The emerging role of class-3 semaphorins and their neuropilin receptors in oncology

Patrick Nasarre
Robert M Gemmill
Harry A Drabkin

Division of Hematology-Oncology,
The Hollings Cancer Center and
Medical University of South Carolina,
Charleston, SC, USA

Abstract: The semaphorins, discovered over 20 years ago, are a large family of secreted or transmembrane and glycosylphosphatidylinositol -anchored proteins initially identified as axon guidance molecules crucial for the development of the nervous system. It has now been established that they also play important roles in organ development and function, especially involving the immune, respiratory, and cardiovascular systems, and in pathological disorders, including cancer. During tumor progression, semaphorins can have both pro- and anti-tumor functions, and this has created complexities in our understanding of these systems. Semaphorins may affect tumor growth and metastases by directly targeting tumor cells, as well as indirectly by interacting with and influencing cells from the micro-environment and vasculature. Mechanistically, semaphorins, through binding to their receptors, neuropilins and plexins, affect pathways involved in cell adhesion, migration, invasion, proliferation, and survival. Importantly, neuropilins also act as co-receptors for several growth factors and enhance their signaling activities, while class 3 semaphorins may interfere with this. In this review, we focus on the secreted class 3 semaphorins and their neuropilin co-receptors in cancer, including aspects of their signaling that may be clinically relevant.

Keywords: semaphorin, neuropilin, plexin

Introduction

Cancers arise from normal cells through a series of genetic and epigenetic changes affecting the expression and function of driver oncogenes and tumor-suppressor genes. The pathways commonly altered in various neoplasms have proven to be crucial for regulation of cell-autonomous functions such as growth and cell cycle, cell survival and senescence, energy production, and immortality, as well as non-autonomous functions such as neo-angiogenesis and evasion of the immune response. Tumor progression is associated with invasive behavior and metastases as well as resistance to therapy. In addition, the recruitment of normal cells into the tumor microenvironment plays a major role by contributing to invasion and metastasis as well as to the proliferative potential of neoplastic cells. Semaphorins and their receptors impact many of these processes. Indeed, a recent review by Rehman and Tamagnone¹ pointed out that the semaphorin/plexin/neuropilin (NRP) signaling axis influences at least seven of ten ‘hallmarks’ of cancer proposed by Hanahan and Weinberg.² Thus, the semaphorins, plexins and NRPs constitute a regulatory system deeply intertwined with multiple functions critical to the pathology of tumors and, as such, present significant opportunities for novel therapeutic interventions.

Correspondence: Harry A Drabkin
Division of Hematology-Oncology,
173 Ashley Ave, Suite 102C BSB,
Charleston, SC 29425, USA
Tel +1 843 792 4879
Fax +1 843 792 0644
Email drabkin@musc.edu

The semaphorin family contains ~25 members grouped into eight classes based on structural similarities. Semaphorins are secreted or anchored to the plasma membrane by either a glycosylphosphatidylinositol (GPI) modification at the C-terminus or a transmembrane domain. Initially described as collapsins, due to their influence on migrating neural growth cones, they all share a common and highly conserved 500 amino acid Sema domain. Aside from their function as guidance molecules in the central nervous system (CNS), semaphorins, NRPs, and plexins have been increasingly associated with both normal and pathological processes. Normal functions include organ development, tissue repair, immune responses, angiogenesis, and bone metabolism. The class 3 semaphorins are the only group of secreted soluble proteins in the semaphorin family, which, as described later, present many advantages from a therapeutic standpoint. In addition, they uniquely and specifically interact with NRPs. Consequently, semaphorin–NRP interaction prevents the binding of multiple growth factors to NRPs and blocks the activation of downstream signaling pathways, affecting, among other aspects, tumor growth, tumor-associated angiogenesis, and metastatic spread. This review specifically focuses on the role of class 3 semaphorins and NRPs in cancer as a major pathology associated with dysfunction of these pathways. In particular, we review the current knowledge about these two groups of molecules, along with potential therapeutic strategies to target them.

Structure, signaling, and function of class 3 semaphorins and their receptors

Structure

Class 3 semaphorins

The seven members of the class 3 semaphorins, SEMA3A through SEMA3G, are secreted, in contrast to all other classes that are either transmembranous or anchored to the membrane via GPI modification. Consequently, SEMA3s can have both autocrine and paracrine functions. They are defined by a 500 amino acid Sema domain, which is common to semaphorins, plexins, and the oncogenic receptor tyrosine kinases, MET and RON (Figure 1A). The Sema domain, with its 7-bladed beta-propeller structure, is crucial for protein–protein interactions and is conserved across species.³ Class 3 semaphorins are characterized by several additional conserved domains including the plexin, semaphorins, and integrin (PSI) domain, an immunoglobulin (Ig)-like and a C-terminal basic domain (BD).

