4^+CD25^+Foxp3^+$ T_{reg} cells derived from the blood, thymus, spleen, peripheral lymph nodes, and tonsils, whereas Nrp1 is highly and specifically expressed on murine T_{reg} cells. Therefore, further careful evaluation will be required to determine whether Nrp1 is a reliable marker capable of distinguishing T_{reg} subsets.

In addition, global gene expression studies showed that Nrp1 is a Foxp3-inducible gene, along with CD25, cytotoxic T lymphocyte antigen 4 (CTLA4), and glucocorticoid-induced tumor necrosis factor receptor-related protein (GITR) [11, 12]. Nrp1, which is expressed by most T_{reg} cells but not by naive helper T (Th) cells, increased the frequency of long interactions between T_{reg} cells and DCs, stabilizing their interaction and preventing naive T cells from interacting with DCs [12]. This observation implies that T_{reg} cells are more capable than naive Th cells of engaging in long interaction with DCs.

Furthermore, recent work revealed a novel function of Nrp1 in maintenance of T_{reg} cells functions (Fig. 9.1) [13]. That study demonstrated that Nrp1 is the receptor of Sema4A, a class IV transmembrane semaphorin preferentially expressed in immune cells including Th cells. Ligation of Sema4A to Nrp1 restrains Akt phosphorylation in T_{reg} cells by recruiting phosphatase and tensin homologue (PTEN), thereby inducing nuclear localization of the transcription factor Foxo3a, which plays a crucial role in the development and programming of T_{reg} cells [14, 15]. In vivo experimental models of inflammatory colitis or T_{reg} cell stability in tumor tissues demonstrated that this Sema4A/Nrp1-dependent pathway is essential for maintenance of immune homeostasis, indicating that Nrp1 is a potential therapeutic target that could limit T_{reg} cell-mediated tumor-induced tolerance without inducing autoimmunity.

Many other reports support the idea that Nrp1 is functionally relevant in T_{reg} cell-mediated immune suppression. Cell-specific deletion of Nrp1 on $CD4^+$ T cells increases the severity of experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis (MS), a neuroimmunological disorder characterized by demyelination in the central nervous system (CNS) [16]. Nrp1 expressed by T_{reg} cells regulates antitumor immunity by guiding T_{reg} cells into the tumor in response to tumor-derived vascular endothelial growth factor (VEGF). $CD4^+$ T

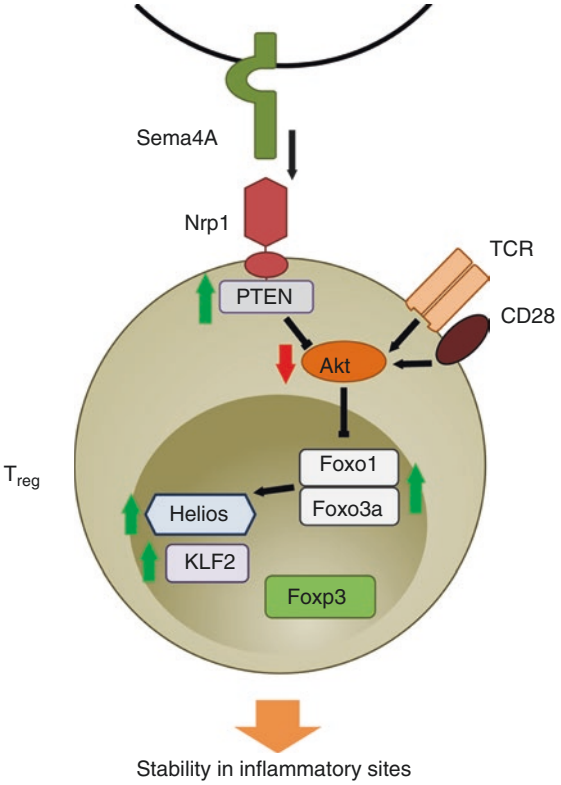

Fig. 9.1 Neuropilin-1 maintains T_{reg} cell stability**.** Ligation of Nrp1 to Sema4A suppresses Akt activation via recruitment of PTEN, thereby inhibiting nuclear exclusion of Foxo transcription factors. This reaction promotes changes in gene expression, including increases in the expression levels of *Helios* and *KLF2*, thus stabilizing and enhancing T_{reg} function. *PTEN* phosphatase and tensin homologue, *Foxo* forkhead box o, *KLF2* Krüppel-like factor 2, *TCR* T cell receptor

cell-specific ablation of Nrp1 expression in mice results in delayed tumor formation and progression in mouse tumor transplantation models, and tumors transplanted into these mice are accompanied by activated intratumoral CD8+ T cells [17]. These observations strongly support the idea that Nrp1 plays a key role in T_{reg} cell-mediated immunosuppression.

9.2.2 Neuropilin-1 in T Cell Development in Thymus

In the human thymus, Nrp1 is expressed in both cortex and medulla of thymus. In situ and in vitro expression profiling revealed that Nrp1 is also expressed in distinct CD4/CD8-defined thymocyte subsets, DCs, and thymic epithelial cells (TECs). Importantly, Nrp1 is recruited specifically at the site of TEC–thymocyte contact, suggesting that it might be involved in thymocyte development [18]. Nrp1 expression in thymocytes is rapidly upregulated after stimulation by T cell receptor (TCR) and IL-7 or after adhesion to TECs. Sema3A is also present in human thymus, both in thymocytes and TECs, and is upregulated in thymocytes after TCR engagement. Sema3A decreases the adhesion of Nrp1-positive thymocytes and induces their migration by exerting a repulsive effect. These results indicate that

Nrp1/Sema3A-mediated interactions play important roles in the control of human thymocyte development.

9.2.3 Neuropilin-1 in Follicular Helper T Cells

A recent report showed that Nrp1 is selectively expressed by follicular helper T (Tfh) cells, a specific T cell subset that aids in B cell differentiation and formation of germinal centers (GCs) in human secondary lymphoid organs, leading to the generation of long-lived plasma cells and memory B cells [19]. The results of that study showed that Nrp1 expressed by Tfh cells contributes to B cell differentiation in vivo and in vitro. In addition, Nrp1 is expressed by malignant Tfh-like cells in angioimmunoblastic T cell lymphoma (AITL), which is associated with elevated terminal B cell differentiation. These findings suggest that Nrp1 could be a specific marker for Tfh cells and a useful prognostic factor for malignant neoplasms associated with Tfh cell activity.

9.3 Neuropilin in Dendritic Cells (DCs)

9.3.1 Neuropilin-1 in Plasmacytoid DCs

Expression of Nrp1 on dendritic cells (DCs) was first reported in 2001. During a search for human dendritic cell (DC) markers, Nrp1 was identified as blood DC antigen 4 (BDCA4), expressed by plasmacytoid DCs (pDCs), and was assigned as CD304 [20]. pDCs are innate immune cells that produce large quantities of type I interferons (IFNs) and play crucial roles in host antiviral responses [21]. Therefore, the function of Nrp1 in DCs has largely been characterized in the context of host antiviral defense. Treatment of pDCs with anti-Nrp1 antibody results in significantly reduced virus-induced IFN-α production, suggesting an immunoregulatory role of Nrp1 [22]. However, the mechanisms underlying this effect remain unclear.

9.3.2 Neuropilin-1 in Interactions Between DCs and T Cells

Nrp1 is expressed on human DCs; specifically, Nrp1 expression can be detected human monocyte-derived DCs in vitro, but not in monocytes [23]. Nrp1 is also expressed by resting T cells. Of particular interest, initial contact between DCs and resting T cells leads to Nrp1 polarization at the contact sites. Additionally, the soluble form of Nrp1 binds DCs and resting T cells and thereby mediates DC−T cell clustering and DC-induced proliferation of resting T cells. These results suggest that Nrp1 mediates interactions between DCs and T cells and is essential for initiation of the primary immune response in the lymph nodes. However, further studies are required to determine how and to what extent such interactions are relevant to physiological immune responses.

9.3.3 Neuropilin-1 in DC Migration Into Lymphatics

Nrp1 is also involved in the transmigration of DCs into the lymphatics [24]. Nrp1 expressed by DCs forms a receptor complex with plexin-A1, and Sema3A secreted by the lymphatics functions as a ligand for the plexin-A1–Nrp1 receptor complex, thereby inducing phosphorylation of the myosin light chain and ultimately leading to actomyosin contraction. In this manner, DCs pass through narrow gaps between lymphatic endothelial cells (Fig. 9.2).

9.4 Neuropilin in Macrophages

9.4.1 Neuropilin-1 in Tumor-Associated Macrophages (TAMs)

A recent study described a function of Nrp1 in immune cells associated with antitumor immunity [25]. Nrp1 expressed in tumor-associated macrophages (TAMs) regulates infiltration of these cells into hypoxic regions and promotes tumor progression. TAMs have two opposing classes of phenotypes: pro-tumor and antitumor. The behavior of TAMs is regulated by specific chemokines and cytokines that polarize macrophages into a pro-immune M1 or immunosuppressive M2 phenotype. Sema3A induced by hypoxia acts as an attractant for TAMs through an association with the Nrp1–plexin-A1–plexin-A4 receptor complex, followed by

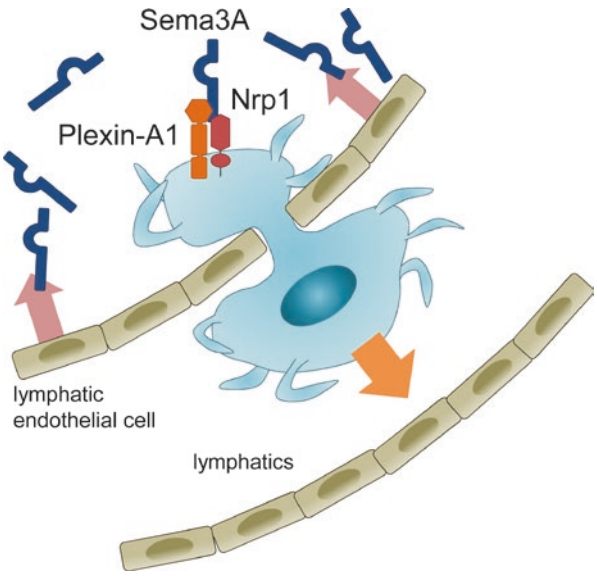

Fig. 9.2 Neuropilin-1 in migration of dendritic cells. During transmigration of dendritic cells (DCs) into the lymphatics, Sema3A secreted by lymphatic epithelial cells (ECs) binds to the plexin-A1–Nrp1 receptor complex expressed on the rear sides of DCs. The interactions among these molecules promote actomyosin contraction in DCs and their detachment from ECs, resulting in elevated DC transmigration into the lymphatics

phosphorylation of vascular endothelial growth factor receptor 1 (VEGFR1), leading to TAM migration into hypoxic areas (Fig. 9.3). Importantly, the expression levels of Nrp1 are downregulated in the hypoxic environment, whereas Sema3A continues to regulate TAMs in an Nrp1-independent manner by eliciting plexinA1/plexinA4-mediated stop signals, which retain these cells inside the hypoxic niche. Indeed, cell-specific *Nrp1* gene deletion in macrophages increases entrapment of TAMs in normoxic tumor regions, thereby decreasing their pro-angiogenic and immunosuppressive functions, leading to attenuated tumor growth and metastasis. These observations study strongly suggest that TAMs' heterogeneity depends on their localization, which is tightly controlled by Sema3A/Nrp1 signaling.

9.4.2 Neuropilin-1 in Osteoimmunology

Osteoimmunology is an interdisciplinary research field focusing on the molecular relationships between cells in the immune and skeletal systems at the molecular level. Homeostasis of bone tissue is maintained by the activities of two types of

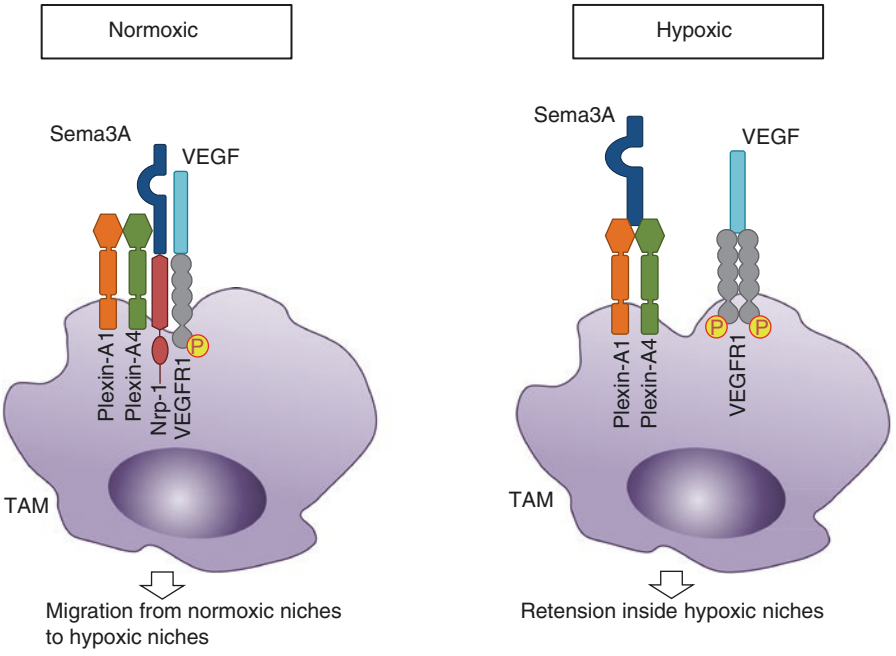

Fig. 9.3 Neuropilin-1 controls the localization of tumor-associated macrophages (TAMs) to hypoxic tumor areas**.** Sema3A and VEGF attract TAMs from normoxic (perivascular) sites to hypoxic (avascular) sites through VEGFR1 transactivation. Although VEGF functions independently of Nrp1, activation of VEGFR1 by Sema3A requires Nrp1 as well as plexinA1 and plexinA4. Upon repression of Nrp1 expression by hypoxia, Sema3A retains TAMs inside the hypoxic areas in a plexinA1/A4-dependent manner. Thus, whereas normoxic TAMs have antitumor and antiangiogenic phenotypes, hypoxic TAMs exert immunosuppressive and pro-angiogenic functions, thereby contributing to tumor growth and metastasis. *VEGF* vascular endothelial growth factor, *VEGFR1* vascular endothelial growth factor receptor 1

cells, osteoclasts and osteoblasts. Bone tissue is broken down by osteoclasts, and new bone tissue is built by osteoblasts. Osteoclasts develop from the self-fusion of progenitor cells in the monocyte/macrophage lineage in response to cytokines such as receptor activator of nuclear factor kappa-B ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) secreted by osteoblasts.

Recently, a breakthrough was made regarding the signals mediated by Nrp1/plexin-A1, and their ligand Sema3A. That study showed that the Sema3A–plexin-A1–Nrp1 axis induces an osteoprotective effect by decreasing osteoclast activities and increasing osteoblast activities. *Nrp1*-deficient mice are embryonically lethal, but knock-in mice in which the *Nrp1* gene is replaced by a mutant allele lacking the Sema-binding site ($Nrp1^{Sema-}$ mice) exhibit an osteopenic phenotype accompanied by enhanced osteoclast differentiation [26]. Notably, this phenotype is identical to that of *Sema3A*-deficient mice. Further analysis revealed that ligation of Sema3A to Nrp1 expressed by osteoclasts inhibits signals mediated by immunoreceptor tyrosine-based activation motif (ITAM) and RhoA, leading to suppression of RANKL-induced osteoclast differentiation (Fig. 9.4). By contrast, binding

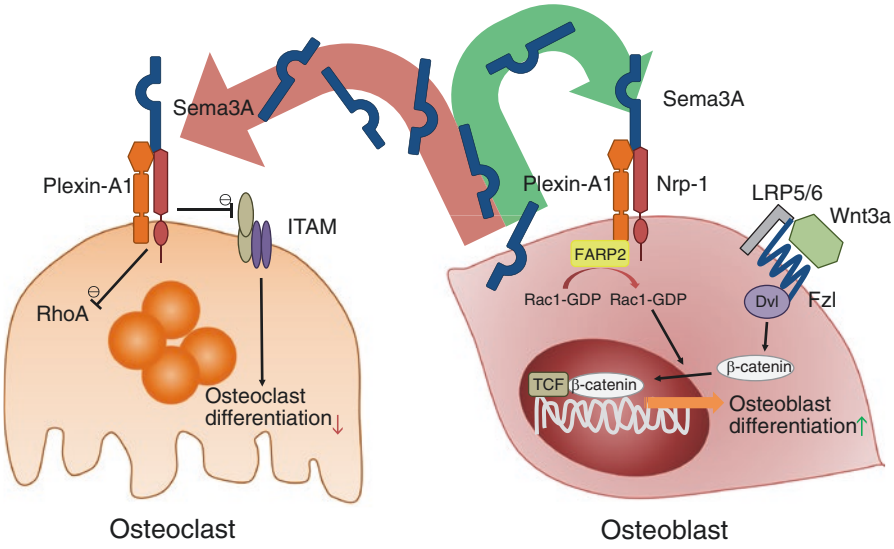

Fig. 9.4 Neuropilin-1-mediated signals in osteoimmunology**.** (*left*) The binding of osteoblast-derived Sema3A to Nrp1 on osteoclasts inhibits ITAM signaling, leading to attenuated osteoclast differentiation. The Sema3A–Nrp1–plexin-A1 axis also inhibits migration of osteoclast precursor cells by inhibiting RhoA activation. (*right*) The Sema3A–plexin-A1–Nrp1 complex regulates osteoblast differentiation. Sema3A secreted from osteoblasts ligates to the plexin-A1–Nrp1 receptor complex on osteoblasts. This reaction leads to activation of the small G-protein RAC1 through FARP2 activation, which subsequently promotes WNT3a-induced accumulation of β-catenin in the nucleus. Thus, the Sema3A–plexin-A1–Nrp1 axis exerts an osteoprotective function by suppressing osteoclast activities and enhancing osteoblast activities. *ITAM* immunoreceptor tyrosine-based activation motif, *RhoA* Ras homologue gene family member A, *FARP2* pleckstrin domain protein 2, *RAC1* RAS-related C3 botulinus toxin substrate 1, *LRP5*/6 low-density lipoprotein receptor-related protein 5/6, Fzl frizzled, *Dvl* disheveled, *TCF* T cell factor

of Sema3A to Nrp1 expressed by osteoblasts stimulates the canonical Wnt/β-catenin signaling pathway through Rac1 activation mediated by FERM, RhoGEF, and pleckstrin domain protein 2 (FARP2), resulting in enhanced osteoblast differentiation. Furthermore, the osteopenic phenotype in *Sema3A*-deficient mice can be rescued by intravenous administration of recombinant Sema3A protein, suggesting that Sema3A is a promising therapeutic agent for osteoimmunological diseases.

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Part III

The Role of the Neuropilins in Cancer and in Immune Disorders

The Role of the Neuropilins in Tumour Angiogenesis and Tumour Progression

10

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Contents

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Abstract

Neuropilins (NRPs) are multifunctional receptors for class 3 semaphorins, which are responsible for axon guidance during the development of the nervous system in vertebrates, and for vascular endothelial growth factors (VEGFs), essential for vascular development and angiogenesis in disease. There is now a large body of evidence that NRPs also mediate tumour angiogenesis and progression, and they have also emerged as novel therapeutic targets in cancer. Many neoplastic cell types express NRPs, and NRP1 and NRP2 upregulation is positively correlated with tumour progression and poor patient prognosis in several cancer types (Pellet-Many et al. Biochem J 411:211–226, 2008). Recently, NRPs have been shown to play novel roles in the tumour stem cell niche and in regulation of tumour immunity. This chapter focuses on the role of NRPs in tumour angiogenesis and tumour progression, focusing on the role of the NRPs as modulators of VEGF function and highlighting approaches to therapeutic targeting of NRPs in cancer.

