Short Conceptual Overview

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Control of cellular motility by neuropilin-mediated physical interactions

Abstract: The neuropilin (Nrp) family consists of multifunctional cell surface receptors with critical roles in a number of different cell and tissue types. A core aspect of Nrp function is in ligand-dependent cellular migration, where it controls the multistep process of cellular motility through integration of ligand binding and receptor signaling. At a molecular level, the role of Nrp in migration is intimately connected to the control of adhesive interactions and cytoskeletal reorganization. Here, we review the physiological role of Nrp in cellular adhesion and motility in the cardiovascular and nervous systems. We also discuss the emerging pathological role of Nrp in tumor cell migration and metastasis, providing motivation for continued efforts toward developing Nrp inhibitors.

Keywords: cellular motility; neuropilin; receptor signaling; semaphorin; VEGF.

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Introduction

Neuropilins (Nrp) are essential vertebrate cell surface receptors that function to convert extracellular stimuli into directional cellular migration in multiple distinct cell types [reviewed in refs. (1–6)]. Two physiologically important Nrp ligand families include vascular endothelial growth factor (VEGF) and class 3 semaphorins (Sema3) (7–9). Underlying its role in regulating cellular motility and migration is the ability of Nrp to integrate multiple physical mechanisms, including ligand binding, cellular activation, adhesion, and cytoskeletal reorganization.

At a molecular level, Nrp functions through specific physical interactions that include binding to the canonical ligands Sema3 and VEGF (1), other heparin-binding growth factors (10), signaling and adhesive cell surface receptors (1, 11–15), and components of the extracellular matrix (ECM), including glycosaminoglycans (GAGs) (16, 17). There are two Nrp genes, *Nrp1* and *Nrp2*, that are conserved in all vertebrates (8, 18). Both Nrp homologues share the same subdomain organization and, in humans, are 44% identical on the amino acid level. Nrp has a large extracellular region composed of two calcium-binding complement binding factors C1s/C1r, Uegf, BMP1 (CUB) domains (a1a2); two coagulation factor V/VIII homology domains (b1b2); a Meprin, A5 antigen, receptor tyrosine phosphatase μ (MAM) domain (c); a single-pass transmembrane domain (TMD) helix; and a short cytoplasmic tail (Figure 1). The Nrp extracellular domain directly binds to a wide array of molecules that are essential for its versatile function in cellular motility. The TMD has been shown to dimerize and is thought to be important for assembling active signaling complexes (19, 20). The Nrp intracellular domain binds to postsynaptic density 95, disk large, zona occludens-1 (PDZ)-domain containing proteins (21) and is important for regulating interactions with other receptors and the cytoskeleton, thus having an essential role in cellular migration (22–25).

In particular, the extracellular b1b2 domains serve a central role in specific binding and competition for a large number of ligands (1, 26–28). Thus, for example, it has been demonstrated that the VEGF-A C-terminus binds to a specific binding pocket formed by the coagulation factor loops of the b1 domain of Nrp1 (29). Sema3 engagement is more complex and involves both the Nrp a1 and b1 domains (26, 30). Current models indicate that the Nrp a1 domain binds the Sema domain of different Sema3 family members, controlling specificity, while the Nrp b1 domain binds to the Sema3 C-terminal basic domain, controlling high-affinity binding (30–35). This model is affected by the recent discovery that the a2 domain of Nrp integrally interacts with b1 and b2 domains forming a stable core (36). Thus, domain deletion experiments, which generally

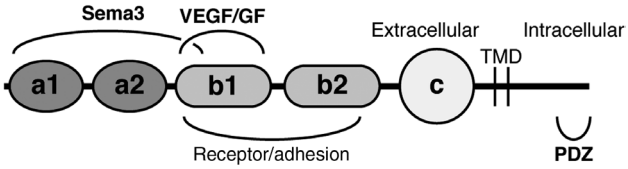

Figure 1 Nrp structure. Nrps contain a large, modular extracellular region that facilitates binding to multiple ligand families in both a competitive and non-competitive fashion. Ligand binding is coupled to intracellular signaling through PDZ-domain-containing adaptor proteins that bind the SEA motif of the Nrp cytoplasmic domain.

delete a1a2 or b1b2 in tandem, may have more complex interpretations. A recently published structure of Sema3A/PlexinA2/Nrp1 complex has begun to elucidate the molecular details of the Sema3 signaling machinery. This structure revealed that the Nrp1 a1 domain cross-braces the Sema domains of Sema3A and PlexinA2, assembling them to form a dimer of heterotrimers critical for the activation of signaling (37, 38).