Early studies revealed that dimerization is required for semaphorin function. The Sema domain, the Ig domain, and disulfide bridges established between the C-terminal BDs are essential for dimerization.^{4,5} Proteolytic cleavage of a C-terminal pro-peptide at RXXXXR consensus sites by furin-like proteases is also necessary for class 3 semaphorin function. This cleavage is believed to result in the stabilization of active semaphorin dimers, as well as the formation of a C-terminal basic motif that has high affinity for an acidic groove in the extracellular domains of NRP1 and NRP2.⁶ The resulting processed end sequence resembles the C-terminus of vascular endothelial growth factor (VEGF), as well as tuftsin, an NRP-binding peptide. The crystal structure of the PSI and Ig-like domains has recently been described, giving new insights into the mode of interaction between class 3 semaphorins, NRPs, and plexins.⁷ Unlike other semaphorins, SEMA3s usually cannot directly bind to plexins, rather requiring NRPs to stabilize a heterotrimeric complex. Low-resolution crystal structure of a semaphorin–NRP–plexin complex revealed that SEMA3 dimers bind to NRP dimers with plexins positioned on each side.⁷ Higher-order structures can also be formed through interactions involving the NRP C (MAM)-domains or transmembrane segments (see below for NRP structure). As a consequence, higher-order multimers and plexin clustering could form, possibly resulting in enhanced semaphorin signaling.

Neuropilins

NRP1 and 2 are 130 kDa type-1 transmembrane glycoproteins that share 44% sequence identity at the amino acid level (Figure 1B). Their extracellular domain contains an N-terminal signal peptide, two calcium-binding C1r/C1s/Uegf/Bmp1 (CUB) domains (designated a1 and a2), two coagulation factor V/VIII-like discoidin domains (b1 and b2), and a juxta-membrane meprin/A5-antigen/ptp-Mu (MAM or c) domain.^{8–10} The ‘a1a2’ region interacts with the Sema domain of SEMA3s,¹¹ while the ‘b1’ domain interacts with the semaphorin PSI and Ig-like domains.¹² Of note, the affinity for NRP1 and 2 varies specifically from one SEMA3 to another; NRP1 preferentially interacts with SEMA3A, SEMA3B, and SEMA3E; NRP2 has higher affinity for SEMA3F and SEMA3G, while SEMA3C binds both NRPs with similar affinity.^{12–16} NRP ‘b1b2’ domains also interact with several growth factors containing heparin-binding domains,¹⁷ including VEGFA-D,^{18–20} placenta growth factor (PIGF)-2,²¹ fibroblast growth factor (FGF),²² galectin,²³ hepatocyte growth factor (HGF),^{22,24–26} platelet-derived growth factor (PDGF),^{27–32} and transforming growth

Figure 1 General domain architecture of class 3 semaphorins and their receptors, neuropilins, and plexins.

Notes: (A) Semaphorins are characterized by a 500 amino acid Sema domain. In addition, class 3 semaphorins contain a PSI, an immunoglobulin-like domain, and a C-terminal basic-rich domain that is unique to this class of semaphorins. (B) Neuropilins are single-pass transmembrane proteins. They contain two complement binding (CUB), or a1 and a2 domains, that interact with class 3 semaphorins. They also contain two FV/FVIII coagulation factor-like domains (or b1 and b2 domains). Class 3 semaphorins bind b1 but not b2 domain, while growth factors containing a heparin-binding domain, such as VEGF, interact with both. The 'MAM', or c domain, is thought to have a role in neuropilin dimerization and does not interact with semaphorins or growth factors. The intracellular sequence of some neuropilin isoforms contains a C-terminal SEA sequence that is thought to be the only motif capable of activating downstream signaling pathways. (C) Plexins are single-pass transmembrane proteins characterized by a 500 amino acid Sema domain. In their extracellular domain, like semaphorins, they also contain PSI motifs and IPT/(G-P)-rich motifs that are involved in the interaction with semaphorins. The intracellular sequence is unique in that it contains a split GAP domain (C1 and C2) separated by a GTPase-binding domain.

Abbreviations: EXT, extracellular environment; GAP, GTPase-activating protein; G-P, glycine-proline; INT, intracellular environment; IPT, immunoglobulin-plexin-transcription; PSI, plexin, semaphorin, and integrin; VEGF, vascular endothelial growth factor.

factor (TGF)- β .^{33–36} In contrast, the 'MAM/c' domain of NRP1 and 2 is not required for ligand binding, but remains essential for signaling.³⁷ The transmembrane helix contains a conserved GXXXG motif important for dimerization and NRP interaction with other co-receptors.³⁸ The cytoplasmic domain is a short ~40 amino acid sequence lacking recognizable enzymatic functions. For this reason, NRPs are often thought to be devoid of direct signaling activity. Instead, plexins or other Sema3 co-receptors, such as L1-CAM or Nr-CAM, were viewed as the sole transducers of Sema3 signaling.^{39,40} However, NRPs also possess a C-terminal SEA motif that binds to the PDZ domain of the scaffolding protein, GIPC/NIP/synectin.^{41–45} This domain may influence signaling through GIPC1-mediated endocytic trafficking of NRPs along with interacting co-receptors, even in the absence of plexins. However, the specific downstream signaling pathways emanating from NRP-GIPC interaction remain undefined. Aside from their ability to bind Sema3-specific co-receptors, NRPs also interact with various growth factor-specific receptors and do so independently of Sema3 signaling. In this context, integrins^{45–47} and growth factor

receptors like VEGF receptor (VEGFR)1–3,^{48,49} TGF β -R1 and 2,³⁴ c-Met,²⁵ endothelial growth factor receptor (EGFR),⁵⁰ FGF receptor (FGFR),²² and PDGF receptor (PDGFR)^{28,29} have all been reported to interact with NRPs. In general, NRPs appear to increase the affinity of each ligand for its cognate receptor and, consequently, to prolong the stimulation of downstream signaling.