10.1 Neuropilin Structure

NRP1 and NRP2 are transmembrane glycoproteins that share a similar domain structure and have 44 % amino acid sequence homology. The structures of NRP1 and NRP2 are divided into large extracellular regions containing two CUB (a1/a2) domains, FV/FVIII (b1/b2) domain, cMAM domains and a single transmembrane domain and a short cytoplasmic region of 44 amino acid residues in NRP1 and 43 in NRP2 [62, 84]. In the extracellular region, CUB (a1/a2) domains are important for binding to semaphorins. The b1/b2 domains are required to bind VEGFA and also contribute to semaphorin binding. The role of the NRP MAM domain is unclear, but it is thought to be important for protein stability and to play a role in NRP1 oligomerisation, largely based on function of other MAM domains present in diverse proteins [75].

NRP1 is a glycoprotein, but its glycosylation varies between different cell types. NRP1 glycosylation occurs by the addition of an O-linked heparin sulphate and/or chondroitin sulphate glycosaminoglycan (GAG) moiety preferentially to serine 612 in the linker region between the b2 and MAM domains. GAG modifications may enhance both VEGF binding to NRP and cell survival and downregulate VEGFR2 expression levels in vascular smooth muscle cells [31, 96]. In glioma cells, overexpression of a non-GAG form of NRP1 (NRP1 S612A) leads to enhanced cell invasion in a 3D matrix and increased levels of tyrosine phosphorylated p130Cas, indicating that GAG-modified NRP1 plays a negative role in regulating invasion. It is suggested that the balance between GAG-modified and unmodified NRP1 might be important for determining invasive potential [31].

10.2 VEGF Signalling

Vascular endothelial growth factor (VEGF) is a potent angiogenic factor, essential for the development of the vasculature. VEGF levels are upregulated in many tumours, and its contribution to pathogenic angiogenesis in cancer, eye diseases and other disorders is well established.

The mammalian vascular endothelial growth factor (VEGF) family consists of five homodimeric polypeptides of ≈40 kDa: VEGFA, VEGFB, VEGFC, VEGFD and placental growth factor (PlGF) [30, 120]. VEGFE is a virally encoded isoform of VEGF. Since its discovery in 1989, VEGF has emerged has an important signalling protein involved in both vasculogenesis (the formation of the circulatory system) and angiogenesis (the growth of blood vessels from pre-existing vasculature) [95]. VEGFA is alternatively spliced to generate VEGFA121, VEGFA145, VEGFA165, VEGFA189 and VEGFA206, which are endowed with different biological properties [76, 86]. VEGFA121 and VEGFA165 are the most abundant isoforms in mammals, which differ in their biological properties. VEGFA121 lacks exons 6 and 7, and VEGFA165 lacks exon 6.

The binding of VEGFs to NRP1 and NRP2 appears to be mediated by two distinct domains. In VEGFA165, these correspond to the basic heparin-binding domain encoded by exon 7 and the carboxy terminus of exon 8 [54, 98]. Binding of VEGFA121, which lacks exon 7, to NRP1 has been more controversial. Gitay-Goren et al. were unable to detect VEGFA121 binding. However, Pan et al. [81] have shown that VEGFA121 can directly bind to NRP1 using in vitro surface plasmon resonance (SPR) analysis. However, it should be noted that the K_D observed for both VEGFA121 (0.2uM) and VEGFA165 (0.1uM) in Pan et al. [81] was significantly lower than previously published work yielding a K_D of ~.5 nM for VEGFA165 binding to NRP1 using cell-free ligand-binding assays [33, 54, 81].

VEGF activity is mediated by high-affinity tyrosine kinase receptors (VEGFR). There are three main subtypes of VEGFR (VEGFR1 (Flt-1), VEGFR2 (KDR/Flk-1) and VEGFR3 (KDR/Flk-4). VEGFR1 is critical in the regulation of migration of endothelial precursors as well as mature monocyte/macrophages. VEGFR2 is the major transducer of VEGF function in vascular endothelial cells (ECs), whereas VEGFR3 is required for lymphatic endothelial function [1].

VEGFs bind with different affinities to VEGFRs. VEGFA binds to VEGFR1 and VEGFR2; VEGFC and VEGFD bind VEGFR2 and VEGFR3; VEGFB and PIGF bind only to VEGFR1; and VEGFE binds only to VEGFR2. VEGFs' binding to NRP1 and NRP2 modulates the biological outcome of VEGF/VEGFR signalling [34, 61, 84, 98]. NRP1 is known to interact with some heparin-binding isoforms of VEGFA, B, E and PIGF, whereas NRP2 interacts with VEGFA, C and D [40, 59]. NRP1 is a high-affinity receptor for VEGFA in both endothelial and tumour cells, and NRP1/VEGFR2 co-expression can enhance VEGF-induced chemotaxis in comparison with cells expressing only VEGFR2 [98]. Co-expression of NRP1 with VEGFR2 also enhances VEGF binding to VEGFR2, VEGFR2 phosphorylation and

VEGF-induced signalling and migration [112], though NRPs are not required neither for high-affinity binding of VEGFA to VEGFR2 nor for VEGFA activation of VEGFR2 and downstream signalling pathways [84].

10.3 Expression of Neuropilins in Cancer

Numerous studies have reported expression of NRPs in diverse human tumours (Table 10.1). NRP expression is detected both on tumour vessels and also on a large variety of cancer cell types. NRP2 is also expressed by a variety of neoplastic cell types (Table 10.1).

NRPs can influence tumour progression in multiple ways. NRPs form complexes with VEGF receptors (VEGFR1 and VEGFR2) and thereby enhance VEGF signalling through VEGFR2 [84]. Thus, NRPs can influence tumour vascularisation. NRPs also regulate other receptor signalling pathways important in stimulating growth of tumour cells, endothelial cells and/or tumour-associated stromal cells (e.g. PDGFR , c-Met, and TGFBR; covered in other chapters of this book) and therefore also have the potential to mediate growth and migration of tumour cells, VEGF-independent

Table 10.1 Neuropilin (NRP) expression in cancer cells

Tumour	NRP1	NRP2
Astrocytomas	x	ND
Neuroblastomas	x	x
Gliomas	x	ND
Glioblastomas	x	ND
Pituitary tumours	x	ND
Endocrine pancreatic tumours	ND	x
Pancreatic adenocarcinomas	x	x
Gastric cancer	x	ND
Colon cancer	x	ND
Acute myeloid leukaemia (AML)	x	ND
Chronic lymphocytic leukaemia B	x	ND
Breast cancer	x	ND
NSCLC	x	x
Lung cancer	x	x
Melanomas	x	x
Prostate cancer	x	x
Ovarian carcinomas	x	ND
Bladder cancers	ND	x
Osteosarcomas	ND	x

Adapted from Grandclement and Borg [33]
Cells from indicated tumours we probed for NRP1 and NRP2 expression
X indicates detection of protein and/or mRNA expression, *ND* indicates no data or inconsistent data

tumour vascularisation and expansion of fibroblasts and other stromal cells. Lastly, the NRPs are expressed in monocytic cells and on regulatory T cells and have been implicated in recruitment of immunomodulatory cells to cancers.

10.4 Neuropilin Function and Cancer

Several studies have highlighted the role of NRP in multiple aspects of cancer biology. In vitro studies have pointed to a role for NRP1 in mediating endothelial and tumour cell motility. Evans et al. have shown that tyrosine phosphorylation of p130Cas, a key molecule required for cell motility, is stimulated by HGF and PDGF in glioma cells and VEGF in endothelial cells via NRP1. Furthermore they showed that knockdown of NRP1 or p130Cas was able to inhibit growth factor-mediated migration of glioma and endothelial cells. This highlights the role of an NRP1/p130Cas pathway in the regulation of endothelial and tumour cell motility, which has implications for the mechanisms involved in angiogenesis and tumour metastasis [27]. Fantin et al. have recently shown that NRP1 is important for actin remodelling and filopodia formation in endothelial tip cells via CDC42. This leads to proangiogenic signals to be converted into tip cell responses that are important for vessel sprouting and branching [29]. Other studies support a role for NRP1 in mediating cancer cell migration. For example, NRP1 knockdown using targeted siRNA inhibited breast carcinoma cell migration [4]. However, the mechanisms mediating the role of NRP1 in cancer cell migration are presently unclear.

Several in vivo and clinical studies have pointed to important roles for NRPs in cancer growth in vivo [37]. Miao et al. reported that inducible overexpression of NRP1 in prostate carcinoma cells in vivo resulted in larger and highly vascular tumours, at least partly driven by VEGF, since NRP1 overexpressing tumours exhibited increased VEGF expression [70]. Parikh et al. showed that subcutaneous xenografts of stably transfected KM12SM/LM2 human colon cancer cells overexpressing NRP1 led to increased tumour growth and angiogenesis in nude mice [82]. Expression of NRP1 is thought to have important implications for tumour metastasis and therapeutic intervention. Studies have shown that NRP1 is predominantly expressed in metastatic cells and its inactivation by the use of an anti-NRP1-binding peptide is sufficient to induce breast cancer tumour cell apoptosis [3, 6]. Evidence that VEGFA binding to NRP1 is important for NRP1's role in tumour growth has come from a study showing that a NRP1 knockin mouse model containing a mutation in the b1 domain, which prevents VEGF binding ($Nrp1^{Y297A/Y297A}$), display reduced growth of syngeneic B16-F1 mouse melanomas [28].

NRP1 and NRP2 have also been linked with tumour growth and disease progression in human cancer [26, 39, 64]. For example, NRP1 is upregulated in gastrointestinal carcinomas, which appears to be correlated with increased invasive behaviour [42]. Co-expression of NRP1 and NRP2 is also associated with NSCLC tumour progression [60].

Despite the majority of NRP studies reporting a pro-tumourigenic role, others suggest that NRP expression in cancer may play different roles in different tumour

types. In Panc-1 cells, overexpression of NRP1 reduced tumour incidence and volume in vivo, and NRP1-targeted siRNA was shown to increase tumour incidence in the same model [36]. In addition to its VEGFR-dependent actions, there is growing evidence that NRP1 has important functions in tumours independent of VEGFRs and possibly receptor tyrosine kinases for other cytokines. Studies in melanomas in which VEGFRs 1 and 2 are absent have shown that NRP1 is able to promote invasion through the activation of selected integrins, which can then recruit VEGFA and metalloproteinases and therefore modulate downstream signalling [37].

10.5 Cancer Biology Stem Cells

Cancer stem cells (CSCs) have been described in various cancers. Recently, studies have been done using a mouse model of skin tumourigenesis in order to understand the role of the vascular niche and VEGF signalling on controlling the stemness (the ability to self-renew and differentiate) of squamous skin tumours during the early stages of tumour progression. In this study, it was observed that VEGF signalling through VEGFR2 in endothelial cells is critical to sustain angiogenesis and to create a vascular niche for CSCs. NRP1 also played an essential role in skin tumourigenesis in this work, as conditional genetic deletion of NRP1 in the epidermis reduced the number of induced squamous skin tumours. Furthermore, specific deletion of NRP1 from the tumour epithelial cell compartment abrogated the ability of VEGF to stimulate tumour cell proliferation. Taken together, these results highlighted the essential role of NRP1 in maintaining a VEGF autocrine loop which contributes to tumour initiation and CSC expansion in skin tumours, with important implications for the prevention and treatment of epithelial cancers [8]. In a further study in 2012, Hamerlik et al. found that NRP1 is important for the proliferation of human glioma stem-like cells by maintaining autocrine VEGF production, allowing for sustained activation of downstream intracellular prosurvival pathways and promotion of glioblastoma tumour growth, invasiveness and enhanced resistance to bevacizumab [41].

10.6 Tumour Angiogenesis

In cancer, angiogenesis plays a fundamental role during the transition of tumours from a benign to a malignant state. So far, several proteins have been identified as angiogenic factors. Among those are vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), angiopoietins (Ang), transforming growth factor (TGF)-α, TGF-β, tumour necrosis factor (TNF)-α, platelet-derived endothelial growth factor, granulocyte colony-stimulating factor, placental growth factor, interleukin 8, hepatocyte growth factor and epidermal growth factor [46].

The recognition of VEGF pathway as a key regulator of angiogenesis has led to the development of several VEGF-targeted agents, including agents that prevent VEGFA binding to its receptors [102], antibodies against VEGFA [113] and small molecules that inhibit the kinase activity of VEGFR2 thereby block growth factor

signalling [17, 22]. In 2004, bevacizumab (Avastin), a humanised monoclonal antibody against VEGFA, became the first anti-angiogenic drug approved by the FDA for the treatment for metastatic colorectal cancer in combination with chemotherapy. However, while in some cancer types, bevacizumab displayed a synergistic effect [20], in others, bevacizumab had an antagonist effect [57]. A possible explanation for this relies on the fact that a treatment that aims to reduce the blood supply of a tumour is also likely to reduce the delivery of any other therapy such as chemotherapy [77]. On the other hand, bevacizumab can induce normalisation of newly formed vessels and thus allow enhanced delivery of chemotherapy to the tumours [50]. Furthermore, Avastin has recently been removed as a breast cancer therapy due to adverse effects associated with increased cardiovascular toxicity, although this decision is still controversial [94]. Thus there is an argument for additional anti-angiogenic therapies displaying reduced adverse effects to anti-VEGF therapy.

There is limited evidence that specifically supports a major role for NRP1 in tumour angiogenesis, though it is very likely that NRP1 contributes to VEGF-dependent tumour neovascularisation consistent with its role in VEGF-induced endothelial cell migration in cell culture studies and in postnatal angiogenesis in genetic models [28, 32]. A peptide that inhibits VEGF binding to NRP1 has been reported to inhibit angiogenesis and growth of tumour xenografts [99].

10.7 Tumour Lymphangiogenesis

The metastatic spread of cancer cells to distant organs is the major cause of morbidity in cancer patients. Recent studies have shown that the lymphatic vascular system is one of the main routes for the spread of tumourigenic cells leading to metastasis [24, 118]. The distribution and structural features of the lymphatic system, as detailed in Chap. 2, make it particularly suited to its emerging role as a major route of metastasis. The process by which the lymphatic system mediates metastasis is called lymph node metastasis and involves the migration of cancer cells from primary tumours to lymph nodes via the lymphatic vessels (Fig. 10.1). Once the cancer cells pass though the lymph nodes, they can then further metastasise to distant organs via the blood vascular system. Studies have shown that about 80 % of metastasis occurs via the lymphatic system [65], starting from the site of the primary tumour, spreading through the lymphatic system via entry of invasive cancer cells into permissive lymphatic capillaries, and then metastasising at regional sentinel lymph nodes before disseminating systematically to distant organs [24].

Lymphangiogenesis (the growth of new lymphatic vessels from pre-existing ones; see Chap. 2 for more details) has been shown to closely correlate with prognosis in various types of cancer. In 2001, a study using a breast carcinoma mouse model revealed that lymphangiogenesis in tumours promoted the metastasis of cancer cells to the sentinel lymph nodes [97]. Tumour lymphangiogenesis has also been found to be a better predictor of cancer metastasis and survival rate in melanoma patients compared to tumour size [19, 68].

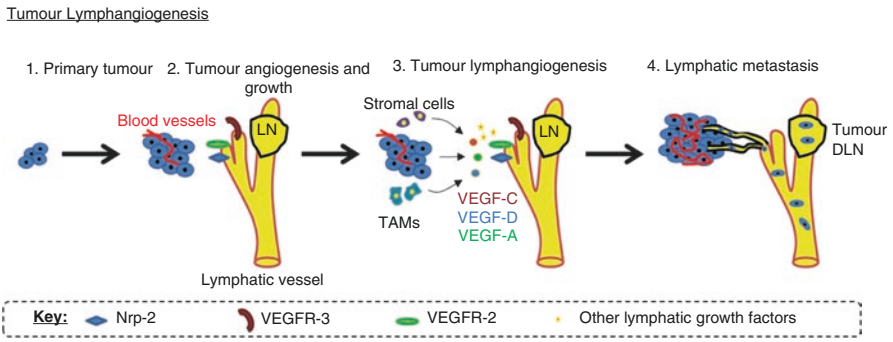

Fig. 10.1 Stages leading to lymph node metastasis. As early-stage tumours grow, they develop their own blood supply via tumour angiogenesis. Tumour lymphangiogenesis arises mainly from the pre-existing lymphatic vasculature. Tumour lymphangiogenesis is regionally induced during tumourigenesis by lymphatic growth factors such as VEGFC/D/A which are secreted by tumour cells, stromal cells and inflammatory cells (e.g. tumour-associated macrophages (TAMs)). These growth factors bind to their specific receptors and mediate the formation of tumour neo-lymphatics, which facilitate the intravasation of tumour cells into the lymphatic vessels. Once tumour cells enter the lymphatic vessels, they can reach the lymph nodes, which are a preferred site for lodgement of metastasising tumour cells. *LN* lymph node, *DLN* draining lymph node, *TAMs* tumour-associated macrophages (Adapted from review by Duong et al. [24])

The most well-established growth factors associated with tumour lymphangiogenesis are VEGFC and VEGFD and, to a lesser extent, VEGFA. These structurally related growth factors are secreted by tumour cells as well as stromal cells, including cancer-associated fibroblasts and macrophages [52, 89, 93]. The secretion of VEGFC/VEGFD correlates with lymphatic metastasis in various cancers such as breast and prostate cancer, which are prone to metastasis [1]. VEGFC overexpression induces the enlargement of tumour-associated lymphatic vessels and induces intercellular gaps which increase lymph flow and facilitate the intravasation of tumour cells into the lymphatics, respectively [97, 104]. Some studies have suggested that primary tumours can induce neo-lymphangiogenesis in the sentinel lymph node even before the arrival of metastatic cells, thereby providing a 'pre-metastatic niche' that may support the survival of incoming metastatic cancer cells [44, 45, 48, 66, 108]. This was shown in transgenic mice overexpressing VEGFA or VEGFC [44, 45]. VEGFD also contributes to the provision of a 'pre-metastatic niche' by inducing the dilation/enlargement of the collecting lymphatics, resulting in increasing lymph flow. This has been shown in a mouse model of VEGFD overexpressing tumours, in which the production of prostaglandins by the collecting lymphatic endothelium is altered leading to inhibition/blocking of smooth muscle cell contraction in these vessels [58].

VEGFC and VEGFD mediate their effects on both physiological and tumour lymphangiogenesis by binding to their cognate receptors VEGFR3 and NRP2. VEGFC/VEGFR3/NRP2 signalling is important for the proliferation, migration and survival of lymphatic endothelial cells [103]. Blocking VEGFR3 signalling has been shown to inhibit tumour lymphangiogenesis as well as lymph node metastasis

in animal models [25]. VEGFC can also bind to VEGFR2, which is expressed by both blood and lymphatic endothelial cells [56]. This could represent an alternative pathway that VEGFC can induce lymphangiogenesis and potentially also angiogenesis in tumours.