Regulatory mechanisms controlling Nrp ligand binding and the coupling of different domains are an active area of research. Post-translational modification of Nrp ligands critically regulates their Nrp binding and activity. Alternative splicing and proteolytic processing of the VEGF family can dramatically alter Nrp binding and ligand activity (9, 39–43). Proteolytic processing of the C-terminal basic domain of Sema3 family members by furin critically regulates binding to the Nrp b1 domain (44, 45) and chemotactic activity (46–48). Despite these data, the importance of furin processing in physiological Sema3 signaling has remained an open question. The recent report that Kallmann's syndrome, a serious genetic disease resulting from defects in axon guidance, can be caused by mutations in a furin-cleavage site in the C-terminal domain of Sema3A (49) argues strongly for the physiological importance of furin processing and Nrp engagement.

Nrp1 was originally identified as a cell adhesion molecule (50, 51). It was shown that expression of Nrp1 conferred adhesiveness to fibroblasts through heterophilic interaction with a protease-sensitive molecule (51). The adhesive function of Nrp was later mapped to the b1b2 coagulation factor domains (52), and subsequent studies demonstrated that the identified region within domain b2 was also responsible for GAG binding (53). In addition to GAG-dependent adhesion, Nrp can couple with other cell surface receptors to modulate cellular adhesion. Specifically, Nrp has been demonstrated to modulate integrin-dependent cellular motility, where the receptors appear to couple through both extracellular and intracellular mechanisms to regulate VEGF-dependent endothelial cell migration in angiogenesis (54).

Nrp-dependent VEGF signaling

VEGF-dependent angiogenesis occurs within the context of a ligand/receptor holocomplex that includes VEGF, Nrp, and the receptor tyrosine kinase, VEGFR (Figure 2). As co-receptors in VEGF-dependent angiogenesis, Nrps function by directly binding ligand and regulating VEGFR signaling and cellular activation [reviewed in refs. (1, 55–58)]. Nrp1 and Nrp2 transduce signals for different VEGF family members. For instance, Nrp1 signaling is critical for VEGF-A-dependent angiogenesis (9, 59) and Nrp2 for VEGF-C-dependent lymphangiogenesis (43, 60).

Genetic studies have demonstrated the importance of Nrp1 within the VEGF-A/VEGFR-2/Nrp1 signaling holocomplex. Knockout of Nrp1 in mice results in embryonic lethality owing to widely distributed defects in vascular patterning (61–63) and overexpression of Nrp1, which is also embryonic lethal, causing hypervascularization within the cardiovascular system (64). The vascular phenotype of the Nrp knockout is similar to that of VEGF-A heterozygous mice (65, 66) and VEGFR-2 null mutant mice (67), although reduced in severity. Interestingly,