Several alternatively spliced isoforms have been identified for NRP1 and NRP2.^{51–54} The secreted isoforms result from the inclusion of an intron containing a STOP codon between the b2 and c domains prior to the transmembrane segment. These secreted forms are endogenous inhibitors capable of trapping growth factors in the microenvironment and blocking interaction with their cognate receptors (see below). The functions of other isoforms remain largely uncharacterized. Furthermore, whether NRP1 and NRP2 variants form homo- or heterodimers, and whether these variants interact preferentially with a specific subset of growth factors or their receptors, is unknown. NRPs are also modified by O- and N-linked glycosylation, and NRP2 is specifically modified by polysialylation.^{31,55–58} These post-translational

modifications affect ligand binding, cell migration, and invasion. Furthermore, glycosylation of NRP1 also affects the tumor microenvironment and is required for the assembly and stiffness of the extracellular matrix.⁵⁹ However, while these recent data suggest that changes in the glycosylation levels of NRPs could affect tumor growth and metastatic spread, the molecular mechanisms involved in this process and the exact consequences of altered glycosylation remain to be defined.

Plexins

Four families of plexins have been identified: plexinA1-4, B1-3, C1, and D1.^{60,61} Only a subset of these, including plexinA1-4, B2, and D1, are known to interact with class 3 semaphorins.^{60,62-67} Like NRPs, plexins are also type 1 transmembrane proteins (Figure 1C), but the cytoplasmic region is far larger, containing protein-protein interaction sites and a split Ras-GTPase activating protein (GAP) domain. The extracellular region of plexins contains Sema, PSI, and Ig-plexin-transcription (IPT)/glycine-proline (G-P)-rich domains and interacts with the Sema domain of semaphorins. In addition, the extracellular domain of plexins shares sequence similarities with MET and RON receptor tyrosine kinases. The plexin cytoplasmic domain contains an R-Ras/M-Ras GAP domain, which is separated into two segments by a Rho GTPase-binding domain (RBD). The GAP domains interact with the R-Ras/M-Ras family of small G-proteins, while the RBD interacts with another family of small G proteins that includes Rnd1/Rac1/RhoD. These effectors are thought to be largely responsible for semaphorin signaling activities.

Signaling

Class 3 semaphorins interact through their Sema domain with both NRPs and plexins. Sema3A/NRP/plexin-A signaling has been extensively studied for its effects on the cytoskeleton, frequently leading to axon repulsion, collapse, or inhibition of cell migration in various cell types. While most studies suggest that plexins are the only molecules capable of triggering an intracellular signal, several reports indicate that NRPs can transduce a signal independently. Thus, NRPs appear to function as more than a simple stabilizing component for the Sema3/plexin complex.

Signaling pathways activated by NRPs

GIPC/NIP/synectin was the first NRP-binding adaptor described that mediates NRP signaling (Figure 2A).⁴¹

The interaction of GIPC with the C-terminal SEA motif of NRP1 regulates vesicle trafficking and internalization of NRP1, VEGFR, and $\alpha 5\beta 1$ integrin.^{41,43,45,68-73} One consequence is that the NRP-GIPC interaction influences VEGF-dependent inhibition of apoptosis in neural and endothelial cells. In cancer models, NRP1 interaction with GIPC and c-Abl promotes $\alpha 5\beta 1$ integrin-dependent fibronectin fibril assembly in the tumor microenvironment. This mechanism increases the stiffness of the extracellular matrix and stimulates tumor growth.⁵⁹ In ischemic models, another effector, the cytoplasmic tyrosine kinase (TK) Fer, interacts with the cytoplasmic domain of NRP1 to induce neuronal apoptosis in response to Sema3A.⁷⁴ Whether the GIPC-binding SEA motif is responsible, at least in part, for Fer interaction with NRP1 is unknown. However, the Fer-binding domain is located within the last 18 amino acids of the extreme C-terminus of NRP1 and does not appear to bind NRP2. Since the SEA motif is completely conserved between NRP1 and 2, these observations suggest that SEA is not required for Fer binding. Fer also interacts with a family of scaffolding proteins called collapsin response mediator proteins (CRMPs).⁷⁵ The increase of NRP1/Fer/CRMP2 in lipid rafts occurs early and transiently during ischemic injury in the brain, but the function of this complex and the molecular pathways activated are unknown. Importantly, while GIPC and Fer allow at least NRP1 to signal independently from other receptors, their function and importance in cancer remain understudied and obscure.