In the vascular system, NRP2 expression is restricted to the veins and lymphatic vessels [119]. NRP2 binding to ligand leads to the internalisation of NRP2 along with VEGFR3, resulting in increased affinity of LECs towards VEGFC gradients during lymphatic development [59]. NRP2 has been reported to mediate VEGFC-induced lymphatic sprouting alongside VEGFR3 by modulating lymphatic endothelial tip cell extension and preventing tip cell stalling and retraction during vascular sprout formation [116]. NRP2 expression is upregulated during tumour lymphangiogenesis, and an anti-NRP2 antibody was shown to reduce tumour lymphangiogenesis and metastasis to the sentinel lymph node and distant organs [15]. More recently, NRP2 was reported to mediate tumour lymphangiogenesis in colorectal carcinoma via activation of integrinα9β1/FAK/Erk signalling independent of the VEGFC/VEGFR3 signalling pathway [79]. NRP2 has also been shown to mediate anti-lymphangiogenic effects in tumours, thereby playing a protective role against tumour metastasis, when mediating signalling by members of the semaphorin family of ligands. A recent study by Mumblat et al. reports that furin cleavage-resistant semaphorin-3C (sema3c) can induce the collapse of the cytoskeleton of LECs in a neuropilin-2-, plexin-D1-, and plexin-A1-dependent manner [74]. This effect is not seen with cleaved sema3C (p65-sema3C). Mumblat et al. generated an active point mutated furin cleavage-resistant sema3C and found that tumours derived from LM2–LM4 cells expressing this recombinant sema3c, implanted in mammary fat pads, grew at a slower rate, had reduced numbers of blood vessels and lymph vessels and metastasised much less effectively to lymph nodes. Semaphorin-3F (sema3F) has also been shown to play a protective role against head and neck squamous cell carcinoma (HNSCC) [21]. Sema3F re-expression in orthotopic HNSCC metastasis mouse models was shown to reduce lymphangiogenesis and lymph node metastasis in these mice, and Sema3F signalling in LECs predominantly required NRP2. NRP1 has not been directly implicated in tumour lymphangiogenesis but has been shown to play an important role in normal lymphatic development. A study by Bouvrée et al. which utilised a mouse model with a mutation in the semaphorin-3A (Sema3A)-binding domain of NRP1, a semaphorin-3A global knockout and a plexin-A1-deficient mouse model reported a direct role for Sema3A-Nrp1-plexin-A1 signalling in regulating lymphatic valve development [12]. For a more detailed review on NRP function in lymphatic development, see Ochsenbein et al. [78] and Chap. 2.

10.8 Role of NRPs in Tumour Immunomodulation

NRP1 plays roles in tumour progression beyond mediating tumour angiogenesis and tumour invasion. As described in Chap. 5, NRP1 plays a role in the immune system and recently has been described to play a role in immune modulation of tumours. This chapter will focus on the role of NRPs in the emerging area of

"cancer immunity" (Fig. 10.2). The interplay between cancer and the host immune system is a dynamic process, sometimes termed cancer immunoediting, which shapes the immunogenicity of developing tumours. Three sequential phases of cancer immunoediting have been proposed, elimination, equilibrium and escape, and these phases represent a continuum of the interplay between tumour and immune system, shifting between elimination, equilibrium and escape depending on the state of the immune system and inherited or acquired properties of the tumour cells [23]. The development of clinical cancer is in part the consequence of the tilted balance between host immunity and immune tolerance/suppression. Multiple pathways of suppression are at play in tumour microenvironments, including macrophages, regulatory T cells (Tregs), regulatory B cells (Bregs), myeloid derived suppressor cells (MDSCs), plasmacytoid dendritic cells (pDCs) and molecules such as checkpoint inhibitors. NRP1 is expressed on macrophages, Tregs and dendritic cells; thus, it is a candidate molecule in eliciting immune tolerance leading to cancer development and progression.

Studies of CD4+CD25+FOXP3+ Tregs, previously called suppressor T cells, have yielded important insights into the role of NRP1 in tumour immunity. Tregs can either directly contact cytotoxic CD8+ T cells or indirectly do so by secretion of immune suppression cytokines such as TGF-β and interleukin 10 (IL-10) to inhibit the activation and proliferation of T effector cells. Before we describe NRP1 function, it should be noted that NRP1 expression patterns between human and mice T lymphocytes appear to be different. NRP1 is expressed on a majority of murine

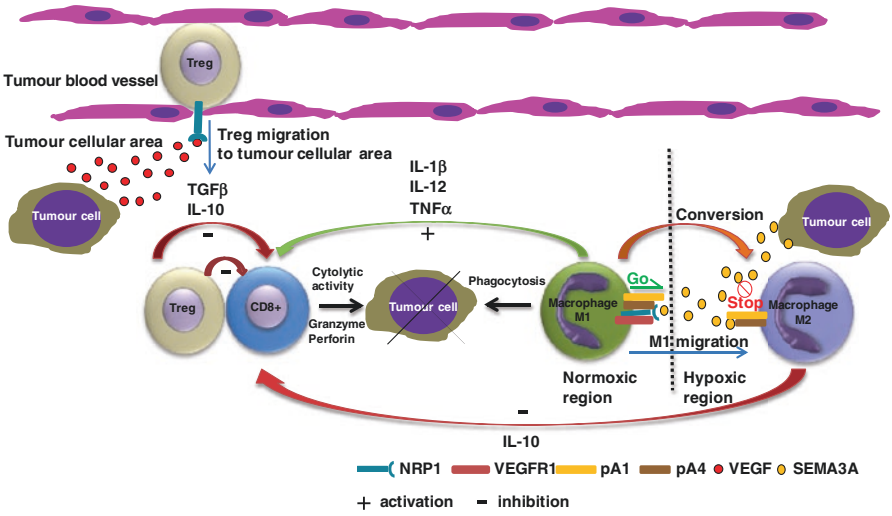

Fig. 10.2 The role of *NRP1* in cancer immunity. Tumour-secreted *VEGF* mediates *NRP1*-expressing Treg migration into tumour, where Tregs either via direct contact or secretion of inhibitory factors such as *TGF-β* and *IL-10* suppress *CD8* T-cell antitumour activity. *NRP1*-expressing *M1* macrophages in normoxic region migrate towards *SEMA3A* secreted by tumour cells in hypoxic region where they lose *NRP1* and convert into *M2* macrophages to suppress anticancer immune response. NRP1 also mediates *M1* to *M2* macrophages conversion

Tregs—up to 70 % of circulating Tregs, whereas it is largely downregulated in tTregs derived from the human thymus [72] and in human pTregs derived from peripheral immune organs in healthy state, although NRP1+ pTregs have been identified in lymph nodes from patients with inflammation [25, 117]. However, several studies show that NRP1 is upregulated in Tregs in human cancer. Thus, it has been reported that NRP1 was significantly upregulated on Tregs isolated from the peripheral blood of chronic lymphocytic leukaemia (CLL) patients in comparison with healthy donors [85]. Chaudhary's group showed that NRP1 was upregulated on Tregs isolated from the peripheral blood of patients with pancreatic adenocarcinoma and colorectal cancer with liver metastasis compared with healthy donors [16]. In metastatic melanoma patients, there was a significant increase in NRP1 expression in tumour-infiltrating CD4+ T cells in comparison with peripheral blood CD4+ T cells [49]. In addition, NRP1 expression in Tregs isolated from metastatic tumour-draining lymph nodes (TDLN) was significantly higher than in metastasis-free lymph nodes in cervical cancer [7]. Interestingly, several lines of evidence indicate that NRP1+ Tregs were reduced after clinical treatment. Reduction of NRP1+ Treg levels was observed in TDLN of patients with cervical cancer following preoperative chemoradiotherapy, and this effect showed a good correlation with the reduction of tumour mass [7]. Piechnik and colleagues found that a significant reduction of NRP1+ Tregs from the peripheral blood of CLL patients followed treatment with the anti-angiogenic drug thalidomide [85], suggesting that the reduced level of the chemoattractant VEGF may result in less NRP1+ Treg migration towards the tumour. Collectively these findings suggest that (1) Treg elimination enhances the generation of T effector cells mediating the destruction of the cancer cells; (2) targeting NRP1 is one of therapeutic approaches to prevent Treg infiltration into tumours; (3) NRP1 may be a useful proof of concept pharmacodynamic (PD) biomarker to assess patient's response following immune, chemoradiation and targeted therapies.

Direct evidence of functions for NRP1 in Tregs came from Hansen and colleagues, who proposed that NRP1 mediates Treg migration towards VEGF cues secreted by tumour and stromal cells in the microenvironment. They generated CD4+ T-cell-specific NRP1 knockout (KO) mice and demonstrated a significant inhibition of tumour immune escape in various transplantation models and in a spontaneous, endogenously driven melanoma model associated with a strong reduction of tumour growth and increased tumour-free survival. They found a significant reduction of tumour-infiltrating Tregs accompanied by increased activation of CD8+ T cells within these tumours. Importantly, the impaired tumour growth in NRP1 CD4+ T-cell-specific KO mice could be restored by adoptive transfer of NRP1+ Tregs from wild-type mice. Furthermore, it was also reported from the same group that NRP1 is not essential for the immune suppression activity in vitro, since when sorted CD4+CD25+ T cells (Tregs) and CD4+ CD25 T cells (T effector) were cocultured, there was no difference in the suppression activity of cells isolated from NRP1 KO mice and wild-type mice. These results indicated that the VEGF/NRP1 axis is a key player of Treg infiltration into the tumour site resulting in a diminished CD8+ cell antitumour immune response and enhanced tumour progression [43].

The function of CD8+ cells is to detect cellular abnormality and to protect the host from pathogenic invasion and malignancy. In addition to Treg-mediated tolerance, another mechanism of the control of adaptive immunity is peripheral T-cell tolerance, which is critical in preventing pathological immune response mediated by excessive CD8+ T-cell activity and is especially important to limit the activation of self-reactive T cells harboured in the periphery of healthy individuals [11]. However, this tolerance also forms a strong barrier to inhibit antitumour immune activities since many cancer antigens are also expressed in healthy tissue [91]. Jackson and colleagues reported that tumour-infiltrating NRP1+ CD8+ T cells were increased in metastatic melanoma patients in comparison with healthy donor peripheral blood cells. Furthermore, they found that NRP1 expression was induced in tolerant self-reactive CD8+ cells in mouse, but was dispensable for the tolerant phenotype since NRP1 KO mice displayed the same functional defects as wild-type self-reactive T cells [49]. Several groups reported that CD8+ T cell tolerance was partially regulated by the co-inhibitory surface markers PD-1 and CTLA-4 [10, 18]. However it is not clear if NRP1 co-expresses with and/or acts like other immune checkpoint inhibitors such as CTLA-4, PD-1 and PD-L1 and contributes to the inhibitory function. It was also reported that NRP1 was one of the most upregulated genes in exhausted CD8+ cells after chronic infection [111]. However it is still unknown if NRP1 expressing CD8+ cells also represent exhausted CD8+ cells in cancer.

Natural killer T (NKT) cells are true T cells, which play a major role in regulating immune responses by bridging the innate and adaptive immune systems. Type I NKT cells, also called invariant NKT (iNKT) cells, express a semi-invariant T-cell receptor (TCR) recognising lipid antigens presented by the nonclassical MHC class molecule CD1d [107]. iNKT cells have been shown to have a role in tumour immunosurveillance. In general iNKT cell numbers are decreased in solid tumours including melanoma, colon, lung and breast cancers, as well as head and neck squamous cell carcinoma [109]. Increased iNKT cells in tumour are associated with a better prognosis, and this may be because iNKT cells produce large amounts of pro-inflammatory cytokine IL-17 [71]. It was found that NRP1 was expressed on thymic recent emigrant iNKT cells, but not on long-lived mature NKT cells [71]. However, ligands and functions of NRP1 on iNKT cells remained to be explored. More details of NRP1 in NKT cells are discussed in Chap. 5.

Tumour-associated macrophages (TAMs) are tissue-resident cells that differentiate from circulating monocytes in peripheral blood and are a major cellular component of murine and human tumours. It has been reported that in most human cancers, macrophage infiltration is correlated with aggressive diseases and poor prognosis. However in colon, gastric and prostate cancers, macrophage infiltration resulted in a better outcome [63]. There are two opposing phenotypes in TMAs. The phenotype of TAMs is regulated by specific tumour-derived chemokines and cytokines that polarise macrophages to a proimmune 'M1' phenotype via toll-like receptor (TLR) agonists and Th1 cytokines (e.g. interferon gamma (IFNγ) and tumour necrosis factor alpha (TNF-α) or to an immunosuppressive/proangiogenic 'M2' phenotype mediated by Th2 cytokines, e.g. IL-4, IL-13 and IL-10 [90, 92]. M1 macrophages can act in a proimmune manner directly, by phagocytosis, and indirectly, by

production of IL-1b, IL-12 and TNFα and reactive molecular species and by presenting antigen via major histocompatibility complex (MHC) class II molecules to activate CD8+ cells to destroy cancer cells. In contrast, M2 macrophages can enhance production of the anti-inflammatory cytokine, IL-10, to reduce expression of pro-inflammatory cytokines; they amplify metabolic pathways that can suppress adaptive immune responses; and they upregulate cell surface scavenger receptors, such as mannose receptor (MRC1/CD206) mechanisms that suppress immunity and promote angiogenesis in favour of tumour growth [92].

Recently Casazza and colleagues found that there are different niches within a tumour, which can be categorised either normoxic or hypoxic regions. In the hypoxic region, hypoxia-induced semaphorin-3A (Sema3A) acts as a chemoattractant for M1 macrophages by triggering VEGFR1 phosphorylation through a heterocomplex of NRP1 and the Sema3A receptor, plexin-A1 (pA1)/plexin-A4 (pA4). Once M1 macrophages arrived in the hypoxic region, NRP1 expression was repressed, and M1 macrophages retained in the hypoxic region where they were educated to become M2 macrophages, allowing them to exert immunosuppression and induction of angiogenesis, thus promoting tumour growth. In NRP1-repressed macrophages, Sema3A continued to regulate M2 in an NRP1-independent manner by eliciting pA1/pA4-mediated stop signals, which retained M2 macrophages inside the hypoxic niche. In macrophage-specific NRP1 KO mice, M1 macrophages were trapped in normoxic regions, where they maintained their immune response and prevented the release of angiogenic factors, hence inhibiting tumour growth and metastasis. Thus the migration of macrophages from normoxic to hypoxic regions of the tumour microenvironment is precisely controlled by the Sema3A/NRP1/VEGFR1/pA1/pA4 signalling pathway. Casazza's study also revealed that Sema3A, not VEGF, is the chemoattractant for macrophage migration and that there were no additive, synergistic or antagonistic, effects when both were added together in an in vivo subcutaneous macrophage chemotaxis assay [14]. Interestingly it has been reported that Sema3A binds to NRP1 and recruits VEGFR1 to induce neural progenitor cell repulsion, and prolonged interaction of Sema3A and NRP1 induces apoptosis in these cells. Furthermore, VEGF antagonised these effects by directly competing with Sema3A binding to NRP1 [5]. However, it is unclear how the Sema3A/NRP1 axis plays two opposite roles in these different cell types.

Unlike NRP1 which acts predominantly as a co-receptor for Sema3A, NRP2 binds to Sema3B, C, D and F [87]. It has been reported that the Sema3F/NRP2 axis mediates repulsive migration on human thymocytes and lymphoblastic leukaemia/lymphoma tumour cells [69]. Otherwise, there are limited reports on the role of NRP2 in the immune system in the literature, and details are described in Chap. 5.

Taken together, the work considered above indicates that NRP1 guides Treg and macrophage migration towards the guidance cues, VEGF and Sema3A, similar to its roles in endothelial migration and neuronal migration/repulsion/outgrowth in development. NRP1 appears to play an immunosuppressive role in cancer and to promote tumour progression. Therefore, NRP1 is of growing interest as a target for anticancer therapy, potentially with a triadic mode of action: anti-angiogenesis, immunomodulation and inhibition of tumour metastasis.

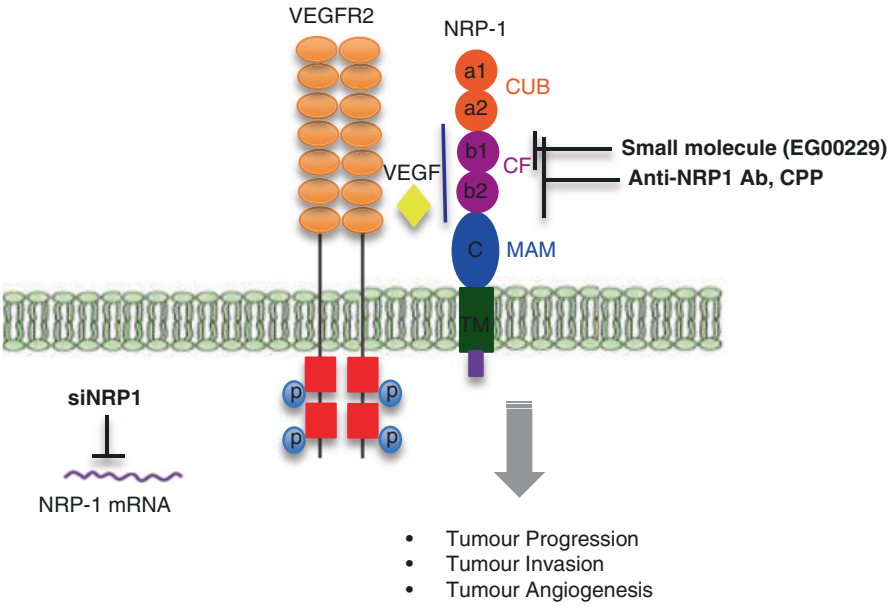

Fig. 10.3 Neuropilin structure and strategies for therapeutic targeting in cancer

10.9 Therapeutic Targeting of Neuropilins in Cancer

Because of its role in mediating several aspects of tumour progression, NRPs are emerging as key targets for development of anticancer therapeutics. A key advantage of targeting NRPs is the expected reduced impact on vascular homeostasis, in contrast to VEGF-targeted therapies such as bevacizumab (Avastin), which are associated with a range of cardiovascular side effects. This chapter provides an overview on the current strategies being explored for development of anti-NRP therapeutics for cancer (Fig. 10.3).

10.10 Therapeutic Targeting of Neuropilin-2 in Tumour Lymphangiogenesis

The VEGFC/VEGFD-VEGFR3-NRP2 axis is the most well-established pathway known to function specifically towards the induction of lymphangiogenesis in pathological conditions such as cancer. A number of studies have shown that targeting this signalling pathway by blocking either VEGFC/VEGFD, VEGFR3 or NRP2 function, using antibodies or soluble constructs, can reduce tumour lymphangiogenesis and inhibit tumour metastasis in experimental models [114].

A monoclonal antibody against the Nrp2 co-receptor was shown to bind exclusively to the b1/b2 domains of Nrp2, thereby directly blocking VEGF binding

without affecting semaphorin binding to the CUB (a1/a2) domains [15]. Caunt et al. showed that by preventing VEGFC binding to Nrp2, this anti-Nrp2 antibody blocked lymphatic endothelial cell migration but not proliferation and resulted in a reduction of VEGFC-driven lymphangiogenesis, but without affecting vascular permeability in vivo. More importantly, using two mouse models of breast adenocarcinoma (66C14) and rodent glioblastoma (C6), they showed that anti-Nrp2 treatment led to a reduction in tumour lymphangiogenesis and inhibited the development of metastasis to sentinel lymph nodes and distant organs in these mice. These data imply that targeting VEGFC binding to Nrp2 may be a promising tool to block tumour metastasis via targeted inhibition of tumour lymphangiogenesis.