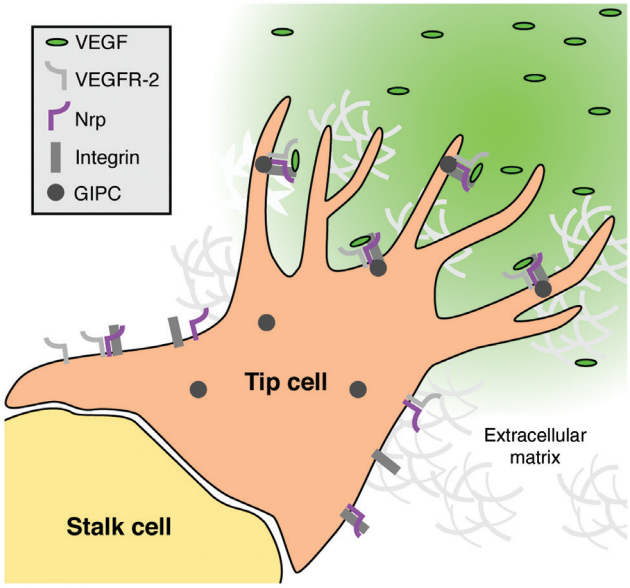

Figure 2 Nrp critically modulates endothelial tip cell function. Nrp is highly expressed in endothelial tip cells where it promotes branching and directional migration in response to VEGF. In tip cells, Nrp serves several essential functions, including ligand binding, holocomplex formation, integrin coupling, and recycling of co-receptors and components of focal adhesions.

a detailed study of the Nrp1 knockout mouse demonstrated that endothelial cell migration, but not proliferation, is defective in the absence of Nrp1 (68). Indeed, Nrp1 knockdown in human umbilical vein endothelial cells (HUVECs) causes impaired F-actin reorganization and focal adhesion distribution during endothelial cell attachment to the ECM (69). A number of studies have also described an essential role for Nrp in endothelial tip cell function (70) (Figure 2). Nrp1 is highly expressed in endothelial tip cell filopodia, and VEGF-A stimulates filopodia extension from the tip cells resulting in branching (71, 72). The same principle has been observed in VEGF-C-dependent lymphangiogenesis where Nrp2 is highly expressed in lymphatic tip cells (73) as well as in tip cell filopodia, and selectively modulates VEGFC/VEGFR3-mediated tip cell extension but not cellular proliferation or survival (74).

The Nrp intracellular domain was initially considered dispensable for its function in angiogenesis. However, it is now understood that the Nrp intracellular domain functions to couple ligand binding to downstream mediators of angiogenesis. Deletion of the intracellular domain leads to impaired arteriogenesis and abnormal retinal artery and vein crossover (75, 76). Additionally, a chimeric receptor, composed of the extracellular domain of epidermal growth factor receptor (EGFR) fused to the TMD and cytoplasmic domains of Nrp1, was found to mediate EGF-induced HUVEC migration but not proliferation (77). The intracellular adaptor protein RGS-GAIP-interacting protein C-terminus (GIPC) has been shown to bind to the intracellular domain of both Nrp (21) and integrin (78). These data suggest a direct mechanism for Nrp-dependent modulation of integrin function through coupling of intracellular domains. Indeed, $\alpha_5\beta_1$ integrin, which binds GIPC, was shown to mediate endothelial cell spreading on fibronectin through a mechanism dependent on the Nrp intracellular domain and GIPC (24). Furthermore, it was recently demonstrated that the Nrp intracellular domain directly binds and traffics protein components of focal adhesions, functioning to promote rapid focal adhesion turnover and cellular migration (79). Similarly, the Nrp intracellular domain has also been found to be essential for ligand-specific receptor localization and trafficking. Binding of VEGF-A_{165} to Nrp1 induces VEGF-A/VEGFR-2/Nrp1 complex formation, receptor internalization, and recycling back to the membrane (12). Nrp1 was found to play a specific role in recycling as the binding of VEGF-A_{165}b, an alternative VEGF-A spliceform that does not bind Nrp, results in VEGFR-2 degradation through an alternative recycling pathway (80).

Nrp-dependent Sema3 signaling

Nrp was initially discovered in *Xenopus* and was shown to be involved in mediating neuronal wiring through cell-cell adhesion (50, 81). The function of Nrps in the nervous system is multifaceted and extends beyond their adhesive properties to regulation of both axon guidance and cellular migration. Nrp functions as an important co-receptor for the Sema3 family (Sema3A-G) of axon guidance molecules, directly physically coupling with plexin receptors to mediate signaling and cellular activation [extensively reviewed in refs. (1, 5, 8, 13, 82, 83)] (Figure 3).