Signaling pathways activated by plexins

The intracellular domain of plexins interacts directly with the Rho and Ras families of small G proteins. This interaction involves a less conserved spacer region (V1) wedged between two highly conserved segments (C1 and C2) that are homologous to GAPs (RasGAPs) responsible for stimulating the intrinsic GTPase activity of small G proteins in the RAS superfamily.⁷⁶ It has now been established that different plexins interact dynamically and preferentially with specific subsets of small GTPases. For example, in the quiescent state, plexin-A1 interacts with and sequesters FARP2, a Rac1 exchange factor (RacGEF) (Figure 2B).⁷⁷ However, when Sema3A binds NRP1/plexinA1, FARP2 dissociates from the plexin C-terminus, increasing the activity of Rac1. Activation of Rac1 by FARP2 leads to the association of Rnd1, a Rho family GTPase 1 protein, with the cytoplasmic domain of plexin-A1. This association increases plexin-A1-intrinsic GAP activity and leads to R-Ras inhibition and cell collapse. However, activated Rac1 also stimulates LIM-kinase-1, which phosphorylates

Figure 2 Main signal transduction pathways activated by class 3 semaphorin binding to neuropilins.

Notes: These pathways involve either neuropilin alone (**A**) or neuropilin/plexin receptor complexes (**B–D**). In the absence of plexins (**A**), semaphorins can signal through neuropilin interactions with Fer and GIPC, which regulate cell viability, matrix stiffness, and tumor growth. This latter mechanism involves integrin activation by the protein c-Abl. However, class 3 semaphorin function has been mainly described to involve plexins (**B–D**). In this context, cell migration (**B**) is regulated by the release and activation of the protein FARP2 from the plexin cytoplasmic domain, which leads to the activation of the small G-protein Rac1. In addition, R-Ras is inhibited by interacting with plexin GAP domain, and this inhibition prevents cell adhesion. The binding of the proteins MICAL (**C**), Fes, and Fyn (**D**) to the cytoplasmic domain of plexins affects actin dynamics and induces cell collapse through molecular mechanisms involving CRMPs. Sema 3 binding to neuropilin-plexin complex is also known to promote cell repulsion (or chemorepulsion) (**C**). This mechanism involves the interaction of a p190RhoGAP-p120RasGAP-FAK complex with plexins and integrins, and inactivates the small G-protein RhoA. Finally, in neural cells (**D**), the protein L1-CAM has been shown to inhibit neurite outgrowth through a mechanism involving interactions of RanBPM with plexins and integrins, and inhibition of the MAP kinases ERK1/2 by L1-CAM. Green arrows: activation; red bars: inhibitory mechanisms.

Abbreviations: CRAM, CRMP-associated molecule; CRMP, collapsin response mediator proteins; MAP, mitogen-activated protein; MICAL, mono-oxygenase molecule interacting with CasL; NRP, neuropilin; VEGFR, vascular endothelial growth factor receptor; oxid, oxidized.

and inactivates cofilin, an actin-depolymerizing molecule.⁷⁸ How this mechanism leads to cell collapse is not well understood, but the involvement of a phosphatase that would activate cofilin has been suggested. Other small G proteins have been involved in *Sema3* signaling. For example, *RhoD* binds *plexin-A1* but antagonizes *Rnd1*,⁷⁹ and would counteract *Sema3*-mediated cell collapse. In the immune system and CNS, *Rap1*, another small G protein of the *Ras* family, is involved in *Sema3A*-mediated signaling that leads to blockade of T-cell activation and growth cone collapse, respectively.^{80,81}

In endothelial cells, the *RhoGAP* protein, *p190a*, which locally converts GTP-bound *RhoA* into the inactive GDP-bound form, is important for chemorepulsion induced by *Sema3A* and *3F* (Figure 2C).^{82,83} Interestingly, *p190a* appears to be a convergence point for adhesion regulation by many pathways, including those involving $\alpha 5 \beta 1$ integrin, *syndecan4*,⁸⁴ *G(alpha)(13)*,⁸⁵ and some kinases such as *Brk*, *Src*, protein kinase C (PKC), and *AB12/ARG*.⁸³ *P190a* also associates with *p120-RasGAP* and focal adhesion proteins to regulate cell migration.^{86,87}

An additional mechanism for semaphorin-mediated collapse of actin filaments involves the mono-oxygenase molecule interacting with *CasL* (*MICAL*).^{88–93} *MICAL* is a flavin adenine dinucleotide (FAD)-dependent mono-oxygenase that interacts with the C-terminal C2 domain of A-type plexins (Figure 2C). When activated by semaphorin signaling, the *MICAL* mono-oxygenase converts two conserved methionine residues of actin (M44; M47) to methionine sulfoxides. Since these methionines are crucial for actin polymerization, their conversion causes rapid depolymerization of filamentous actin and prevents its re-polymerization. Remarkably, the oxidation reaction is reversible by methionine sulfoxide reductases (*MSRB1–3*), potentially resulting in fine control of filamentous actin polymerization – depolymerization. *MICAL* also generates hydrogen peroxide, which oxidizes *CRMP2*, leading to its dimerization, interaction with thioredoxin (*TRX*), phosphorylation by *GSK-3 β* and microtubule collapse. Semaphorins control *MICAL* activity by releasing the auto-inhibition of mono-oxygenase activity mediated by the *MICAL* C-terminal domain.⁹⁰ However, the exact mechanism of this release has not been established, although it clearly involves interaction between the C2 region of *plexinA* and the *MICAL* auto-inhibitory C-terminal domain.