10.11 Anti-NRP1 mAbs

Research has been done by Genentech on the development of antibodies that target the b1 domain of NRP1, thus blocking VEGFA binding to NRP1 and inhibiting VEGFR2-NRP1 complex formation, VEGF-induced migration and sprouting of endothelial cells. Pan et al. [80, 81] showed that an antibody targeted to the b1 domain of NRP1 caused a range of effects in endothelial cell cultures, including inhibition of VEGFR2 complex formation, VEGF-induced migration and vascular sprouting, reduced angiogenesis in a neonatal retinal neovascularisation model and inhibition of tumour growth and tumour vascularisation in mouse xenograft models [39, 80]. NRP1 inhibition, on its own, had a small effect on tumour growth, but the combination of anti-NRP1 antibody with bevacizumab had an additive effect, leading to a stronger reduction of tumour growth. These findings suggested that the combination of anti-NRP1 and anti-VEGF agents could improve the survival of patients with advanced malignancies [39, 80, 84].

Genentech has generated several additional anti-NRP monoclonal antibodies that block NRP1 interactions with VEGFA and semaphorins [2, 13, 80]. In 2014, Genentech conducted a phase I clinical trial using an anti-NRP1 antibody, MNRP1685A, in patients with advanced solid tumours. Results showed that MNRP1685A was well-tolerated as a single agent, but had modest clinical activity. MNRP1685A was also used in combination with bevacizumab and paclitaxel in patients with advanced solid tumours [110] and, when co-administered with bevacizumab, caused a high rate of clinically significant proteinuria, resulting in the cessation of the phase I clinical trial [83].

10.12 Small Interfering RNAs or microRNAs

Small interfering RNAs are small pieces of double-stranded RNA that can be used to ‘interfere’ with the translation of proteins by binding to and promoting the degradation of messenger RNA (mRNA) at specific sequences, thus inhibiting the production of specific proteins. siRNA has been used to target NRP1 resulting in reduction of human tumour growth, angiogenesis and metastasis formation in

models of hepatocellular carcinoma [9, 88], acute myeloid leukaemia [67] and lung cancer [47].

NRP1 is also involved in targeting several microRNAs (miRNAs) that are known to be involved in angiogenesis and invasion: miR-9, miR-181b and miR-320 (Wu et al. [115] #75, Zhang et al. [121] #74). MicroRNAs (miRNAs) have been implicated in regulating diverse cellular pathways, and there is evidence that various miRNAs function as oncogenes or tumour suppressors. Zhang et al. in 2012 observed that miR-320a may suppress the invasion and metastasis of colorectal cancer (CRC) by directly binding to the 3'UTR of NRP1. Thus, miR-320a might work as a novel potential marker to identify CRC patients that are at an elevated risk for developing liver metastasis [121]. These findings support the possible development of siRNA and microRNA-based agents as anti-angiogenic and/or anticancer drugs.

10.13 Cell-Penetrating Peptides

Another approach to NRP1-targeted therapy is cell-penetrating peptides (CPPs). CPPs express a C-terminal consensus sequence (R/KXXR/K), referred to as the C-end rule (CendR) motif that interacts with the b1/b2 domain of NRP1 [100, 105]. This interaction induces the internalisation of the CPP into NRP1-expressing cells via an endocytic mechanism. Once inside NRP1-expressing cells, CPPs are able to target NRP-1 expressing tissues [105]. Importantly, CPPs can also be modified in order to create tumour-penetrating peptides (TPPs) and be able to deliver drugs into tumours [106]. As NRP1 is expressed in several cancer types, co-administration of TPPs with cancer drugs is emerging as an attractive approach as it could allow cell internalisation of high-molecular-weight drugs that cannot cross the plasma membrane and selective targeting of tumour tissues [37, 101].

10.14 Small-Molecule Inhibitors

There has been growing interest in developing inhibitors of VEGFA interactions with NRP1 [54]. Jia et al. developed a peptide antagonist of VEGF binding to NRP1 (EG3287), capable of binding specifically to NRP1 through the b1 domain. Thus, EG3287 inhibited VEGFA165 binding to endothelial cells and to breast carcinoma cells endogenously expressing NRP1, but not KDR or Flt-1. This study also demonstrated that the C-terminus of VEGFA encoded by exon 8 plays a key role in EG3287 (and VEGFA165) binding to NRP1. In particular, a critical role is played by the C-terminal arginine encoded by exon 8 in VEGFA binding to the NRP1 b1 domain [54]. EG3287 also antagonised VEGFA165 binding to NRP1 in human tumour cells and enhanced their sensitivity to cytotoxic chemotherapeutic agents [55]. Another peptide based on a modification of EG3287 (EG00086) reduced the viability of A549 lung cancer cells and, similar to EG3287, enhanced the cytotoxicity of the chemotherapeutic agents, 5-fluorouracil (5-FU) and paclitaxel [53]. Based on

structure-function analysis of EG3287, Jarvis et al. developed the first small-molecule inhibitor for NRP1, called EG00229 [51]. In this study, data generated by analysis of NRP1 b1 domain mutants, X-ray crystallography and NMR spectroscopy of NRP1 bound to EG00229 showed that EG00229 is able to bind the NRP1 b1 domain at a defined site and thereby reduce VEGFR2 phosphorylation in endothelial cells and as cell migration in vitro [51]. In addition, EG00229 enhanced the potency of the chemotherapeutic drugs paclitaxel and 5-fluorouracil in tumour cells [51].

Recently, two groups reported antitumour activity of EG00229 in vivo. Grun et al. performed a study using a subpopulation of epidermal cancer stem cells (ECS cells), which form rapidly growing, invasive and highly vascularised squamous cell carcinomas. These cells produce high levels of VEGFA, which is important for aggressive tumour growth, and, accordingly, treatment with bevacizumab reduces tumour vascularity and growth. However, these cells lack VEGFR1 and VEGFR2, and therefore VEGF signalling appears to occur principally via NRP1. EG000229 treatment of tumour-burdened mice reduced tumour spheroid size and tumour invasion and attenuated tumour growth [38]. These findings suggest that antagonism of VEGF binding to NRP1 may inhibit tumour growth via a mechanism independent of VEGFR activation.

Miyauchi and colleagues examined effects of NRP1 inhibitors in glioma models. NRP1 is expressed on glioma-associated microglia and macrophages (GAMs) from glioma patients of varying grades. Genetic ablation of NRP1 specifically in mouse CNS microglia and macrophages delayed glioma progression accompanied by reduced tumour growth pace, longer survival period, less vascularity and increased M1/M2 GAM ratio. Strikingly, the inhibitory effect of genetic loss of NRP1 in these cells was reproduced by dosing glioma-bearing wild-type mice with EG00229 [73]. These two studies demonstrate proof of concept in targeting NRP1 with small-molecule inhibitors as a potential therapy for suppression of cancer progression in vivo.

10.15 Summary

Both NRP1 and NRP2 play important roles in the regulation of vascular development during tumour progression, but whereas NRP1 is primarily important for angiogenesis, NRP2 plays a key role in tumour lymphangiogenesis. Recent studies implicate NRP1 as a mediator of cancer immunomodulation functioning as a regulator of tumour-infiltrating lymphocytes, including T-regulatory cells and tumour-associated macrophages. Due to its pleiotropic effects in tumour progression, NRP1 and NRP2 provide promising molecular targets for cancer therapies. Though at the time of writing this chapter we are not aware of any NRP-targeted therapeutic in ongoing clinical trials, several approaches to targeting NRP1 in cancer are currently being developed, and preclinical proof-of-concept studies suggest that some of these may offer future promise as anticancer therapeutics.

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The Role of Neuropilins in TGF-β Signaling and Cancer Biology

11

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Abstract

The neuropilins (Nrps) interact with a number of growth factors (GFs) and/or their receptors. This includes vascular endothelial growth factor (VEGF), transforming growth factor β1 (TGF-β1), hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF), and epidermal growth factor receptor (EGFR). These interactions can involve one or both homologues, Nrp1 and Nrp2, and generally enhance the response to these GFs. Here, we will review non-VEGF interactions, with emphasis on TGF-β. We found that both Nrp1 and Nrp2 bind active TGF-β1 and its latent form denoted latency associated peptide (LAP)-TGF-β1. The Nrps also bind to the signaling TGF-β receptors (TβRI and TβRII) and the co-receptor betaglycan (TβRIII). Studies by us and others established that Nrp1 and Nrp2 augment TGF-β canonical (Smad2/3-dependent) or noncanonical signaling. This was observed in fibroblasts, hepatic stellate cells, lymphocytes, endothelial cells, cardiomyocytes, and several types of cancer cells. TGF-β1-mediated effects that were reduced by Nrp1 or Nrp2 knockdown and/or enhanced by their overexpression include collagen production, epithelial-to-mesenchymal transition (EMT), endothelial-to-mesenchymal transition (EndMT), cancer cell activities (e.g., migration and invasion), and regulatory T-cell-mediated suppression. TGF-β markedly upregulated the expression of Nrp2 on cancer cells, which promoted EMT. Conventional CD4+ T lymphocytes induced to express Nrp1 acquired immunosuppressive activity. These effects appear cell type and context dependent, and in some cases Nrps did not enhance or reduced canonical signaling. In conclusion, the Nrps impact on the stimulatory capacity of TGF-β and other GFs, and this is relevant to angiogenesis, wound healing, cancer biology, immunity, and other processes. As such, the Nrps are important targets for drug development.

11.1 Introduction

Neuropilin-1 (Nrp1) and its homologue neuropilin-2 (Nrp2) are co-receptors that enhance responses to several growth factors (GFs) [1, 2]. The neuropilins (Nrps) are expressed by endothelial cells, vascular smooth muscle cells, neurons, hepatocytes,

melanocytes, osteoblasts, immune cells (regulatory T cells and dendritic cells), some epithelial cells, and several other cell types [3–5]. In addition, the Nrps are frequently expressed by cancer cells of several organs (e.g., skin, lung, breast, kidney, pancreas, GI tract, and brain), and this correlates with a poor prognosis [1, 2, 6–9]. As detailed in other chapters, the Nrps are best known as co-receptors for class 3 semaphorins (SEMA3) and several members of the vascular endothelial growth factor (VEGF) family. However, studies performed over the last decade have revealed many additional interactions. Nrp1 interacts with transforming growth factor β1 (TGF-β1) [10–13], hepatocyte growth factor (HGF) [14–16], platelet-derived growth factor (PDGF) [16–18], epidermal growth factor receptor (EGFR) [19], fibroblast growth factors (FGFs) [14], L1-CAM [20, 21], galectin-1 [22], Glut-1 [23], fibronectin [24], and integrins [24–27] (Table 11.1). In fact, new Nrp ligands are constantly being reported. In the case of GFs, the Nrps characteristically interact with both the GF and its receptors and in most cases act as co-receptors to enhance responses (Fig. 11.1). Interestingly, the Nrps also contribute to signaling in the Hedgehog pathway, which is linked to natural stem cell and cancer stem cell (CSC) differentiation and survival. The molecular basis for such a large number of interactions is largely unknown, but crystal structure and binding studies have provided valuable information. Peptides that have affinity for the Nrps have been studied extensively [28–41], and many bind through a consensus C-terminal motif mimicking the VEGF sequence, denoted the C-end rule (CendR). The CendR is relevant to GF binding at least in some cases, including latent TGF-β, and applicable to cancer

Table 11.1 Non-VEGF/non-SEMA3 interactions with the neuropilins

Ligand	Nrp1	Nrp2	References
TGF-β1 and LAP TβRI and TβRII	+ +	+ +	[10–13, 55]
HGF and c-Met	+	+	[14–16]
PDGF and PDGFR	+	?	[16–18]
FGF-1, 2, 4, 7 FGF receptor-1	+ +	? ?	[14]
EGFR	+	?	[19]
Integrins	+	+	[24–27, 132]
Fibronectin	+	?	[24]
Galectin-1	+	?	[22, 71]
L1-CAM	+	+	[20, 21]
Glut-1	+	?	[23]
CendR and other peptides	+	+	[28–41, 98–102]

+ the ligand binds to neuropilin, ? unknown/not reported

Abbreviations: *CendR* C-end rule peptides (R/K-X-X-R/K motif at C-terminal), *c-Met* hepatocyte growth factor receptor, *EGFR* epidermal growth factor receptor, *HGF* hepatocyte growth factor, *Nrp1* neuropilin-1, *Nrp2* neuropilin-2, *PDGF* platelet-derived growth factor, *PDGFR* PDGF receptor, *SEMA3* class 3 semaphorin, *TGF-β* transforming growth factor-β, *TGF-βRI* TGF-β receptor type 1 (also denoted ALK5), *TGF-βRII* TGF-β receptor type 2, *VEGF* vascular endothelial growth factor

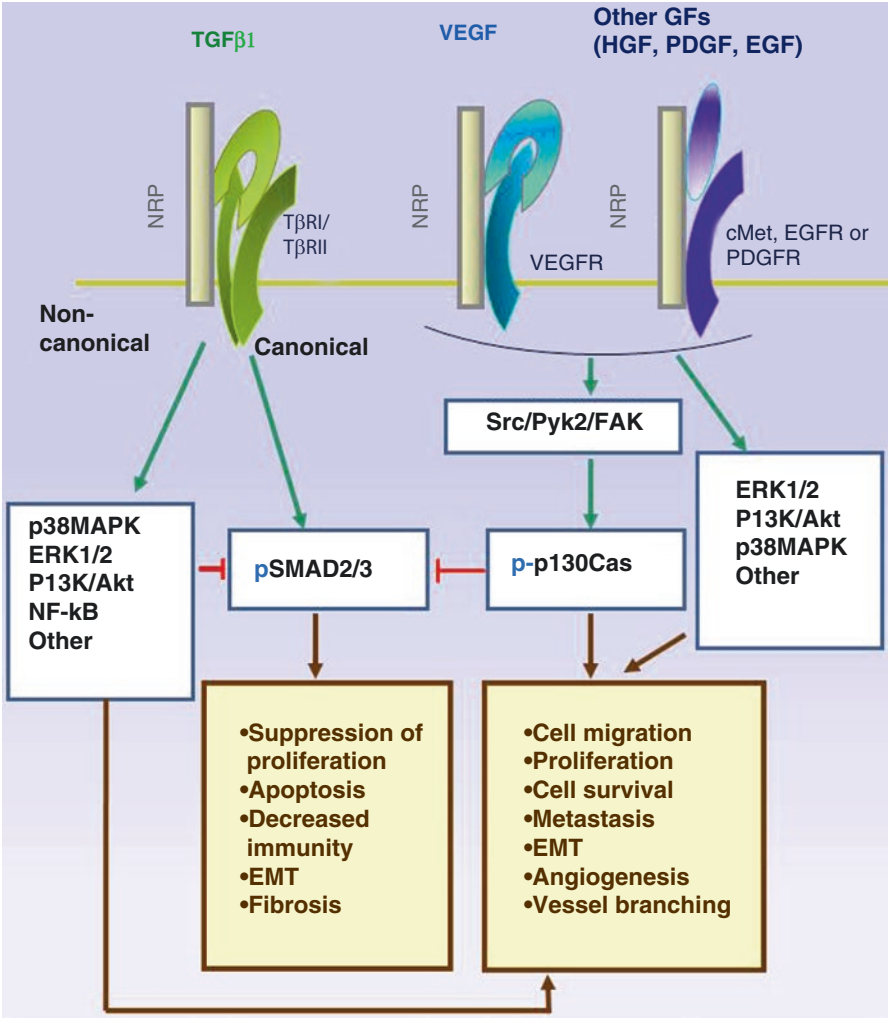

Fig. 11.1 Nrp/GF interactions, signaling, and cellular response. The Nrps interact with several growth factors and/or their receptors, i.e., TGF-β1, VEGF family, HGF, PDGF, and EGFR. This figure presents a hypothetical model of interacting signaling pathways and the ensuing cellular response. TGF-β mediates antiproliferative and immunosuppressive activities through Smad2/3 canonical signaling. TGF-β noncanonical signaling (or other GF pathways) can either antagonize or cooperate with Smad2/3 signaling depending on the context. As an example, p130Cas is phosphorylated as a result Nrp/GF/receptor engagement and signaling and can interfere with Smad2/3 signaling while enhancing noncanonical signaling. *Abbreviations*: *c-Met* hepatocyte growth factor receptor, *EMT* epithelial-to-mesenchymal transition, *FAK* focal adhesion kinase, *GF* growth factor, *HGF* hepatocyte growth factor, *p-* phosphorylated form, *p130Cas* Crk-associated substrate, *EGF* epidermal growth factor, *EGFR* epidermal growth factor receptor, *PDGF* platelet-derived growth factor, *PDGFR* PDGF receptor, *Pyk2* proline-rich tyrosine kinase 2, *TGF-β* transforming growth factor-β, *TGF-βRI* TGF-β receptor type 1, *TGF-βRII* TGF-β receptor type 2, *VEGF* vascular endothelial growth factor, *VEGFR* VEGF receptor

therapy. In this chapter, we will focus mainly on Nrp/TGF-β interactions, and related signaling, particularly in terms of immunologic, cardiovascular, and cancer-related effects.

11.2 TGF-β Signaling

TGF-β signaling and its regulation, as well as interactions with other signaling pathways, are highly complex, and only a brief overview is provided here. There are three TGF-β isoforms (TGF-β1 is the most abundant), which are homodimers that bind to the same signaling receptor. The major components of this receptor [42] are TβRI (or ALK5) and TβRII. TGF-β binds to TβRII and TβRI, resulting in the formation of a serine/threonine kinase complex. This signaling complex consists of two mature TGF-β molecules (homodimers), two TβRI, and two TβRII chains (six units) [43, 44]. In the canonical pathway [45], TβRII phosphorylates TβRI, which in turn phosphorylates Smad2 and Smad3 (receptor-activated Smads [R-Smads]) in their C-terminal segment. Phosphorylated Smads 2 and 3 form a heteromeric complex with Smad4 (the common or Co-Smad). This Smad complex translocates into the nucleus, binds to DNA, and regulates transcriptional events. A third TGF-β-binding chain denoted betaglycan (or TβRIII) is also expressed by many cell types. It is thought that this chain captures TGF-β and presents it to TβRII, and this primarily enhances the response to TGF-β2, which has a lower affinity for TβRII than either TGF-β1 or TGF-β3 [46]. However, this interpretation is complicated by the observation that in some studies, betaglycan suppressed the response to TGF-β [47].

The ALK5/Smad2,3 pathway outlined above represents the typical route of signaling; however, endothelial cells can also signal through the alternative ALK1/Smad1,5,8 pathway [48, 49]. In this case, ALK1 couples with TβRII to phosphorylate Smads 1, 5, and 8, which form a complex with the common Smad (Smad4) to mediate downstream signaling. This pathway is intricate and requires ALK5, which recruits ALK1 into the TGF-β receptor complex, and activates ALK1 through its kinase action, before Smad1,5,8 signaling can occur. Furthermore, the co-receptor endoglin, which is expressed primarily by endothelial cells, interacts with the receptor complex and favors a shift from ALK5/Smad2,3 signaling to the ALK1/Smad1,5,8 signaling [50]. Both pathways are active in endothelial cells and appear to be antagonistic. Generally, the Smad2,3 pathway suppresses endothelial cell migration and proliferation, whereas Smad1,5,8 has the opposite effect [48–50]. However, there have been contradictory reports on the endothelial response induced by these pathways [49].