Nrp1 and Nrp2 have a unique Sema3 binding specificity and perform distinct biological functions in the nervous system (84, 85). Nrp1 null mice show defects in neuronal axon pathfinding in both the central and peripheral nervous systems (61, 62, 86). Additionally, Nrp1 knockdown impairs the migration and invasion of cranial neural crest cells into the branchial arches, leading to the failed development of the peripheral architecture (87). Nrp2 null mice (88, 89) have impaired spinal sensory axon projection and hippocampal mossy fiber axon projection (90), along with abnormal sensory neuronal axon innervation and fasciculation in the olfactory bulb (91). Specific signaling is accomplished by tissue-specific expression and action of different Sema3, plexin, and Nrp family members (30, 88, 89). Additionally, there are exceptions to the canonical Sema3/Nrp signaling, with Sema4A reported to have Nrp-dependent function in the immune system (92) and Sema3E having Nrp-independent function (93).

Physically, signaling through Sema3 involves interaction in extracellular, membrane, and intracellular regions of the cell that control adhesive interactions and cytoskeletal dynamics (94) (Figure 3). The intracellular region of plexins contains a GTPase-activating protein domain (GAP) and directly binds to Rho and Ras family GTPases, including, but not limited to, Rnd1, RhoD, R-Ras, M-Ras, and Rap1. Rnd1 binding initiates Sema3A-induced actin depolymerization, leading to sensory neuron growth cone turning and collapse. Conversely, RhoD antagonizes Rnd1 and inhibits Sema3 function (95). Plexin-mediated regulation of both R-Ras and M-Ras is critical for semaphorin-mediated signaling in both neuronal and vascular cells (96–98). While multiple GTPases interact with the intracellular region of plexin family members, recent work demonstrating the dimerization-dependent interaction of plexin with Rap1 indicates that multiple physical interaction mechanisms distinguish the different families (99). Additionally, plexins bind to molecule interacting with CasL (MICALs), an oxidoreductase that functions as an

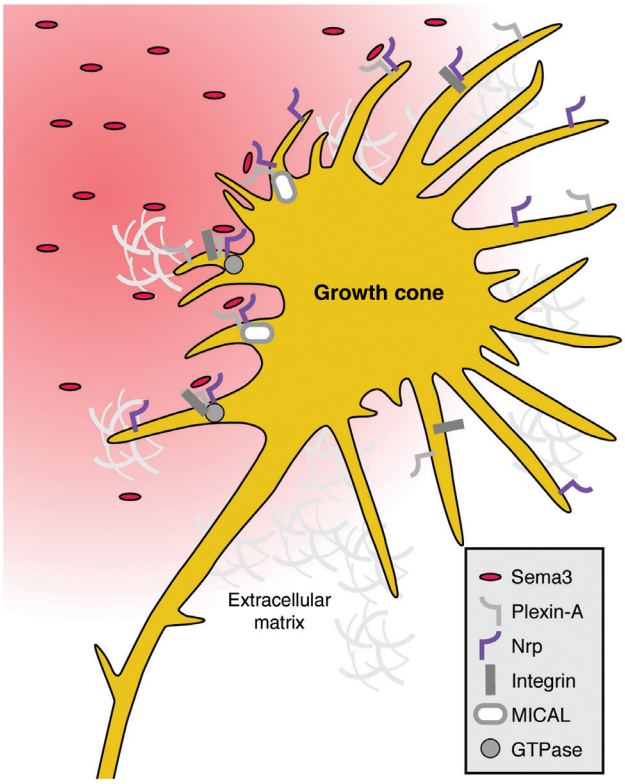

Figure 3 Nrp critically modulates neuronal growth cone function. Nrp is expressed in the axonal growth cone of migrating neurons where it couples with plexin receptors in responding to the guidance cue Sema3. Nrp functions by coupling high-affinity ligand binding to plexin-dependent cytoskeletal rearrangement through different GTPases and MICAL.

F-actin disassembly factor (100). The cellular response to Sema3 stimulation also depends on the intracellular environment. For example, the availability of the second messenger cGMP has been demonstrated to switch Sema3-dependent repulsive cues into an attractive response in neurons (101). Similar to the discussion above, the coupling of Nrp-dependent function to integrin interactions and signaling is an important aspect of the integrated cellular and physiological response to Sema3 signaling.