As noted previously, the semaphorin-NRP axis also interacts with *Src* family tyrosine kinases. For example, *Fes/Fsp* (*Fes*) interacts with and mediates signaling downstream of activated *plexin-A1* (Figure 2D).⁹⁴ In the presence of

Sema3A/NRP1/plexA1 complex, the activation of *Fes* by *plexin-A1* stimulates *CRMP*-associated molecule (*CRAM*), as well as *CRMP1* and *2*, leading to cell contraction and growth cone collapse.^{94,95} Another *Src*-tyrosine kinase, *Fyn*, regulates the phosphorylation of *CRMP1*, and, through a *Sema3A*- and *plexinA2*-dependent mechanism, activates *Cdk5*, leading to growth cone collapse (Figure 2D).^{63,96} Similarly, in dorsal root ganglion cells, *Sema3A* induces phosphorylation of *CRMP2* by *GSK-3 β* after priming by *Cdk5*, which reduces the ability of *CRMP2* to bind tubulin, thereby destabilizing microtubule structure and impairing cell migration.⁹⁷ Moreover, increased *GSK-3 β* activity occurs as a result of *Sema3A*-mediated growth cone collapse. Mechanistically, this involves decreased phosphatidylinositol (3,4,5)-trisphosphate levels with inhibition of phosphoinositide 3 kinase (*PI3K*) by *R-Ras* inhibition and phosphatase and tensin homolog (*PTEN*) activation.^{98–100}

Additional scaffolding proteins mediate the effects of *Sema3/NRP/plexin* on the actin cytoskeleton and microtubules (Figure 2). They include *RanBPM* (Figure 2D),¹⁰¹ as well as the Myeloid translocation gene 16b (*MTG16b*).¹⁰² In some circumstances, these proteins interact together,^{90,101} or with co-receptors such as β -integrin, *Met*, and *L1-CAM*¹⁰³ to prevent non-neuronal cell spreading and inhibit axon outgrowth.

While NRPs are typically necessary to stabilize the *Sema3/plexin* complex, it has been found that *Sema3E* interacts with *plexin-D1* and controls vascular patterning independently of NRPs.⁶⁵ *Plexin-D1* expression is regulated by *VEGF* through a notch-mediated signaling pathway,¹⁰⁴ and *plexin-D1* antagonizes *VEGF* signaling by increasing the levels of the *VEGF* decoy receptor *sFlit1*.¹⁰⁵ The signaling cascade induced by *Sema3E* binding to *plexin-D1* involves *Rnd1* and *Rnd2*, which are required for the activation of *plexin's* *Ras-GAP* function, although the mechanism of interaction is not clear.¹⁰⁶ As with other class 3 semaphorins, *Sema3E* signaling results in an anti-angiogenic effect on endothelial cells, which is mediated by inhibition of *R-Ras* and disassembly of adhesive structures involving integrins.¹⁰⁷ Furthermore, *Sema3E* stimulates *PI(4)P-5* kinase activity, increasing the levels of phosphatidylinositol 4,5-bisphosphate, and activates the *Arf6* exchange factor protein, *GEP100/Brag2*, leading to *Arf6* activation, $\beta 1$ -integrin internalization, and reduced cell adhesion.¹⁰⁸

It is important to note that while *Sema3s* often cause depolymerization of actin filaments and microtubules, leading to repulsion of growth cones, this can be reversed into an

attractive effect by activating adenosine 3',5'-cyclic monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) pathways.¹⁰⁹ Other factors have also been shown to reverse the effects of *Sema3s*. For example, in the CNS, soluble L1-CAM converts an NRP1-dependent repulsive guidance response to an attractive one by activation of the nitric oxide (NO)/cGMP pathway.³⁹ The *cis* and *trans* interaction of L1-CAM with the NRP1/SEMA3A complex also controls its endocytosis, a mechanism that is mandatory for SEMA3A-mediated cell contraction.¹¹⁰ Interestingly, p53-dependent expression of cGMP-dependent protein kinase type I (cGKI) is required to enable cGMP to counteract *Sema3A*-induced growth cone collapse.¹¹¹

Function during development and injury

Class 3 semaphorins were first identified as guidance molecules in the CNS, regulating cell migration, and axon and dendrite elongation and guidance during development or after injury.^{112–115} During neural development, SEMA3s generally act as repulsive cues that block neural growth cones from progressing toward the semaphorin source. This results in the turning of axons into new directions, such as crossing the commissure. More recently, the developmental role of SEMA3s has been expanded to include angiogenesis/lymphangiogenesis,¹¹⁶ and organ morphogenesis during development of bone,^{117–119} lung,^{120–123} teeth,^{124–126} and kidney.¹²⁷ Like SEMA3s, NRPs and plexins are expressed in a wide variety of cell types and tissues, including endothelial cells, neurons, pancreatic islet cells, T-cells, hepatocytes, melanocytes, and osteoblasts, and in epithelial cells of the skin, breast, prostate, gastrointestinal tract, lung, kidney, and bladder.^{128–131} NRP1 is also expressed in the immune system by thymocytes,^{132–135} plasmacytoid dendritic cells (pDCs),^{136,137} and activated regulatory T-cells (Tr or T_{reg} cells).^{33,138–141} At the cellular level, class 3 semaphorins regulate signaling pathways that control not only cell adhesion, cytoskeletal dynamics, and migration/invasion, but also cell survival and proliferation.^{142,143}