Betaglycan and endoglin have long been considered co-receptors for TGF-β [46], but despite numerous investigations, their role and function are not fully understood. The identification of Nrp1 and Nrp2 as co-receptors is much more recent. Neuropilins, betaglycan, and endoglin are expressed by endothelial cells and various other cell types and have affinity for TGF-β and its receptors [46]. They also interact with other receptors and share some features such as large extracellular domains, short cytoplasmic segments that bind synectin/GIPC, and ectodomain

shedding. It seems likely that these co-receptors interact as part of the TGF-β receptor complex and in other ways, but at least as far as the neuropilins are concerned, these interactions have not been studied.

In addition to the pathways outlined above, TGF-β receptor can activate several noncanonical (non-Smad2,3-dependent) pathways. The mechanisms initiating noncanonical signaling are not completely elucidated, and there are cooperative and antagonistic interactions with the canonical pathway. Noncanonical signaling occurs through the MAP kinase pathways (ERK, JNK, and p38), PI3K/Akt, and Rho-like GTPases [51, 52]. Of note, TRAF6 interacts with the TGF-β receptor to activate TGF-β-activated kinase 1 (TAK1), which in turn activates the MAP kinase and NF-κB pathways [52]. In some circumstances, the noncanonical pathways inhibit canonical signaling, as discussed below.

11.2.1 Regulation of the TGF-β Response

TGF-β activity is regulated at multiple levels, as recently reviewed [53], such as by microRNAs (miRNAs), TGF-β receptor endocytosis and recycling, co-receptor expression, dephosphorylation by phosphatases, Smad7 (inhibitory Smad), inactivation of Smads by various kinases, as well as ubiquitination and proteasomal degradation of receptors and signaling components. Notably, TGF-β induces Smad7 expression, which competes with R-Smads for interaction with TβRI and inhibits R-Smad phosphorylation. R-Smads can be negatively regulated by phosphorylation, particularly in the linker region and MH1 domain of these molecules. This can be accomplished by MAP kinases (ERK, JNK, p38), cyclin-dependent kinases (CDKs), TAK1, protein kinase C (PKC), and several other kinases. R-Smads are also deactivated by phosphatases that remove C-terminal phosphorylation. Furthermore, TGF-β is secreted in a latent form, as described later, and its activity depends on a number of potential activation processes controlling the availability of this cytokine.

11.3 Nrps Have a High Affinity for TGF-β1 and Its Receptors

Nrp1 and Nrp2 bind both mature (active) TGF-β1 and LAP-TGF-β1 (the small latent complex [SLC]) [10, 11, 13]. The binding sites for these interactions are not fully characterized, but there is overlap with VEGF binding. Indeed, we observed that free LAP, LAP-TGF-β1, and TGF-β1 all competed with VEGF165 for binding to Nrp1. Cells that expressed Nrp1 had an increased ability to capture soluble LAP-TGF-β1 [10, 11]. Nrp1 was found to be a marker for mouse Treg cells [54], and we hypothesized that it might be enhancing regulatory function by capturing TGF-β1. This hypothesis was supported by the finding that CD4+ T-helper cells (that lack Nrp1) acquired regulatory (suppressive) function when coated with Nrp1-Fc and LAP-TGF-β1 [10]. In these experiments, we also found evidence of Nrp1-dependent LAP-TGF-β1 activation, as discussed in another section.

In subsequent studies, we observed that Nrp1 and Nrp2 have affinity for both components of the TGF-β signaling receptor, i.e., TβRI and TβRII [11]. In accord

with our findings, other investigators have reported that Nrp1 interacts with TβRII [12, 55] and Nrp2 interacts with TβRI [13]. Our further studies suggested that the Nrps act as co-receptors for TGF-β. Using a Cignal Smad reporter system, we demonstrated that the Nrps augment TGF-β1-induced Smad2/3-dependent canonical signaling in breast cancer cells [11]. Other investigators similarly found that Nrp1 [12] and Nrp2 [13] enhance TGF-β canonical signaling in other cell types, based on increased R-Smad phosphorylation and/or a TGF-β reporter assay. Taken together, these findings suggest that the Nrps, as cell membrane co-receptors, capture active or LAP-TGF-β1, activate the latent form of the cytokine, and augment TGF-β-mediated receptor signaling.

11.3.1 Nrp1-Dependent Activation of LAP-TGF-β

TGF-β is primarily secreted in the large latent complex (LLC) form, which is produced by the covalent linking of LAP (as part of the SLC) to one molecule of latent TGF-β-binding protein (LTBP) [42, 56, 57]. In some cells, such as regulatory T cells, the LTBP is replaced by a membrane-bound protein denoted GARP [56, 58–61]. Importantly, TGF-β is not covalently linked to LAP in either the SLC or LLC. In the process of activation, mature (active) TGF-β is released, and it can then bind to and activate the TβRI/TβRII receptor complex. The activation of TGF-β can be enacted by several different molecular mechanisms, and this provides a post-secretion and extracellular level of regulation [62–66].

The LTBP chain attaches the LLC to extracellular matrix (ECM) constituents (e.g., fibronectin and fibrillin) [66], which produces a tissue-based reservoir of latent TGF-β. In contrast, GARP retains covalently bound SLC on the cell membrane [56]. The RGD-binding integrins (especially the α_V type) and Nrp1 are other cell membrane molecules that bind LAP-TGF-beta, but in non-covalent fashion. In the case of integrins, LAP is bound at its RGD motif [62–66], and interactions with other proteins result in the release of free TGF-β (activation). Note that TGF-β2, unlike the other isoforms, does not have an RGD motif and is not activated by integrins. The activation of latent TGF-β1 or TGF-β3 by integrins is thought to occur by one of two mechanisms [66]. In some cases (notably $\alpha_V\beta6$), the LLC attached to ECM components binds simultaneously to the integrin. In that configuration, traction forces can induce a conformational change in LAP, which releases active TGF-β. In other cases, typically involving $\alpha_V\beta8$-bound LAP-TGF-β, activation appears to be induced by MMP enzymes.

We hypothesize that because LAP has both neuropilin-binding sites and an RGD motif (in either latent TGF-β1 or TGF-β3), that activation can be induced by a third mechanism. In this case, we postulate that Nrp1 or Nrp2 promote the activation of latent TGF-β after it binds to other molecules. In accord with this, we have observed in bioassays that Nrp1 activates LAP-TGF-β1 attached to either αvβ3 integrin or GARP [11]. This would be most relevant in cancer cells, which express both RGD-binding integrins and neuropilins.

The mechanism by which Nrp1 activates latent TGF-β1 has not been elucidated. However, both Nrp1 and Nrp2 have a b2 domain motif (RKFK) that in soluble

peptide form activates LAP-TGF-β1 [10]. This peptide sequence is closely similar to the ^{94}RKPK sequence of TGF-β1 that binds to LAP and activates LAP-TGF-β1 in soluble peptide form [63]. Remarkably, thrombospondin-1 also has a similar LAP-TGF-β1 activating motif (KRFK) [10, 63]. We speculate these similar basic peptides compete with TGF-β1 for binding to LAP or act by inducing a conformational change in LAP, and this partially or completely releases active TGF-β.

It is important to note that our observations of TGF-β activation were obtained strictly from in vitro assays, and it has not been demonstrated that neuropilins activate TGF-β in vivo. In contrast to our in vitro findings, there is recent evidence that Nrp1 might reduce αvβ8-mediated TGF-β activation. Indeed, Hirota et al. [55] employed biochemical, cell biological, and molecular genetic methods to show that β8 integrin and Nrp1 work together to promote CNS angiogenesis. β8 integrin in the neuroepithelium is thought to activate ECM-bound latent TGF-β. This releases active TGF-β, which then binds to its receptors on endothelial cells. They report that Nrp1 expressed by endothelial cells binds in trans to β8 integrin expressed by the neuroepithelial cells, and this appears to interfere with latent TGF-β activation. Decreased TGF-β action promotes angiogenesis in their CNS angiogenesis models. However, although Smad3 phosphorylation was decreased in the presence of Nrp1 in these experiments, the activation of TGF-β and the availability of active TGF-β in vivo were not examined and cannot easily be determined. Therefore, it is difficult to draw conclusions about activation. The mechanism of TGF-β activation by αvβ8 integrin is not completely clear. It has been postulated to depend on cleavage by the transmembrane metalloproteinase MMP14 [66], which might somehow be blocked by Nrp1, and this question merits further investigation.

11.4 Neuropilins Enhance the Response to TGF-β

The ability of Nrps to enhance TGF-β-induced responses has been demonstrated in several cell types including a hepatic stellate cells (HSCs) [17], mouse embryonic fibroblasts (MEFs) [12, 17], stromal fibroblasts [12], T lymphocytes [10], endothelial cells [67], cardiomyocytes [68], and cancer cells of various types, as reviewed below. In some cases, this was clearly associated with increased TGF-β canonical signaling, as measured by phosphorylation of R-Smads (immunoblotting), and/or luciferase reporter assays of Smad activation [11–13, 67]. At least in some cell types, the promotion of Smad2,3 phosphorylation coincided with decreased Smad1,5 phosphorylation [12]. In some cases, it appears that noncanonical pathways are activated, rather than Smad2,3 [69]. Notably, Nrp/TGF-β interactions have been linked to increased EMT and EndMT, as well as increased cancer cell migration and invasion.

However, Nrp-mediated effects appear to be context dependent, and in some studies, for reasons that remain unclear, Nrp1 expression has been linked to decreased TGF-β pathway activation [55, 70]. This is a relatively recent area of research, and the molecular mechanisms by which Nrps alters responsiveness to TGF-β have not been elucidated and require further investigation.

11.4.1 Enhanced Responses to TGF-β in Fibroblasts and HSCs

In a study of hepatic fibrosis [17], the authors examined whether Nrp1 regulates TGF-β-induced collagen deposition. Nrp1 overexpression in HSCs promoted TGF-β-induced collagen production as determined by immunoblotting and a colorimetric assay. Moreover, MEFs from Nrp1-deficient mice exhibited much reduced collagen deposition. In another study [12], the role of Nrp1 on TGF-β-induced Smad signaling in stromal fibroblasts was investigated. The elimination of Nrp1 enhanced Smad1/5 phosphorylation and downstream responses. In contrast, it decreased Smad2/3 phosphorylation (activation) as demonstrated by immunoblotting, a Smad3-sensitive reporter assay, and reduced α-smooth muscle actin (α-SMA). Thus, Nrp1 expression favored canonical Smad2,3 signaling. In this model, the Smad1,5 and Smad2,3 pathways appear to be antagonistic, with the former favoring a quiescent state, whereas the latter promotes fibroblast activation and fibrosis. Moreover, Nrp1 was shown to bind to TβRII, suggesting that it is a co-receptor regulating TGF-β signaling. Taken together, these results suggested a role for Nrp1 in promoting fibrosis, at least in part by raising TGF-β-dependent canonical signaling.

11.4.2 Nrp1 Increased Smad2/3 Phosphorylation in Cardiomyocytes

Nrp1 is expressed at high levels in cardiomyocytes, and the significance has been unclear; however, a recent study points to an important function. The conditional deletion of Nrp1 in cardiomyocytes resulted in mitochondrial accumulation and induction of hypertrophy and stress markers [68]. These mice displayed cardiomyopathy, greater sensitivity to ischemia-induced cardiac injury, and shortened survival. The authors report that Nrp1 downregulates peroxisome proliferator-activated receptor γ coactivator 1 α (PGC1α) and peroxisome proliferator-activated receptor γ (PPARγ) in cardiomyocytes. Signaling studies implicated cross talk between the Notch and Smad2 pathways. Importantly, Nrp1 deletion resulted in decreased Smad2 and Smad3 phosphorylation in cardiomyocytes and cardiac tissues. It was found that Nrp1 contributed to the TGF-β-induced downregulation of PGC1α and PPARγ. These findings are in accord with other reports showing that Nrp1 enhances signaling in the TGF-β pathway.

11.4.3 Galectin-1 (Gal-1) Binds to Nrp1 and Promotes Smad3 Activation

Gal-1 is a carbohydrate-binding protein with affinity for β-galactosides, and it binds to a wide variety of ligands [68]. It plays an important role in cancer, wound healing, and many other pathological conditions [71–74]. Gal-1 binds to Nrp1, but not the classical VEGF receptors (VEGFR-1, VEGFR-2, or VEGFR-3) [22]. The binding

of Gal-1 to NRP1, in conjunction with VEGF, stimulates the migration and other activities of endothelial cells. This appears related to increased VEGFR-2 phosphorylation and downstream signaling.

Gal-1 also induces myofibroblast activation, but the mechanism has been unclear. In a wound healing study [75], Gal-1 knockout was found to delay wound healing. The authors reported that Gal-1 enhanced myofibroblast activation, migration, and proliferation by stimulating reactive oxygen species (ROS) production. The binding of Gal-1 to Nrp1 on the membrane of human fibroblasts stimulated Smad3 phosphorylation/activation, whereas knockdown of Nrp1 had the opposite effect. Surprisingly, this effect did not appear to be mediated by TGF-β, because an ALK5 (TβRI) inhibitory drug had no effect. The authors concluded that this Nrp1/Smad3 signaling pathway upregulated a ROS-producing protein, NADPH oxidase 4 (NOX4). Of note, the injection of Gal-1 in tissues accelerated wound healing in a model of diabetes.

11.4.4 Knockout of Nrp1 Decreases Smad2/3 Phosphorylation in Microglial Cells

Some authors have produced mice that lack expression of Nrp1 in glioma-associated microglia and macrophages (GAMs) [76]. Interestingly, gliomas in mice with Nrp1-deficient GAMs exhibited tumors with reduced vascularity, slower growth, and higher numbers of anti-tumorigenic GAMs. Survival was improved in the Nrp1-deficient tumor-bearing mice. When incubated with glioma-derived factors, microglia lacking Nrp1, or treated with the Nrp1 blocking drug EG00229 (blocks b1-domain-binding site), demonstrated a bias toward an inflammatory/antitumor (M1) phenotype. This was associated with decreased Smad2/3 activation. In contrast, the presence of Nrp1 favored an anti-inflammatory (M2) phenotype. The authors postulate that in the absence of Nrp1, the response of microglia to TGF-β is defective, resulting in an inflammatory bias. They conclude that inhibition of Nrp1 represents a potential strategy for suppressing gliomas.

11.5 Nrps and Epithelial-to-Mesenchymal Transition (EMT)

EMT is a multifaceted, extreme display of epithelial cell plasticity, as recently reviewed [77]. During EMT, epithelial cells are converted into fibroblastoid cells or fibroblasts capable of migration [77] (Fig. 11.2). The fibroblast-like phenotype resulting from EMT is characterized by the acquisition of mesenchymal markers such as vimentin, α-SMA and neural (N)-cadherin, and the corresponding loss of epithelial markers such as cytokeratins, occludins, and epithelial (E)-cadherin. It occurs as a normal developmental process during embryogenesis and in adults contributes to wound healing; however, it is also observed as a pathological feature in adult tissues. EMT occurs in neoplastic disease and has been linked to aggressive tumor behavior [77]. In chronic fibrotic disorders of the kidney, liver, and other

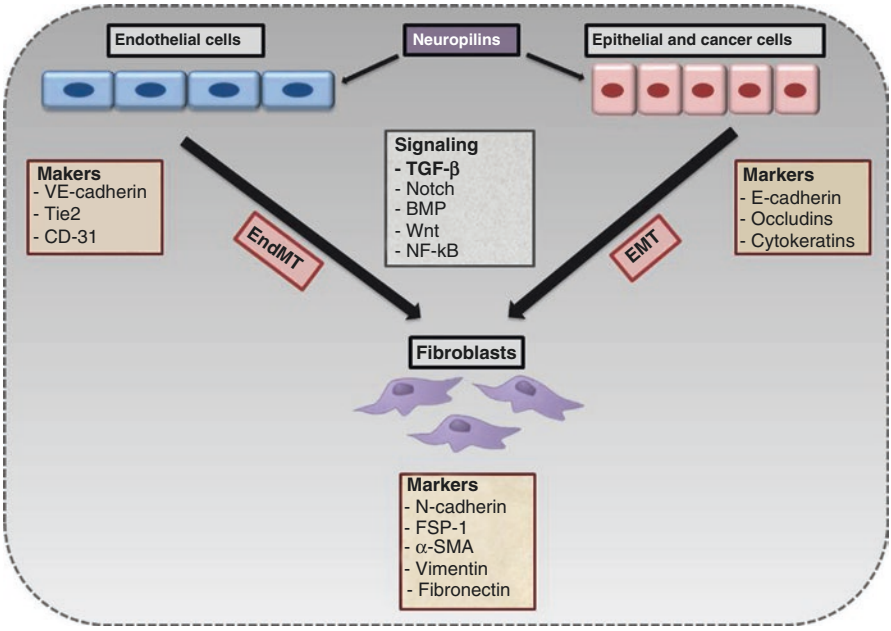

Fig. 11.2 Nrps and TGF-β in EMT and EndMT. Schematic illustration of role of Nrps in the process of EndMT and EMT. Nrps are expressed on endothelial cells, some normal epithelial cells, and many types of cancer cells, where they are able to bind TGF-β1 and/or the signaling TGF-β receptors (TβRI and TβRII). TGF-β signaling is a primary mediator of the process of EndMT and EMT that contribute to fibrotic disorders and cancer progression. Studies by others and us demonstrate a role for Nrps through increased TGF-β signaling in these processes of cell differentiation (see text). The endothelial or epithelial cells lose their respective cell surface markers and cell-cell adhesion junctions and gain the mesenchymal or fibroblastic cell markers, accompanied by a spindle-shaped morphology as depicted in the figure

organs, EMT may contribute to the loss of mature epithelial structures and replacement with fibroblasts and related with accumulation of extracellular matrix [78].

TGF-β is by far the most potent factor that induces EMT, although other GFs also contribute [79, 80]. A number of studies point to an important role of Nrp1 and Nrp2 in EMT. Due to its multiple ligand-binding ability, Nrp1 modulates four major signaling pathways that have been linked to EMT, i.e., TGF-β, Hedgehog, HGF/c-Met, and PDGF. TGF-β signaling, the primary mediator of EMT, regulates the expression of multiple genes and pathways such as Wnt, Ras, Hedgehog, and Notch to facilitate EMT [81]. During TGF-β signaling, Smads interact with EMT-associated transcription factors (e.g., Snail1, Zeb1/2, Twist, β-catenin). The Smad transcription factor complexes further regulate the expression of EMT-associated genes. Nrps also bind integrins that appear to play a significant role in EMT. For example, the depletion of αv integrin or β5 integrin blocked TGF-β-induced EMT in breast carcinoma cells [82]. Additionally, the depletion of β5 integrin significantly reduced invasiveness of the breast cancer cells, thus supporting an important role for integrins in EMT and tumorigenicity. These findings are relevant to Nrps,

because they bind some integrins and Nrp/integrin complexes might contribute to the EMT process. Using immunohistological techniques, Adham et al. [83] demonstrated a positive correlation between Nrp1 and EMT markers E-cadherin and Slug, suggesting a significant relationship between Nrp1 and the process of EMT. Recently, Chu et al. [84] showed that Nrp1 overexpression using plasmid DNA promoted EMT and was associated with enhanced invasive and metastatic properties in oral squamous cell carcinoma cells. Moreover, selective chemical inhibition of NF-κB suppressed the Nrp1-mediated EMT, supporting a link between Nrp1 and this pathway, which is important in tumor progression.