Nrp function as a versatile co-receptor depends on its ability to integrate multiple extracellular cues that can result in either stimulation or inhibition of the cytoskeletal dynamics (1, 102, 103). For example, VEGF-A stimulates axon outgrowth (104, 105), whereas Sema3F blocks endothelial cell migration (106). Thus, Nrp function allows cross-talk between different ligand families in different tissues and provides exquisite control of cellular migration (107, 108). The nature and regulation of this cross-talk involves competitive binding of the two ligands to the b1 domain of Nrp and is dependent on the furin processing of Sema3 (26, 44, 109, 110). While the binding of the two ligands to Nrp appears to be mutually exclusive in most cases, it is theoretically possible to support some level of Sema3 binding either to only the Nrp a1 domain or, given recent data indicating a bivalent binding mode for the Sema3 C-terminal domain with the Nrp b1 domain, partial engagement in the absence of processing (111). The unique ability of Sema3 family members to inhibit cytoskeletal dynamics is seen in both physiological and pathological settings. Indeed, Sema3A has been shown to disrupt the actin cytoskeleton of breast cancer cells, thus decreasing their migration and invasion (112).

Nrp in tumor metastasis

In addition to the important role of Nrp in development and homeostasis, aberrant Nrp pathway activation has been observed in diverse tumors, including those of the prostate, lung, gastrointestinal tract, numerous additional organs, and even hematopoietic tumors (113, 114). Importantly, both Nrp1 and Nrp2 expression contribute to the invasiveness and metastatic potential of these tumors. For example, Nrp1 functions in an autocrine pathway to promote colon carcinoma migration, lymph node metastasis, and tumor cell survival (115). As an additional example, Nrp2 expression has been reported in gastrointestinal cancer cells but not in the normal surrounding mucosa and Nrp2 knockdown leads to decreased migration and invasion *in vitro* (116). Nrp2 expression is also observed in salivary adenoid cystic carcinoma and is highly correlated with microvessel density, tumor size, invasion, and metastasis (117). A number of factors make Nrp2 a particularly promising target for therapeutic intervention (Figure 4). Specifically, Nrp2 expression is seen in a significant number of tumors where it contributes to tumor metastasis and progression. Indeed, use of an Nrp2 monoclonal antibody has been reported to decrease tumor metastasis (73, 117). Additionally, Nrp2 knockout does not result in embryonic lethality, indicating that therapeutic inhibition of Nrp2 function may be well tolerated *in vivo*. Finally, Nrp2 functions in controlling pathological lymphangiogenesis, where it contributes to tumor metastasis through both direct and indirect means (114).

Pathological Nrp activation can result from either Nrp overexpression or from the deregulation of ligand activity, such as overactive VEGF or a loss of Sema3 signaling (118). Indeed, the relative level of the opposing Sema3 and VEGF signals has been shown to regulate Nrp1-dependent cancer cell migration (119). The connection to aberrant Nrp function extends beyond stimulation by ligands.

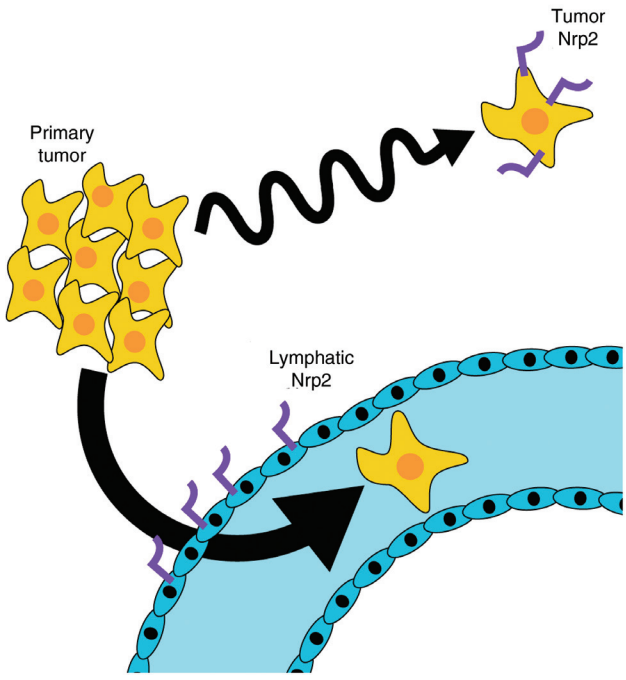

Figure 4 Nrp2 is an important therapeutic target in cancer. Nrp2 is highly expressed in a number of different cancers where it promotes cellular migration, invasion, and metastasis. Additionally, lymphatic vessel expressing Nrp2 provide a route for tumor invasion and local metastasis.