In the adult CNS and after tissue injury, semaphorins and their receptors generally function as an impediment to nerve regrowth. *Sema3A*, in particular, has been associated with neural cell apoptosis, and class 3 semaphorins are often induced in neural and glial scars.¹⁴⁴ Both *Sema3A* and *Sema3F* seem to favor re-myelination by attracting oligodendrocyte precursors to the injured area in a model of multiple sclerosis.¹⁴⁵ Conversely, SEMA3A secreted by ischemic neurons prevents neovascularization of the injured area.¹⁴⁶ *Sema3A*, plexin-A1 and plexin-A2, and CRMP2 can form

a complex that affects Cdk5 and GSK-3 β phosphorylation in Alzheimer patients and mouse models.^{97,147} *Sema3A* and CRMP4 are up-regulated in motor neurons during pre-symptomatic stages in a model of familial amyotrophic lateral sclerosis.^{148,149} Mutations of *Sema3D* and plexin-A2 are associated with the development of schizophrenia and heightened anxiety in mice.^{150–152} Mutation of SEMA3E is associated with the CHARGE syndrome, a non-random pattern of multiple congenital anomalies usually associated with deafness/blindness that occurs in 1:10,000 births worldwide. The lack of expression of *Sema3A*, *Sema3C*, *Sema3F*, and NRP2 causes a predisposition to epileptic seizures and could be related to autism.^{153–157} *Sema3A* repulses nerve growth factor (NGF)-expressing C-fibers, which extend abnormally in the epidermis of patients affected by atopic dermatitis.¹⁵⁸ *Sema3A* levels decrease in the epidermis of patients affected by this disease. The intracutaneous or topical application of recombinant *Sema3A* reduced the density of C-fibers present in the epidermis and decreased the symptoms associated with this pathology.^{159–163}

Class 3 semaphorins and their receptors affect bone as well as cartilage development and reconstruction. *Sema3A* knockout causes abnormal bone and cartilage formation.¹⁶⁴ In fact, *Sema3A* inhibits osteoclastic bone resorption and increases osteoblastic bone formation.^{118,165} Conversely, *Sema3B* promotes osteoclastogenesis and osteopenia in a mouse model.¹¹⁷ Plexin-A2 polymorphisms have been associated with increased fracture risk and bone mineral density in a postmenopausal population.¹⁶⁶

The role of NRPs and semaphorins in the immune system has been recently reviewed.¹⁶⁷ *Sema3A*/NRP1 and *Sema3E*/plexin-D1 complexes regulate the migration of thymocytes and their interaction with thymic epithelial cells.^{133,168} *Sema3A*/NRP1/plexin-A4 also inhibits monocyte and T-cell migration and negatively affects the immune response by impairing T-cell activation and cytokine secretion.^{169,170} In addition, *Sema3A* produced by lymphatic vessels binds plexin-A1 at the surface of dendritic cells (DCs) and promotes actin-myosin and cell contraction to facilitate the transmigration of DCs through the lymphatic wall.¹⁷¹ NRP1 is a marker of T_{reg} cells,¹³⁸ and recent work by Delgoffe et al¹⁷² has shown that Nrp1 is required for T_{reg}-mediated inhibition of anti-tumor immune responses and the ability of these cells to limit or eliminate inflammatory colitis in an experimental mouse model. However, Nrp1 was not required for limiting autoimmunity or for immune homeostasis. Nrp1 effects were mediated by binding to Pten, which reduced protein kinase B (Akt) activity and led to nuclear localization of

FoxO3a and expression of survival and quiescence factors. Curiously, these investigators found that this axis required *Sema4A*, which is not known to bind *Nrp1*. However, whether *Sema4A* receptors, *plexinB1*, *B2*, or *D1* are required was not reported.

Class 3 semaphorins and cancer

Because *Sema3s* are diffusible factors, they can affect the overall growth of tumors by direct mechanisms that influence tumor cell physiology, and/or by indirect mechanisms affecting the tumor microenvironment (TME). Some class 3 semaphorins appear to function almost entirely in an anti-tumor manner (*SEMA3-D*, *-F*, and *-G*); conversely, *SEMA3-C* is primarily pro-tumorigenic. In addition, several (*SEMA3-A*, *B* and *E*) can have both positive and negative influences on tumor growth and metastases, depending on the tissue-specific context, stage of development of the tumor, the receptors expressed at the surface of the cytoplasmic membrane, the panel of growth factors with which it competes, or its ability to be cleaved by furins or proteases. Furthermore, as described in this section and discussed in the conclusion (see also Table 1), several class 3 semaphorins affect the same type of cancers, suggesting overlapping functions and raising questions about their interchangeability.