Similarly, Nrp2 has been identified as an important mediator in promoting EMT. Grandclement et al. [13] showed that Nrp2 is a co-receptor for TGF-β1 and promotes Smad-dependent signaling. Indeed, Nrp2 induced EMT in a TGF-β1-dependent fashion. They showed that Nrp2 significantly promotes tumor formation in a colon cancer xenograft model. Importantly, the expression of Nrp2 was associated with constitutive canonical TGF-β signaling. Interestingly, Nasarre et al. [69] reported that Nrp2 is upregulated in a TβRI-dependent and Smad-independent fashion during TGF-β1-driven EMT in lung cancer cells. Nrp2 inhibition attenuated TGF-β1-driven EMT, migration/invasion, and ERK activation in these cells. Another study determined that Nrp2 is induced by canonical TGF-β/Smad signaling in hepatocellular carcinoma (HCC) cells, where it regulates HCC cell migration and invasion [85]. In vitro, Nrp2 associates with a mesenchymal-like phenotype and, correspondingly, correlates with a higher tumor grade in vivo indicating a functional role of Nrp2 in epithelial and mesenchymal-like HCC cells. Taken together, these findings indicate that the Nrps promote EMT.

EMT has long been thought to promote tumor invasiveness and metastasis. However, some recent studies contradict this view and suggest that EMT is not required for metastasis but mainly contributes to chemoresistance [86, 87]. This might be due to the fact that EMT markedly decreases cell proliferation, which should protect the cells against many chemotherapeutic agents. Furthermore, EMT has also been linked to the acquisition of a CSC-like phenotype [88], which is also associated with chemoresistance. In accord with this, Ma et al. [89] reported a critical role of EMT in drug resistance of hepatocellular carcinoma cells to oxaliplatin, a commonly used chemotherapeutic drug. This opens new avenues of investigation and points to a potential role of Nrps in EMT-related cancer cell resistance to drugs.

11.6 Nrp1 and TGF-β-Induced Endothelial-to-Mesenchymal Transition (EndMT)

Other than lining the vessel wall, endothelial cells perform additional functions in distinct physiological and pathophysiological contexts. Recent findings have suggested the transition of endothelial cells into mesenchymal cells as an interesting example of such a function. This phenomenon is known as endothelial-to-mesenchymal

transition (EndMT) and shares many features with the more widely known EMT (Fig. 11.2). During EndMT, endothelial cells lose adherence junctions, delaminate from the organized layer of cells in the vessel lining, and possibly enter the underlying tissue due to their increased invasive capacity [90]. The mesenchymal phenotype resulting from EndMT is characterized by the acquisition of markers such as α-SMA and N-cadherin and the corresponding loss of endothelial markers such as CD-31, Tie2, and vascular endothelial (VE)-cadherin [90]. Although several discoveries were made initially in the area of EndMT largely from the viewpoint of embryonic development, EndMT has now been associated with a wide variety of pathological conditions such as fibrosis and cancer.

Similarly to EMT, TGF-β signaling through Smads plays a significant role in regulating maladaptive EndMT. The Snail family of transcription repressors has been shown to be an integral part of the molecular machinery responsible for TGF-β-induced EndMT [91]. In addition to TGF-β signaling, Notch and canonical (β-catenin-dependent) Wnt signaling have also been shown to participate in the induction of EndMT.

Although there have been no previous reports, our recent results indicate a novel regulatory role of Nrp1 in mediating EndMT [67]. Pancreatic ductal adenocarcinoma (PDAC) is an aggressive form of cancer with a very low 5-year survival rate. PDAC is characterized by a distinct microenvironment consisting of an intense fibrotic reaction called tumor desmoplasia, primarily composed of cancer-associated fibroblasts (CAFs) along with other stromal cells. The CAFs are thought to contribute to alterations in the tumor microenvironment by releasing oncogenic factors such as TGF-β. Although the origin of CAFs depends on several factors, the importance of EndMT is emerging [92, 93]. Nrp1 is aberrantly expressed in PDAC [94, 95] and correlates with accelerated tumor progression. Given the role of TGF-β and Nrp1 in cancer, we investigated the precise function of Nrp1 in TGF-β1-mediated EndMT.

Our in vitro studies in HUVECs using loss and gain of function approaches demonstrated that Nrp1 modulates the process of EndMT in the presence of TGF-β1, at the morphological and molecular level. Nrp1 being a co-receptor for TGF-β1, manipulating the levels of Nrp1 led to significant changes in TGF-β1 signaling. Thus, Nrp1 knockdown decreased canonical (pSmad2-dependent) TGF-β signaling, whereas Nrp1 overexpression resulted in increased canonical signaling. Importantly, we observed that Nrp1 regulated the levels of the signaling receptors, i.e., TβRI and TβRII. Nrp1 knockdown downregulated the expression of TGF-β receptors, whereas Nrp1 overexpression did the opposite. Previously, it has been demonstrated that Nrp1 plays a crucial role in VEGFR-2 endocytosis, internalization, trafficking, degradation, and signaling [96]. Given these results, it will be of importance to investigate how Nrp1 modulates the expression of TGF-β receptors in endothelial cells. Of note, in human PDAC orthotopic xenografts, we observed a significant correlation between Nrp1 expression, EndMT, and fibrosis markers. Furthermore, we observed that knockdown of Nrp1 in vivo produced a substantial reduction in tumor growth, EndMT, and fibrosis.

11.7 Tuftsin Binds Nrp1 and Signals Through the TGF-beta Pathway

Tuftsin is a small peptide (TKPR) long known to have immunomodulatory activities, particularly on macrophages and microglial cells, but its receptor and mechanism of action have been difficult to establish [97]. Tuftsin was found to bind to Nrp1 [98], and this is consistent with the fact that it is a peptide mimetic of the C-terminal motif of $VEGF_{165}$ (KPRR). However, tuftsin had a relatively low affinity for Nrp1, and a modified peptide, TKPPR, had much higher affinity and competed effectively with VEGF for binding. This is in accord with the C-end rule (described in another section), but surprisingly other authors recently reported that tuftsin has a high affinity in a docking study [99]. Analysis of the crystal structure of the b1/b2 domains of Nrp1 associated with tuftsin [100] revealed that tuftsin binds to an electronegative b1-domain pocket. Interestingly, the terminal arginine residue of tuftsin was essential for binding. A subsequent report on the crystal structure of VEGF bound to Nrp1 [101] confirmed that VEGF binds in the same electronegative pocket as tuftsin, with its exon-8 C-terminal KPRR motif, and at a second site involving a VEGF exon-7 sequence. As in the case of tuftsin, the C-terminal arginine of VEGF was necessary for high-affinity binding to Nrp1.

Recently, Nissen et al. [102] reported that the binding of tuftsin to Nrp1 induces TGF-β signaling. Tuftsin promotes an anti-inflammatory effect in microglia, manifested as an M2 shift, which was reversed by an inhibitor (EG00229) that blocks the binding tuftsin to Nrp1. The blockade of TGF-β signaling with a TβRI (ALK5) inhibitory drug had the same effect as EG00229. Moreover, tuftsin increased Smad3 phosphorylation and decreased Akt phosphorylation. Notably, tuftsin treatment stimulated the secretion of TGF-β, and this was prevented by EG00229 or a TβBRI inhibitor. The increased production of TGF-β in these assays may account for the signaling observations. However, it is unknown how tuftsin promotes the secretion of TGF-β. The authors conclude that tuftsin acts by binding to Nrp1 and activating the TGF-β pathway.

11.8 Nrp1 and the Immune System

Nrp1 appears to contribute to the development of the immune system and thymocyte differentiation [103–107]. It might assist in the formation of the immune synapse between antigen-presenting cells (APCs) and T cells [108]. However, this is likely to be a limited role because most T cells (Teff) do not express Nrp1 and, similarly, most APCs lack this marker, except for the interferon α (IFN-α)-producing plasmacytoid dendritic cells (pDCs) that are positive [54, 109–112].

11.8.1 Regulatory T Cells

There is evidence that Nrp1 contributes to regulatory T cell activity. The majority of mouse Treg cells express Nrp1 [54], and these have been identified as thymus-derived (tTreg) cells [113, 114], whereas peripherally derived Treg cells (pTreg) are

negative. This distinction appears valid for mouse cells, but not human Tregs because most are Nrp1 negative [115]. Nevertheless, some investigators have reported that there is a small subpopulation of human Tregs that express Nrp1 and that are highly suppressive [111].

Forced expression of Nrp1 in conventional (non-suppressive) T cells induces a regulatory phenotype. Indeed, we observed that CD4+ T cells lacking regulatory activity, and negative for Treg markers, acquired strong suppressive activity when coated with both Nrp1-Fc and LAP-TGF-β1 [10]. Solomon et al. [116] using a conditional knockout approach showed that the lack of Nrp1 on mouse CD4+ T cells was associated with increased severity of experimental autoimmune encephalomyelitis (EAE). The absence of Nrp1 promoted the preferential differentiation of Th17 cells over Treg cells. Moreover, Treg cells expressing Nrp1 were more suppressive than those lacking this marker. Importantly, the expression of Nrp1 by CD4+ T cells was associated with a suppressive phenotype, independently of conventional Treg markers (negative for CD25). This suppressive activity was attributed to TGF-β production. These authors concluded that Nrp1 is required to maintain immune tolerance and prevent autoimmunity.

In a recent study, Nrp1 on Tregs was found to bind SEMA4a, and this enhanced Treg function and survival [117]. This is an exception to the rule that Nrps bind the SEMA3 group and highlights the fact that semaphorins have important immunoregulatory effects. Nevertheless, the physiological role of Nrp1 in Treg cells is not well understood. We hypothesize it can capture LAP-TGF-β or active TGF-β, and interact with the TGF-β signaling receptors, resulting in increased responsiveness to this cytokine. This model does not exclude the possibility that other LAP-binding proteins such as integrins or GARP are involved. A caveat is that Nrp1 is not expressed by the majority of human Foxp3+ Tregs or is low. Indeed, in contrast to studies in mice, reports of Nrp1 expression by human Tregs have been inconsistent. Some authors reported human Tregs to be Nrp1 negative, at least in the resting state [115]. In contrast, Delgoffee et al. [117] identified Nrp1 at a low level on human Treg cells. In a colon cancer study, Chaudhary and Elkord [118] observed that Nrp1 was highly expressed on CD4+ tumor-infiltrating lymphocytes (TILs), and this correlated with CD25+ expression, irrespective of the Treg markers Helios and Foxp3. Other investigators have reported expression primarily in tumor-draining lymph nodes and TILs. Battaglia et al. [119, 120] found that the tumor-draining lymph nodes of metastatic tumors were enriched for Nrp1+Foxp3+ Treg cells. Furthermore, they established that Nrp1-expressing Tregs were more strongly suppressive than their Nrp1-negative counterparts. The numbers of Nrp1+ Tregs were increased by high-dose radiation. A caveat is that the percentage of Nrp1+ Tregs in these studies was small, and the physiological significance of these observations requires further investigation.

11.9 Nrp1/TGF-β1 Interactions in Cancer Cells

As we have previously reviewed [1], Nrps promote cancer progression. Clinically, a high level of Nrp1 or Nrp2 expression correlates with a poor prognosis in several types of tumors [1, 6–9, 121, 122]. This might be due to increased tumor

angiogenesis, but there is strong evidence for other mechanisms as well. Indeed, the expression of Nrps promotes tumor survival, migration, invasion, and metastasis. The mechanisms are not fully elucidated, but GFs are likely to contribute. For example, Nrp1 boosts the response to TGF-β, VEGF, HGF, and PDGF. As noted in another section, this stimulates EMT and EndMT. Nrp1 also interacts with galectin-1, to exert some tumor-promoting effects. In addition, Nrps contribute to Hedgehog signaling, which is instrumental in the generation of cancer stem cells. Thus, Nrp expression is likely to promote the activation of several signaling pathways that interact widely, with detrimental outcomes in the context of cancer.

11.9.1 Cross Talk with Other Pathways and Putative Signaling Functions of the Nrps

TGF-β signaling interacts with many other signaling pathways including bone morphogenetic proteins (BMPs), Notch, Wnt, Hedgehog, Hippo (TAZ/YAP) [1, 123, 124], and GF receptor tyrosine kinases (e.g., HGF, EGF, and PDGF). Thus, Nrp/TGF-β interactions could impact directly or indirectly on a very large number of biological processes. These pathway interactions are too numerous to describe here in detail, but they are relevant to development, stem cell fate, angiogenesis, wound healing, EMT, and cancer progression.

It is not well understood how the binding of ligands to Nrps initiates signaling. For instance, tuftsin is a very small Nrp1-binding peptide that seems to initiate a response. We postulate that the Nrps have to interact with other receptors to initiate signaling, but this is not entirely clear, and there is evidence they can signal directly. A potential mechanism involves cytoplasmic binding to the PDZ protein synectin (also denoted GIPC), although the consequence of this binding remains poorly defined [125]. In a mechanism not requiring synectin, Nrp1 has been found to enhance phosphorylation of the integrin adaptor molecule p130Cas and subsequent signaling [16, 18]. p130Cas has a very large interactome and contributes to transformation by several oncogenes [126]. It can stimulate cell proliferation, migration, survival, and invasion [126]. This is likely relevant to TGF-β signaling, because p130Cas can inhibit canonical TGF-β signaling by direct action on Smad3 [127, 128], while it enhances noncanonical signaling [128].

The cytoplasmic domains of Nrp1 and Nrp2 interact with phosphodiesterase 4D (PDE4D) [129]. This enzyme inactivates cAMP and, therefore, has a major physiological function. SEMA3 promotes the Nrp1/PDE4D interaction, leading to PDE4D translocation to the cell membrane and cAMP degradation. The consequent inhibition of protein kinase A (PKA) enhances Hedgehog transduction, and this stimulates the growth of medulloblastoma in a mouse model [129]. Since cAMP is a key secondary messenger for a large number of hormones and other mediators, this type of interaction could have widespread consequences and merits further investigation.

Nrps influence stem cell pathways. Hillman et al. [130] reported that Nrp1 and Nrp2 have a key regulatory function in Hedgehog signaling. Nrp1 transcription was induced by Hedgehog, and the overexpression of Nrp1 stimulated Hedgehog target

gene activation, in what appears to be a positive feedback circuit. Nrps were found to mediate Hedgehog signal transduction between the activated Smoothened (Smo) protein and the negative regulator Suppressor of Fused (Sufu). Nrp1/PDE4D/PKA/Hedgehog effects were mentioned above, and Nrp2 has been shown to contribute to tumorigenicity in Hedgehog pathway medulloblastoma [131]. Goel et al. [132] observed that Nrp2 signaling promoted breast cancer initiation through activation of α6β1 integrin, focal adhesion kinase (FAK)/Ras/MEK, and Hedgehog pathways. Cao et al. [133] found that Nrp1 fosters renal cell carcinoma by activating Akt and Hedgehog.

Some studies point to a role of Nrp1 in CSCs [134]. CSCs are self-renewing cells that express characteristic stem cell genes and are highly tumorigenic [135]. They form tumor spheres in low-adherence cultures and strongly resist anticancer drugs [135]. Some of the key mediators and pathways of CSCs include TGF-β, EGFR, NF-κB, and Hedgehog [135–138], and all of these are influenced by Nrps. We observed expression of Nrp1 in breast CSCs [139]. To examine a potential role of Nrps in breast cancer cell lines, we knocked down their expression with siRNA. This inhibited differentiation to a CSC phenotype in culture, prevented tumor sphere formation, and blocked NF-κB activation [139]. These findings all point to an important function of Nrp1 in CSCs.

11.10 The C-End Rule and Latent TGF-β

Peptides and other ligands that bind to Nrp1 are rapidly internalized, as recently reviewed [41]. Teesalu et al. [41] observed that many cell-penetrating peptides (CPPs) bound to Nrp1 at high affinity, and this appeared to be required for efficient cell membrane penetration. When these peptides were conjugated to larger molecules, vascular permeability and tissue penetration were markedly increased. These CPPs had a C-terminal consensus motif ($^{R}/_{K}XX^{R}/_{K}$), with a C-terminal arginine (R) or less frequently lysine (K). They denoted this binding characteristic the C-end rule (CendR). Indeed, this is in accord with peptide binding to the electronegative pocket of the b1 domain of Nrp1, as reported for tuftsin and other peptides mimicking the C-terminal motif of VEGF. The precise mechanism of internalization has not been completely elucidated, but it appears to differ from other known pathways of endocytosis. It is dependent on Nrp1/synectin coupling and shows similarities with macropinocytosis [40]. Importantly, this internalization mechanism has been exploited for the intracellular delivery of large anticancer drugs.

Several natural proteins have a CendR motif, which can be either constantly exposed, as in VEGF or LAP of TGF-β1, or revealed by enzymatic cleavage (e.g., some viral capsid proteins) [28–32]. We found that LAP-β1, which has the RHRR CendR motif, is quickly internalized into tumor cells after binding to Nrp1 [10]. It is currently unknown how this Nrp-mediated internalization process affects the response to TGF-β, either in terms of the availability of the cytokine on the membrane of cells or signaling events. Based on previous studies with CendR peptides, it could impact on endothelial cells and result in increased vascular permeability.

Interestingly, CendR motifs might be important for the internalization of some bacterial toxins [28], viruses [140], and animal toxins such as imperatoxin A of scorpions [141]. Numerous recent studies point to the CendR as an efficient method of delivering large anticancer drugs [41] and provide a new clinical dimension to Nrp-targeted therapy.

11.11 Concluding Remarks

The Nrps are multifunctional receptors that interact with several GFs, GF receptors, integrins, galectin-1, as well as Hedgehog signaling components. As a consequence, they are involved in a wide variety of biological phenomena ranging from angiogenesis, cardiac embryogenesis, fibrosis, immune regulation, and cancer biology. In this chapter, we reviewed primarily Nrp/TGF-β interactions, which is an area that is still at an early stage of investigation. The Nrps have been found to have high affinity for TGF-β1, as well as its signaling receptors. Moreover, we have shown that Nrp1 can bind latent TGF-β1 and activate this cytokine. The Nrps have been shown to have co-receptor function for TGF-β in various studies and share some properties with two other well-known co-receptors, i.e., betaglycan and endoglin.

Interestingly, a common feature of Nrps is their ability to interact with both GFs and their signaling receptors. Indeed, this is the case for VEGF, TGF-β, HGF, and PDGF. The Nrps are not essential for the induction of a response in these pathways, but they usually enhance or modify the response. The molecular bases for these numerous interactions are poorly understood and require further study. However, it is apparent that Nrps can capture the GF ligand, enhance receptor expression, and promote signaling. Moreover, some peptide ligands of Nrp1, such as tuftsin, appear to directly stimulate TGF-β signaling by mechanisms that have not been elucidated.