Coupling between Nrp and integrin was first recognized under pathological conditions. Nrp1 is highly expressed in pancreatic ductal adenocarcinomas where it associates with β1 integrin to promote cellular adhesion and invasion (120). An interaction between Nrp2 and $\alpha_6\beta_1$ integrin was demonstrated and was shown to regulate integrin interaction with the cytoskeleton and focal adhesion formation on laminin in breast cancer cells (121). Likewise, Nrp2 has also been demonstrated to bind α_5 integrin and mediate tumor cell extravasation, vascular adhesion, and metastasis (122).

Given their pathological roles, it is clear that Nrp receptors represent a promising therapeutic target. Initial attempts at inhibition focused on the production of a series of monoclonal antibodies against both Nrp1 and Nrp2. Intriguingly, phase I clinical trial results for MNRP1685A, a Nrp1 monoclonal antibody, showed unexpected platelet activation and thrombocytopenia (123). Other inhibitory modalities have been reported, including peptides and small molecules (124–126). Challenges with these classes of inhibitors include limited potency and selectivity. However, recent findings suggest that this is not an inherent limitation and continued development of diverse inhibitory modalities should be pursued (111, 127). Additionally, labeled Nrp-binding molecules could potentially be useful for diagnostic purposes. Also, emerging data indicate that Nrp/integrin interactions function as important autocrine tumor initiation and survival factors and thus inhibition may have additional benefit (3, 120, 128, 129). Future biochemical and structural studies will be important to guide inhibitor design.

Summary

Nrps function as critical cell surface receptors in the physiological and pathological control of cellular motility and migration. Initially, Nrp was described as an adhesive receptor in the nervous system and has been additionally shown to possess critical roles in the cardiovascular system, immune system, and beyond. Nrp functions as a versatile co-receptor in these processes by binding to multiple ligands and facilitating molecular cross-talk between the different ligand families. Understanding the nature of Nrp activation and receptor coupling is critical for understanding its function.

Continued work is needed to define the physiological role of the multiple Nrp ligand and co-receptor families of proteins, and to understand how these protein families physically engage Nrp. The basis for ligand binding has been a source of intensive study, and fundamental insights about the importance of a C-terminal arginine residue have proven fruitful (29, 53, 130–132). However, with the identification of a host of potential Nrp ligand families, continued work is needed to define the physiological role of Nrp signaling by these ligands.

In VEGF signaling, where both VEGFR and Nrp receptors are required, Nrp is intimately connected to the ability of endothelial cells to respond to ligand concentration gradients leading to directional migration. Similarly, Nrp function in the axon growth cone allows directional migration in response to Sema3 concentration gradients. In these processes, Nrp functions in ligand-dependent control of cellular motility and migration. The downstream functions of Nrps involve both direct physical coupling with integrins and regulation of cytoskeletal dynamics. Finally, Nrp function plays a critical role not only in physiological processes but also in pathological cellular migration. Aberrant activation of both Nrp1 and Nrp2 are associated with tumor aggressiveness and metastasis.

Finally, the basis for receptor/co-receptor coupling remains an important area for future research. In particular, the nature of Nrp binding to signaling receptors in both the presence and absence of ligand remains unclear. The architecture and differential specific coupling to VEGFR

family members, plexins, and other receptors remains to be determined. These insights will be particularly important in understanding additional fundamental aspects of Nrp function. Additionally, while current therapeutic strategies focus on blocking ligand binding, inhibition of receptor coupling may, in fact, prove superior to traditional inhibition strategies (133).

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