Pro-tumoral functions of class 3 semaphorins

SEMA3A, *NRP1*, and *plexinA1* messenger RNAs (mRNAs) are highly expressed in metastases from patients with pancreatic cancer, and their levels correlate with a poor outcome.¹⁷³ In addition, *SEMA3A* in pancreatic cells activates multiple pathways, including *Rac1*, extracellular signal-regulated kinase (*ERK*)-1/2, and *GSK-3 β* . *SEMA3A* is also expressed by many tumor cells and inhibits anti-neoplastic immune response by blocking T-cell proliferation, cytokine production, induction of cytotoxic activity, and T-cell adhesion to tumor cells.⁸⁰ Mechanistically, *Sema3A* inhibits cluster of differentiation (*CD*)-3/*CD28*-mediated *Ras*/mitogen-activated protein kinase (*MAPK*) activation in T-cells. This antagonistic effect of *Sema3A* involves the activation of a small GTPase, *Rap1*, which interacts with *Raf-1*. Sequestration of *Raf-1* away from *Ras* is suspected to cause *Ras*/*MAPK* inhibition. In colon cancer cells, *SEMA3A* induces cell invasion through a *Rho*-independent mechanism that involves *MAPK* signaling and *Rac1* activation.¹⁷⁴ *Rac1* is also involved in a mechanism leading to *Sema3A* infiltration in glioblastoma models.¹⁷⁵ Interestingly, in this model, it was reported that the *Sema3A* effects could switch from chemorepulsive

to chemoattractive by affecting the ratio between *NRP1* and *NRP2*.¹⁷⁶ Casazza et al¹⁷⁷ recently demonstrated that the *SEMA3A*/*NRP1* signaling axis was responsible for recruitment of tumor-associated macrophages (TAMs) into hypoxic regions of tumors. Using macrophage-specific *NRP1* knock-out mice, these investigators found that *NRP1* was essential for recruiting TAMs into hypoxic regions. This recruitment was dependent upon *SEMA3A*, *plexinA1/A4*, and the *VEGF* receptor, *VEGFR-1*. Remarkably, after entry into hypoxic areas, *NRP1* expression was suppressed by hypoxia-inducible factor (*HIF*)2 α -driven nuclear factor (*NF*)- κ B activity. This suppression, in combination with induced expression of *SEMA3A* and *VEGFR-1*, prevented further migration, trapping the TAMs in hypoxic environments. Under these conditions, the TAMs shifted their phenotype away from the pro-immune, anti-tumor 'M1' differentiation toward the 'M2' immune-suppressive and pro-angiogenic differentiation state. Thus, the *SEMA3A*/*NRP1* signaling axis in TAMs controls entry and retention in hypoxic domains of the tumor and ultimately controls immune response, neo-angiogenesis, and metastasis.

SEMA3B is overexpressed in several metastatic cell lines.¹⁷⁸ This is consistent with observations that its expression increases metastatic spread, despite the ability to inhibit primary tumor growth. These effects involve binding to *NRP1*, with activation of *p38-MAPK* and interleukin (*IL*)-8 secretion. In this context, *SEMA3B* leads to increases in tumor-associated macrophages and promotes tumor cell dissemination.

SEMA3C has pro-migratory and pro-adhesive properties on cancer cells in vitro and promotes tumor growth, angiogenesis, and metastasis in vivo.^{179–183} *SEMA3C* cleavage by a metalloproteinase, *ADMTS1*, allows its release from the extracellular matrix and promotes the migration of breast cancer cells.¹⁸¹ Recently, *SEMA3C* expression was associated with an increased risk of recurrence in prostate cancer patients.¹⁸⁴ Its overexpression was accompanied by reduced levels of *E-cadherin* and β -catenin, and by increased levels of α -integrins at the cell membrane.¹⁷⁹ Interestingly, *SEMA3C* is up-regulated by the oncogene *ERBB2*, which is amplified or overexpressed in many breast tumors and other cancers.¹⁸⁵ The *SEMA3C* gene contains a binding motif for *Sox4*, a transcription factor involved in metastatic spread in hepatocellular carcinoma and other cancers.¹⁸⁰ Thus, *SEMA3C* expression may be a component of the pro-tumorigenic program induced by *SOX4*. The promoter region of *SEMA3C* also contains a conserved *E-box* element that can bind *Twist1*, a transcription factor

Table 1 Summary of class 3 semaphorin functions in cancer models and in cell types that influence tumor growth