In the immune system, the expression of Nrp1 serves as a marker of mouse tTregs, and it appears to contribute to Treg suppressive activity. We present evidence that increased Treg-mediated suppression is related to TGF-β1 binding and activation. In this role, the Nrps may contribute to the generation of Treg cells and other aspects of TGF-β immunosuppression that interfere with antitumor immunity. In cancer, there have been numerous studies showing that Nrp expression is a negative factor for prognosis. Although this has frequently been attributed to increased angiogenesis, this is clearly not the only factor. TGF-β plays a dual role in cancer, by inhibiting the growth of early neoplasms and paradoxically enhancing the aggressiveness of more advanced tumors. This contrasting role is not well understood, but we hypothesize that Nrps contribute to this transition. For instance, there is strong evidence that the Nrps promote TGF-β-induced EMT and EndMT, which have both been linked to cancer progression. EMT, in particular, has been extensively studied and is associated with aggressive tumor behavior, as well as differentiation of tumor cells to a CSC phenotype. The EMT/CSC sequence results in tumor cells that are highly resistant to chemotherapy and are likely to cause cancer relapse. It is also plausible that the Nrps promote CSC differentiation through their role in Hedgehog and NF-κB signaling.

In conclusion, Nrp/TGF-β interactions are likely to contribute to several medically relevant processes ranging from fibrosis, angiogenesis, immunity, and cancer and represent important targets for drug development.

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Neuropilin-1-Expressing Monocytes: Implications for Therapeutic Angiogenesis and Cancer Therapy

12

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Abstract

Besides acting as a common receptor for both vascular endothelial growth factors and semaphorins on the endothelial cell surface, neuropilin-1 is also expressed by a variety of circulating immune cells. We have characterized a population of circulating myeloid cells, characterized by the expression of neuropilin-1, able to promote vessel maturation and contributing to cancer vessel normalization. These neuropilin-1-expressing monocytes (NEMs) represent a specific subset of CD11b+NRP1+Gr1-resident monocytes, producing several factors involved in vessel maturation, including PDGF-B, TGF-β, and thrombospondin-1, acting as chemoattractant for vascular smooth muscle cells. NEMs can be isolated from either the bone marrow or Sema3A-expressing muscles. Their direct injection at sites of neoangiogenesis results in the maturation of growing capillaries into functional arterial vessels. When directly injected into growing tumors, NEMs exert potent antitumor activity despite having no effect on cancer cell proliferation. Instead, NEMs promote mural cell coverage of tumor vessels and reduce vascular leakiness, resulting in smaller, better perfused, and less hypoxic tumors. We conclude that NEMs represent a novel, unique population of myeloid cells that can be exploited for multiple therapeutic applications. On the one hand, they can improve functional maturation of neo-vessels for the induction of therapeutic angiogenesis, while, on the other hand, they promote tumor vessel normalization, thereby inhibiting tumor growth.

12.1 AAV Vectors as an Investigational and Therapeutic Tool in Therapeutic Angiogenesis

Our laboratory has a long-standing interest in the use of vectors derived from the adeno-associated virus (AAV) for cardiovascular gene therapy and in particular for the induction of therapeutic angiogenesis in ischemic tissues [1].

AAV is a small parvovirus that owes its name to the fact that it has been originally discovered as a contaminant of an adenoviral preparation. Its genome contains two open reading frames, *rep* and *cap*, flanked by two 145-bp inverted terminal repeats (ITRs), which are required for the major functions of AAV (DNA replication, particle assembly, integration/excision from the host genome) and are the only viral sequences maintained in the recombinant vectors. Any exogenous gene cassette (shorter than 4.5 kb) can be inserted between the two ITRs to obtain a backbone suitable for recombinant vector production.

The traditional method for AAV vector production is based on the co-transfection of the backbone plasmid (containing the transgene controlled by a polymerase II promoter or a short RNA, expressed from a polymerase III promoter) with a second plasmid, supplementing the rep, cap, and helper gene functions. The newly generated AAV genomes are packaged into preformed capsids within the nucleus of suitable packaging cells (traditionally HEK 293 cells), from which the recombinant viral particles can be purified by biochemical methods.

For some not completely understood reasons, AAV transduction occurs preferentially in postmitotic tissues, including the brain (neurons), liver, pancreas, and muscles (skeletal, cardiac, and smooth muscle cells) [2]. Naturally existing AAV serotypes, varying in the amino acid sequence of the capsid protein, show a peculiar tropism for some of these tissues. To date, 13 distinct human or nonhuman primate AAV serotypes have been fully characterized, and their primary receptors have been identified in most instances. While the prototype AAV2, as well as AAV3 and AAV13, binds to heparan sulfate proteoglycans, AAV1, AAV4, AAV5, and AAV6 bind N-linked or *O*-linked sialic acids, and AAV9 binds terminal galactose residues. A variety of serotype-specific co-receptors further increase the efficiency of these vectors in specific cell types.

The gaining popularity of AAV vectors can be attributed principally to their ability to sustain prolonged transgene expression in their target tissues and to their lack of pathogenicity [1]. The safe profile of AAV vectors stems from two major properties. On the one hand, AAV has never been associated to any human disease, while, on the other hand, the recombinant vectors are completely replication deficient. In addition, these vectors offer the unique opportunity to define synergistic, antagonistic, or complementary effects exerted by various angiogenic molecules, as they transduce the target cells at high multiplicity, thus allowing the delivery of gene cocktails. The possibility to deliver different combinations of genes appears of paramount importance in the study of the complex angiogenic process, which requires the coordinated action of multiple molecules in time and in space [3].

Thus, AAV vectors stand as precious tools not only for gene therapy applications but also for their utility in basic research, as they permit in vivo phenotypic assessment of gene function in adult organisms. Being able to carry either coding transgenes or short hairpin RNAs targeting specific genes, these vectors represent an alternative to the use of conditional and knockout animals, with the advantage of shorter and easier production, as well as possible application to any animal species.

12.2 Exploiting AAV Vectors to Decipher the Mechanisms of Blood Vessel Formation

Our laboratory has extensively used AAV vectors to overexpress angiogenic molecules and assess their functional effect in vivo, starting from the members of the vascular endothelial growth factor (VEGF) family.

The molecular history of angiogenesis started with the pioneering works by Donald Senger and Harold Dvorak, who identified and purified a protein able to induce vascular leakage and named it vascular permeability factor (VPF) in 1983 [4], followed by those by Ferrara and Henzel, who cloned a protein promoting the proliferation of endothelial cells and thus named it VEGF [5]. Subsequent studies showed that VPF and VEGF were the same molecule, able to exert both effects.

To date, the term VEGF indeed refers to a family of five mammalian factors (VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placenta growth factor) and to

additional, related proteins of viral (VEGF-E) and snake venom (VEGF-Fs) origin. The proteins encoded by these genes act as homo- or heterodimers through their interaction with three structurally related tyrosine kinase receptors (VEGFR-1/Flt-1, VEGFR-2/Flk-1/KDR, and VEGFR-3/Flt-2) expressed on the surface of endothelial cells as well as on that of several other cell types. In addition, the various VEGF family members also exert their variegated functions by interacting with the co-receptors neuropilin-1 (NRP1) and neuropilin-2 (NRP2) and by the modulation of VEGFR activity through their interaction with other cell surface molecules, such as integrins and heparan sulfate proteoglycans [6]. Additional complexity derives from alternative splicing of the VEGF pre-mRNAs. In particular, the VEGF-A gene consists of eight exons that can be alternatively spliced to generate at least seven different protein isoforms, composed by 206, 189, 183, 165, 148, 145, and 121 amino acids. Through their common N-terminal portion, all these proteins can interact with VEGFR-2 and, with an affinity that is ten times higher, with VEGFR-1. In contrast, bioavailability, biodistribution, and the capacity to bind the neuropilin co-receptors depend on the structure of the alternatively spliced C-terminus and in particular on the inclusion of exons 6 and 7. VEGF-A165, the prototypic and most abundant VEGF-A isoform, differs from VEGF-A121 only by the inclusion of exon 7; as a consequence, the former protein binds heparan sulfate proteoglycans in the extracellular matrix and NRP1 co-receptor, whereas the latter does not [6].

Starting from the initial evidence that the AAV-mediated overexpression of the 165 isoform of the vascular endothelial growth factor by the normoperfused skeletal muscle results in a massive formation of new capillaries and arterioles [3], we have successfully utilized the AAV-VEGF165 vector to promote new blood vessel formation and preserve tissue viability in small and large animal models of cardiac and peripheral ischemia, skin flaps, diabetic wounds, and trauma [7–13].

Other molecules variably control the angiogenic process, including angiopoietin-1 [3], PlGF [14], insulin-like growth factor 1 [15], and VEGF-B [16, 17], and their function in vivo could be effectively investigated using AAV vectors. It is when we started comparing the angiogenic effect of various vectors expressing molecules involved in angiogenesis, and in particular VEGF165, VEGF121, and semaphorin 3A, that we wanted to start dissecting the complex interplay between VEGFs and neuropilins.

12.3 VEGF165 and Sema3A Are Pleiotropic Molecules at the Crossroad Between the Vascular and the Nervous System

Over the last decade, emerging evidence has highlighted the importance of the neurovascular link during both development and disease. Not only blood vessels and nerves grow and branch in a similar manner, but also they share common mechanisms for cell signaling and pathfinding. Specialized endothelial “tip” cells are present at the forefront of navigating cells, which share many similarities with the

axonal growth cones [18]. Through dynamic cycles of extension and retraction of filopodia, the growth cone continually explores and responds to the appropriate set of cues, reassessing its spatial environment and accurately selecting a correct trajectory among the maze of possible routes. Similarly, endothelial tip cells extend and retract numerous filopodia to explore their environment, suggesting that they direct the extension of vessel sprouts. The key function of the tip cells appears to "pave the path" for the subjacent "stalk" endothelial cells. Tip cells proliferate minimally, whereas stalk cells proliferate extensively while migrating in the wake of the tip cell, thus permitting extension of the nascent vessel. Nerves and vessels face similar challenges in finding their trajectories, which are staked out with multiple checkpoints that divide navigation over a long trajectory into a series of shorter decision-making events [19]. In some cases, the same cues that control axon guidance also function to pattern blood vessels. In the 1990s, genetic, biochemical, and molecular approaches identified four families of axon guidance cues, acting over a short-range (cell- or matrix-associated signals) or long-range (secreted diffusible signals): netrins and their DCC and Unc5 receptors, semaphorins and their neuropilin and plexin receptors, slits and their Robo receptors, and ephrins and their Eph receptors (reviewed in [20–22]).

In particular, semaphorins are grouped into eight classes based on their structural domains, with classes 3–7 comprising the vertebrate semaphorins [23]. Although initially identified as potent axon chemorepellents, several semaphorins can provide bifunctional guidance cues, functioning as repulsive or attractive molecules depending on the target cell type, by affecting focal adhesion assembly/disassembly and inducing cytoskeletal remodeling, thus consequently regulating cell shape, attachment to the extracellular matrix, cell motility, and cell migration. While membrane-bound semaphorins bind directly to plexins, secreted semaphorins (class 3 semaphorins; Sema3s) bind to a holoreceptor complex consisting of NRPs as ligand-binding and plexins as signal-transducing subunit. Sema3A has been shown to control endothelial cell migration and survival in vitro [24, 25] and tumor-induced angiogenesis in vivo [26]. The phenotype of Sema3A knockout mice indicates that it may not be essential for the early stages of developmental angiogenesis but rather that Sema3A reshapes the postnatal vasculature, contributing to the formation of a mature vascular network [27].

On the other hand, VEGF, widely recognized as the main angiogenic factor, has been shown to regulate the migration of various neuronal cell types to their final destination [28, 29]. In retrospect, this should not be surprising, as both VEGF and its receptors first appeared in the evolution in the central nervous system in species, such as the worm and the fruit fly, which lack a well-developed vascular system [6]. Consistent with the widespread presence of its receptors, VEGF is emerging as a pleiotropic molecule, able to exert direct effects on an ever-growing list of cell types and tissues, including the bone marrow, liver, bone, kidney, and all types of muscles [6].

Thus, both VEGF and Sema3A stand as pleiotropic molecules, able to control the growth and the pathfinding of both growing nerves and vessels and sharing the NRP1 receptor.

12.4 NRP1 Acts a Shared Receptor for VEGF165 and Sema3A and Is Expressed by a Population of Myeloid Cells (NEMs) that Are Recruited by Both Factors

NRP1 was originally discovered as the main receptor for Sema3A, providing repulsive guiding cues to axonal growth cones in the developing nervous system [30]. However, subsequent evidence showed its abundant expression on endothelial cell surface, where it significantly enhances the affinity of VEGF165 to its Flk-1 receptor [25]. Notably, NRP1 preferentially interacts with VEGF165 rather than with VEGF121, which lacks exon 7.

We first investigated the functional consequence of this dissimilar NRP1-binding capacity of the two main VEGF isoforms, with a particular focus on the recruitment of accessory, circulating cells during the angiogenic process [31]. Over the last two decades, we and others have observed that the sites of prolonged VEGF165 expression become infiltrated by a large number of cells bearing myeloid markers (Fig. 12.1) [32–35]. By exploiting AAV vectors, we assessed the effect of the long-term expression of VEGF165, VEGF121, and Sema3A in the normoperfused skeletal muscle and provided the first evidence that the recruitment of myeloid cells of bone marrow origin by both Sema3A and VEGF165 (but not VEGF121) occurs through the NRP1 receptor [31]. We therefore referred to these cells as NRP1-expressing monocytes (NEMs) [36]. We also demonstrated that NEMs are essential for the process of arterial formation, since they provide chemoattraction for resident smooth muscle cells, contributing to proper vessel maturation. Indeed, their direct injection at the sites of ongoing angiogenesis resulted in the appearance of structurally and functionally mature arteries (Fig. 12.2) [31].

These discoveries led us to propose a new model for the generation of arterial vessels in adult organisms, according to which NEMs, attracted from the circulation to the site of neoangiogenesis, recruit smooth muscle cells around the growing capillaries, leading to the formation of new arteries. Consistently, NRP1 silencing in

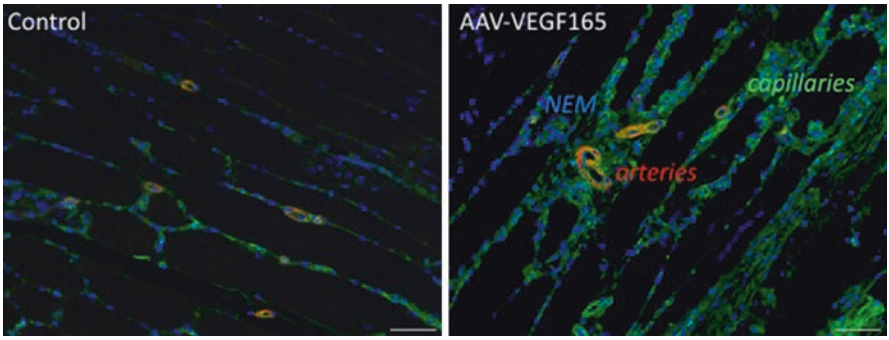

Fig. 12.1 AAV-VEGF165 injection into the mouse skeletal muscle induces endothelial cell proliferation and sprouting of new capillaries (shown in *green* by lectin staining), formation of new arterioles (shown in *red* by anti-α-SMA staining), and infiltration of NEMs (shown in *blue* by nuclear DAPI staining). Scale bar: 100 μm

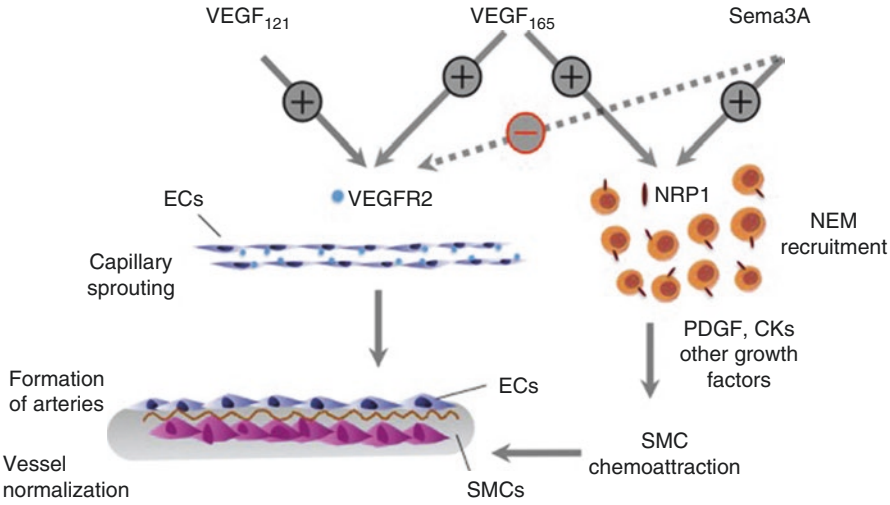

Fig. 12.2 The formation of arterial vessels relies on two concomitant events, namely, the activation of the endothelial cells (*ECs*), essentially through the canonical *VEGFR2*, and the recruitment of *NEMs*. *NEMs* in turn engage smooth muscle cells (*SMCs*) to the sites of endothelial activation. Only *VEGF165* is able to stimulate both events and is thus able to induce the formation of arterial vessels. In contrast, *VEGF121* activates the endothelium, thus inducing capillary sprouting, but is not able to recruit *NEMs*, nor does it form arteries. *Sema3A*, a high-affinity ligand for *NRP1*, is a potent *NEM* recruiter, but it exerts an inhibitory effect on endothelial cells and therefore is not angiogenic. In the context of therapeutic angiogenesis, *NEMs* promote the maturation of newly formed capillaries into arterial vessels. Within tumors, *NEMs* promote the normalization of the tumor vasculature, thereby inhibiting tumor growth

NEMs impaired their migration in response to both VEGF165 and Sema3A in vitro and in vivo. The observation that VEGF121 neither recruits NEM nor forms arteries in vivo points to an essential role of NEM in the specific process of arterial formation, in perfect agreement with the previous notion that knock-in mice expressing only VEGF120 exhibit major defects in arterial development [37, 38]. An appreciation that VEGF121 has different angiogenic properties compared with VEGF165, at least in part because of its ability to recruit NEMs, appears of particular relevance for future gene therapy trials aimed at the induction of therapeutic angiogenesis, since formation of larger arterial vessels in addition to capillaries is an essential requisite to obtain functional neovascularization.

A still unresolved issue is to what extent NEMs also participate to the traditional concept of "arteriogenesis," as referred to the remodeling of preexisting vessels to form larger, collateral conductance arteries. Although arteriogenesis is believed to be mainly triggered by shear stress, it is indeed accompanied by extensive myeloid cell infiltration [39]. Thus, NEMs could be potentially involved in arteriogenesis and also explain, at least in part, the minimal but real benefits conferred to by the injection of autologous bone marrow cells in patients with myocardial or peripheral ischemia, even in the absence of transdifferentiation of the transplanted cells [40, 41].

12.5 NEMs Potently Inhibit Tumor Growth by Inducing Tumor Vessel Normalization

An interesting question, left unsolved by our initial studies, refers to the molecular mechanisms mediating the paracrine activity of NEMs on smooth muscle recruitment and arterial maturation. Additional work by our group has addressed this relevant issue, showing that NEMs partially share their flow cytometry profile with mouse resident monocytes, including low expression of Gr1/Ly6C and CD14, high levels of CD16, intermediate levels of F4/80, as well as expression of low levels of CXCR4 and CD31 [42]. Other studies also indicated that NRP1 is expressed by subsets of pro-angiogenic myeloid cells both in cancer and during embryonic development [43, 44]. However, gene expression and surface protein analysis clearly set NEMs apart from any other monocyte population exerting pro-angiogenic effect, including tumor-associated macrophages (TAM) [45], which produce a panel of cytokines distinct from those expressed by NEMs, and Tie2-expressing monocytes (TEM), which are positive for the surface marker Tie2 [46].

Once injected into preexisting tumors in vivo, NEMs markedly inhibited tumor growth in different tumor models, including B16-F10 melanoma, the more aggressive, highly metastatic 4T1 breast cancer subcutaneous model Carrer 2012 #1156} and the spontaneous pancreatic neuroendocrine tumors in RIP-Tag2 mice or cervical carcinomas in HPV16/E2 mice (unpublished data). Notably, NEMs did not exert any direct activity on cancer cell proliferation, but rather they profoundly modified the structure of tumor vessels, consistent with their property to secrete several factors involved in vessel maturation, such as PDGF-B, TGF-β, and thrombospondin-1. Tumor vasculature upon NEM injection showed increased mural thickness and pericyte coverage, more regular shape, reduced permeability, and, most importantly, improved function, consistent with the capacity of NEMs to secrete factors able to chemoattract vascular smooth muscle cells (Fig. 12.2). The ultimate outcome was improved tumor perfusion and reduced tumor hypoxia, which is emerging as a major determinant of tumor progression [47]. The morphological and functional effect exerted by NEMs on tumor growth fits the concept of "vessel normalization," as originally put forward as a goal to improve blood supply and enhance delivery of chemotherapy drugs by antiangiogenic agents at low dose [48, 49].

To what extent NEMs also contribute to the potent antiangiogenic and anticancer activity of Sema3A? We and others have shown that the delivery of an AAV vector expressing Sema3A to the pancreas of RipTag2 mice significantly inhibited angiogenesis and tumor progression, substantially extending animal survival [26, 50]. Consistent with the hypothesis that this effect could be at least partially due to NEM recruitment, we observed that NEMs, purified by Sema3A-expressing muscles, provided a potent antitumoral effect. Thus, NEMs are likely to mediate the effect of Sema3A on tumor growth and vascularization. Not only, they could also justify the differential activity of the two main VEGF isoforms on tumor growth. Indeed, we have recently observed that while VEGF121 speeds up tumor progression, as expected for an angiogenic factor, VEGF165 instead inhibits it (Kazemi et al., in press). Since neither VEGF isoform directly affected cancer cell proliferation, the

differential tumor growth rate in vivo can be reasonably ascribed to their differential effect on the tumor microenvironment. In line with our previous observations, VEGF165 recruited a significant number of NEMs at the site of tumor angiogenesis, resulting in a normalized tumor vasculature. Is the same happening in human cancer? By analyzing a cohort of 80 patients affected by colorectal cancer, we found that the vascular network of tumors, expressing the highest levels of VEGF121, was essentially formed by small capillaries, whereas a large amount of α-SMA+ arteries was detected in patients expressing higher levels of VEGF165, which is able to recruit NEMs.

Thus, the two major VEGF isoforms are not redundant in inducing and regulating tumor angiogenesis, as they can exert even opposite effects on the growth and invasion of cancer cells in vivo, likely as a consequence of their differential ability to recruit NEMs. Also considering additional literature evidence of the not redundant role of the various VEGF splicing isoforms on tumor progression [51, 52], some of the studies on VEGF (i.e., data obtained using pan-VEGF antibodies unable to distinguish the various isoforms) should be challenged and considered at least inaccurate and potentially misleading.

Conclusions

In conclusion, our experience in the use of AAV vectors as a tool to study the function of angiogenic genes in vivo has led to the identification of NEMs, a novel population of myeloid cells, characterized by NRP1 expression. On the one hand, NEM can be exploited to improve functional maturation of neo-vessels for the induction of therapeutic angiogenesis, while, on the other hand, they promote tumor vessel normalization, thereby inhibiting tumor growth.

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Neuropilin-1 in Immune-Mediated Diseases

13

Zahava Vadasz

Contents

Abstract

In recent years, it was realized that NP-1 is important for maintaining normal immune response homeostasis. It was found that NP-1 is highly expressed on both murine and human T regulatory cells (Tregs), thus increasing Treg cell properties, especially their contribution to the maintenance of self-tolerance [4]. Little is known today regarding the contribution of NP-1 in autoimmunity. In this section we will discuss the role of NP-1 in several autoimmune/immune mediated diseases. We will focus mainly on its regulatory/protective role in autoimmune diseases, such as Systemic Lupus Erythematosus (SLE) and Rheumatoid Arthritis (RA), which are prototypic autoimmune diseases.

13.1 Introduction

Neuropilin-1 (NP-1), first identified as a neuronal receptor, was subsequently characterized as a receptor for secreted class 3 semaphorins. Following this initial characterization, it was also found to be expressed by endothelial cells, serving as a receptor for vascular endothelial growth factor (VEGF) family members, indicating

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that the expression of NP-1 is required for normal vascular development [1]. In addition, increased NP-1 expression on tumor cells was shown to stimulate tumor growth by enhancing angiogenesis and by downregulating tumor cell apoptosis, resulting in increased metastasis and a poor prognosis [2]. In recent years, it was realized that NP-1 is important for maintaining normal immune response homeostasis. In this respect, NP-1 was reported to participate in the interaction between activated dendritic cells (DCs) and resting T cells, initiating by this a primary immune response [3]. Moreover, NP-1 is highly expressed on both murine and human T regulatory cells (Tregs), thus increasing Treg cell properties, especially their contribution to the maintenance of self-tolerance [4]. In this section, we discuss the role of NP-1 in autoimmune-/immune-mediated diseases focusing mainly on its regulatory/protective role in many autoimmune diseases.

13.2 NP-1 and Systemic Lupus Erythematosus (SLE)

In an earlier study, we demonstrated that NP-1 staining is significantly increased in the glomeruli of patients with lupus nephritis when compared to its low expression in glomeruli of normal individuals and its absence in disease control groups (minimal change nephropathy) [5]. In that study we demonstrated that NP-1 staining is much higher in focal glomerulonephritis (GN), and it is expressed only in limited areas of diseased foci (Fig. 13.1a). However, in diffuse GN, NP-1 was expressed widely in the kidney and found to have a positive association with glomerular damage (Fig. 13.1b). In addition, we also showed that there is increased deposition of VEGF in the glomeruli of patients with GN vs. those in whom the glomeruli are considered to be normal. VEGF is considered to be a protective factor, maintaining normal glomerular integrity and homeostasis [5]. Taken together, these observations suggested that the increase in NP-1 expression represents a synergistic mechanism that supports a possible protective effect of VEGF on glomerular endothelial cells, thus preventing their damage and apoptosis [5]. The positive correlation between increased NP-1 expression and extent of GN may fit within the frame of its protective compensatory effect, aimed at limiting advanced GN.

The typical localization of NP-1 in focal GN suggests that NP-1 may be a reliable histological marker for focal GN. Taking into consideration that increased NP-1 expression was found to be in positive correlation with glomerular damage (increased proteinuria and serum creatinine levels), one should consider the possibility that in this case, increased NP-1 expression is damaging rather than protective. Whether it has a protective role or contributes to glomerular damage, NP-1 is definitely involved in the pathogenesis of lupus nephritis. One could expect NP-1 expression to be downregulated on T regulatory cells of SLE patients, contributing to increased pro-inflammatory cytokine production and inflammation; however, reports on this issue are still lacking, and this issue should be the subject of future studies.

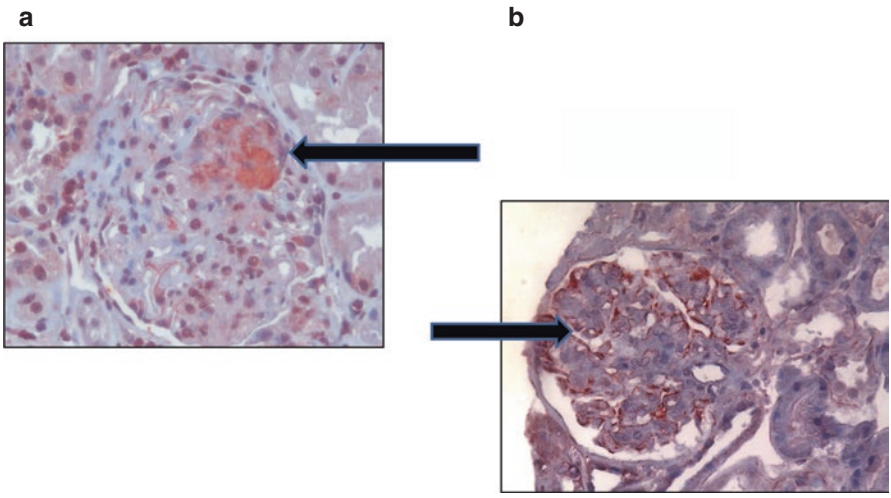

Fig. 13.1 (**a**) Increased expression of NP-1 staining in focal glomerulonephritis mainly in limited areas of disease foci. (**b**) A wide expression of NP-1 in diffuse glomerulonephritis found to be in positive association with glomerular damage

13.3 Neuropilin-1 in Sjogren's Syndrome

Appreciating the importance of inflammatory angiogenesis in many autoimmune diseases including Sjogren's syndrome (SS), the involvement of pro-angiogenic cytokines and their receptors in SS was examined [6]. Sjogren's syndrome (SS) is a chronic, autoimmune disease characterized by mononuclear cell infiltrate and progressive injury to the exocrine glands. SS typically manifests with dryness of the mouth and eyes, but may affect any exocrine gland as well as a wide variety of organs and systems. SS is considered one of the common autoimmune diseases, affecting between 1 and 3 % of the general population. While all ages can be affected by this disease, it generally becomes overt during the fourth and fifth decades of life with a female-to-male ratio of 9:1 [7]. Little is known about the expression of NP-1 in cells of epithelial origin, such as the epithelial cells of human salivary glands. The involvement of NP-1 in regulating angiogenesis in human salivary glands from primary SS patients was compared to that in healthy individuals. The levels of NP-1 mRNA were significantly higher in biopsies of labial salivary glands of patients with active disease than in those obtained from healthy controls. Protein levels of NP-1 were also overexpressed in biopsies taken from active SS patients. The increased NP-1 expression was shown to be mediated by pro-inflammatory cytokines such as IL-6, IL-8, and TNF supporting the hypothesis that inflammatory mediators can promote angiogenesis, which in turn contributes to promoting

autoimmune-mediated inflammation [6]. In addition to the above, it was shown that NP-1 mediated production of pro-angiogenic factors through NF-kB activation. Blocking NP-1 function by a monoclonal antihuman NP-1 antibody induced the nuclear translocation of NF-kB p65 subunit binding to the DNA site and was associated with a remarkable decrease of NF-kB activation. In addition, even when NP-1 upregulation in the salivary gland epithelial cells of SS patients was induced by the pro-inflammatory cytokines IL-6 and TNF, the blockage of NP-1 by inhibitory antibodies resulted in a marked decrease in NF-kB activation [6]. The interference with NF-kB activation through NP-1 becomes relevant because NF-kB regulates the expression of chemokines known to be involved in inflammatory angiogenesis [6].

13.4 Neuropilin-1 and Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by infiltration of cells into the synovial tissue and progressive destruction of cartilage and bone. The most common cell types known to be involved in this inflammatory process in the joint are mononuclear immune cells and fibroblasts. The symptomatic chronic inflammation results in bone and joint erosion and destruction [8, 9]. The main pathophysiological process in RA is characterized by the proliferation of synovial cells and increased angiogenesis, leading to the formation of pannus. As the major cellular component of the invasive pannus, synovial fibroblasts (SF) contribute to the chronic inflammatory responses in the inflamed joints. They highly express a variety of activation molecules, efficiently present antigens to effector T cells, and have the potential to produce several pro-inflammatory cytokines and matrix-degrading enzymes [10, 11]. Moreover, synovial fibroblasts proliferate abnormally, invade local environments, and exhibit characteristics of tumor cells, including somatic mutations in H-Ras and p53 [12, 13]. Angiogenesis is also a prominent process in RA, particularly during the early stages of the disease. Newly formed vessels can maintain a chronic inflammatory state by transporting inflammatory cells to the sites of synovitis, as well as by supplying nutrients and oxygen to the pannus [14, 15]. In RA, VEGF is expressed abundantly in the synovial fluids and tissue. Immunohistochemical studies of RA synovial tissue demonstrated that VEGF expression levels in synovial tissues correlate with the degree of joint destruction [16–19]. Furthermore, VEGF serum levels, especially at onset of the disease, appear to correlate closely with the disease activity of RA, particularly with the numbers of swollen joints [16, 20]. It was found that in the cultured RA synoviocytes, the generation of VEGF expression in these cells can be induced by a variety of pro-inflammatory cytokines, including IL-1 and IL-6 [21]. It was demonstrated that NP-1, rather than VEGFR-1 and VEGFR-2, is the major $VEGF_{165}$ receptor in the synovial fibroblasts. Here, it was shown that the binding of $VEGF_{165}$ to NP-1 prevented the synoviocyte apoptosis induced by serum starvation and sodium nitroprusside (SNP). Furthermore, the expression of phospho-Akt (pAkt), phospho-ERK (pERK), and Bcl-2 was increased by adding $VEGF_{165}$ to cultured synoviocytes. In contrast, SNP-induced Bax translocation from the cytosol to the

mitochondria was blocked by $VEGF_{165}$ treatment. Inhibition of NP-1 expression by short interfering RNA (siRNA) resulted in spontaneous synoviocyte apoptosis, which was associated with the modulation of Bcl-2 expression and Bax translocation. These findings indicate that the binding of $VEGF_{165}$ to NP-1 is crucial for the survival of rheumatoid synoviocytes and suggests that NP-1 could become a potential treatment target in preventing chronic inflammation [17]. In support of the abovementioned study, Yoo and colleagues sought to determine whether a transformed phenotype of RA-FLSs is associated with placental growth factor (PlGF), another angiogenic growth factor induced by hypoxia, rather than with VEGF. PlGF-deficient RA-FLSs demonstrated decreased cell proliferation, migration, and invasion abilities, but an increase in apoptotic death rate in vitro. PlGF gene overexpression in these cells resulted in opposite effects. Moreover, exogenous addition of PlGF-1 and PlGF-2 increased survival, migration, and invasiveness of RA-FLSs by binding their VEGFR-1 and NP-1 receptors and upregulated the expression of the antiapoptotic molecules phospho-ERK (pERK) and Bcl-2. Furthermore, the inhibition of PlGF expression also reduced RA-FLS proliferation in a xenotransplantation model [22].

Based on the above findings, it was hypothesized that blocking the interaction between $VEGF_{165}$ and NP-1 may induce enhanced apoptosis of RA synoviocytes and reduce angiogenesis and experimentally induced arthritis. The experimental $VEGF_{111–165}$ peptide was shown to have a selective anti-NP-1 predisposition, possibly by competing with full-length $VEGF_{165}$. This anti-NP-1 peptide decreased the $VEGF_{165}$-induced upregulation of synoviocyte survival, Bcl-2 expression, and ERK phosphorylation, which suggests that this peptide may be effective for impeding synoviocyte hyperplasia. These effects were probably the result of NP-1 being the major receptor for $VEGF_{165}$ in synoviocytes [17, 23, 24]. The experimental $VEGF_{111–165}$ peptide also demonstrated a novel function of NP-1 in that it enhanced synoviocyte adhesion and migration. In this study, the addition of $VEGF_{165}$ to fibroblast-like synoviocytes (FLSs) enhanced synoviocyte adhesion, migration, and chemotaxis, all of which were almost completely blocked by the anti-NP-1 peptide [15]. Considering that synoviocyte adhesion and migration are pivotal processes for synoviocyte survival, activation, and, presumably, joint destruction [25–27], these results suggest that the anti-NP-1 peptide may regulate RA inflammation by intervening in these processes. It was also found in this work that the use of the anti-NP-1 peptide improved the severity of experimentally collagen-induced arthritis (CIA), synovial hyperplasia, and angiogenesis in the inflamed joints. It was speculated that the therapeutic benefit of the anti-NP-1 peptide derived not only from its inhibitory actions on synoviocyte pro-inflammatory influence or from excessive angiogenesis but were also due to an effect on DC-T cells since NP-1 is known to mediate DC-T cell clustering and DC-induced proliferation of T cells [3].

NP-1 also promotes long-term interactions between Treg cells and immature DCs, resulting in a higher threshold to antigen stimulation, thus suppressing the effector function of DCs [28]. However, the number of NP-1-positive Treg cells in synovial biopsies from RA patients was not observed to increase significantly, a finding that may be attributed partly to the fact that Treg cells cannot fully

suppress/regulate the autoimmune inflammation in RA [28, 29]. Co-localization of DCs and Treg cells was demonstrated by double staining in synovial biopsies, indicating that Treg cells and DCs may interact with each other. In the in vitro model, it was found that Treg cells suppressed the function of DCs through surface molecules such as CTLA-4, LAG-3, and NP-1 [28, 29]. XQ et al., in their study, found that the expression of LAG-3 and NP-1 in Treg cells did not increase significantly during the inflammation progress. In contrast, the number of DCs increased significantly, which may be attributed to the fact that Treg cells cannot fully suppress the function of DCs [29].

Based upon these results, it is thought that NP-1 plays a role in antigen-specific autoimmune responses in RA. The additional finding in the work of Jin-Sun Kong et al., namely, that the serum levels of anti-CII antibodies dropped following treatment with the anti-NP-1 peptide, supports this notion. Overall, these findings provide new insights into the role of NP-1 in RA pathogenesis and suggest that the anti-NP-1 peptide may offer a new approach for the treatment of chronic autoimmune arthritis [15].

Early detection of inflammatory clinical signs in RA is important because it raises the probability of successful treatment. Many attempts have been made to define biomarkers for strong local inflammatory processes in synovial tissue that can be used in the future for radio diagnostic approaches that would allow caregivers to differentiate between patients with active synovitis and those in remission. Fueldner et al. found differences in the expression of investigated markers linked to the activity/severity status of synovitis in long-standing RA. An increased expression of CD64, CD11b, and NP-1(CD304) was found to be highly specific for patients with high activity synovitis, making them suitable markers to monitor active inflammation processes. On the other hand, an elevated expression of CD90 and CD29 was found to indicate mild activity in RA synovitis. Thus, the researchers' conclusion was that for identification of highly active synovitis, a panel including CD64 and the plasmacytoid dendritic cell marker NP-1 (CD304) or the combination of CD11b and CD38 was more suitable. They also concluded that CD64 and HLA-DR, as well as the biomarker panels CD64/CD304 and HLA-DR/CD90/CD29, were found to be clearly discriminative between RA (including mild- or high-current activity in synovitis) and acute non-RA [9].

In conclusion, NP-1 is a unique regulatory molecule found to be expressed on a wide spectrum of cells, namely, T and B cells and other cell types (e.g., synoviocytes) participating by that in immune-mediated and autoimmune processes. The involvement of NP-1 in the pathogenesis of autoimmune diseases is still poorly investigated. The better understanding of its regulatory mechanism may raise new therapeutic options which should be the subject of many future studies.

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