Class 3 semaphorin	Organ/tissue/cell type	Levels in patient samples and/or function	References
SEMA3A	Pancreatic cancer	Induces cell scattering, invasion High levels associated with poor outcome	173
	Colon cancer	Increases invasion and tissue infiltration	174
	Glioblastoma	Increases invasion and tissue infiltration	175
	T-lymphocytes	Inhibits anti-neoplastic immune response	80
	Tumor-associated macrophages	Stimulates recruitment to the tumor and differentiation into immune-suppressive and pro-angiogenic 'M2' state	177
	Breast cancer	Levels correlate positively with sensitivity to chemotherapy and decrease with stage Inhibits adhesion, motility, and cell invasion	192,193,196,198,200
	Tongue cancer	Levels correlate positively with patient survival and negatively with lymph node metastases	194
	Prostate cancer	Inhibits motility and cell invasion	179,182
	Multiple myeloma	Inhibits neo-angiogenesis Prevents excessive proliferation induced by growth factors	197
	Mesothelioma	Prevents excessive proliferation induced by growth factors	199
	Melanoma	Inhibits tumor growth and metastases Sensitizes cells to anti-tumor agents	201
	Pancreatic cancer	Reduces invasion and metastases induced by the resistance to sunitinib	202
	Cervical cancer	Reduces invasion and metastases induced by the resistance to sunitinib	202
	Breast cancer	Increases metastasis	178
	Lung cancer	Increases metastasis	178
SEMA3B	Ovarian cancer	Reduced levels in tumors Inhibits tumor growth, colony formation, adhesion, invasion, and cell viability	204,214,217
	Lung cancer	Reduced levels in tumors Inhibits cell and tumor growth, induces apoptosis	203,206,213,215,216
	Gallbladder cancer	Reduced levels in tumors	205
	Liver cancer	Reduced levels in malignant tumors	207
	Breast cancer	Reduced levels Inhibits cell growth and induces apoptosis	193,215,216
	Gastric cancer	Reduced levels in tumors	209
	Neuroblastoma	Reduced levels in tumors	208
	Prostate cancer	Predictive marker for biochemical recurrence Increases cell adhesion and invasion	179,182,184
	Hepatocellular cancer	Increases cell invasion	180
	Breast cancer	Increases cell migration	181
SEMA3C	Gastric cancer	Highly expressed in tumors Increases tumor growth and metastasis, reduces apoptosis, and increases proliferation in tumors Increases tumor-associated angiogenesis	183
	Breast cancer	Inhibits tumor growth	218
	Glioblastoma-multiforme	Inhibits tumor growth Inhibits tumor-associated angiogenesis Inhibits cell survival	219
SEMA3D	Ovarian endometrioid carcinoma	Levels correlates with high-grade tumors	189
	Melanoma	Expressed in invasive tumors	190
	Cervical cancer	Increases cell migration	
SEMA3E	Lung	Inhibits primary tumor growth Promotes metastasis Increases cell migration and invasion	190,191

(Continued)

Table 1 (Continued)

Class 3 semaphorin	Organ/tissue/cell type	Levels in patient samples and/or function	References
	Breast	Inhibits primary tumor growth by reducing vessel density Enhances tumor cell survival and extravasation and promotes metastasis Increases cell migration and invasion	188,190
	Colon	Inhibits primary tumor growth, reduces vessel density, and increases tumor cell apoptosis Increases cell migration	190
	Prostate cancer	Inhibits migration and invasion	182
	Glioblastoma	Inhibits tumor growth	219
	Melanoma	Absent in metastases Inhibits metastases	220
SEMA3F	Lung cancer	Reduced levels or loss of expression in tumors Inhibits tumor growth and angiogenesis Reduces colony formation, cell growth	213,223–225,227,229, 230,236,239,240,246
	Kidney cancer	Reduced levels in tumors Inhibits tumor growth and angiogenesis	227,235
	Cervical cancer	Reduced levels in tumors	226
	Ovarian cancer	Reduced expression in carcinoma and cancer cell lines Inhibits colony formation, cell adhesion, cell invasion, and endothelial tube formation	204,217
	Breast cancer	Reduced expression in invasive tumors Reduces tumor growth and tumor invasion Inhibits tumor angiogenesis Inhibits cell–cell contacts, cell spreading, cell adhesion, and has chemorepulsive activity	193,218,227,233,237, 243
	Prostate cancer	Single polymorphism associated with increased cancer risk and poor prognosis	228
	Endometrial cancer	Reduced levels in tumors Inhibits cell growth, colony formation, and cell invasion	231
	Glioma/glioblastoma	Induces cell collapse, inhibits cell proliferation, migration, and invasion Inhibits tumor growth	83,219,241
	Melanoma	Reduced levels in metastatic cell lines Inhibits tumor angiogenesis and metastasis Inhibits cell adhesion, migration, invasion, and proliferation	234,238,241
	Colorectal cancer	Inhibits tumor growth and metastases	242
	Schwannomas	Induces vessel normalization in the tumors	244
	Malignant mesothelioma	Loss of expression	247
	Glioma	Expression correlates with patient survival	248
	Glioblastoma-multiforme	Inhibits tumor growth and angiogenesis Inhibits migration and invasion	219,250

involved in the epithelial to mesenchymal transition (EMT); in the heart, Twist1 was shown to up-regulate *Sema3C* expression.¹⁸⁶ Therefore, by facilitating mesenchymal transformation, *Sema3C* could promote tumor cell invasion and metastasis.

Sema3E was identified as a gene commonly expressed in murine mammary adenocarcinoma cell lines capable of spreading to the lung and bones, but only rarely expressed in non-metastatic cells.^{187,188} In addition, *SEMA3E* levels correlate with high-grade ovarian endometrioid carcinoma.¹⁸⁹

While *SEMA3E* has anti-angiogenic properties and inhibits tumor growth, its overexpression in several cancer models promotes transendothelial migration and metastatic spread.¹⁹⁰ This depends on plexinD1-associated human epidermal growth factor receptor (HER)-2/Neu (ERBB2) oncogenic kinase activity. Moreover, the furin-cleaved 61 kDa form of *Sema3E* converts its repulsive activity into a pro-migratory/invasive function in tumor cell lines.¹⁹¹ Supporting a pro-invasive function for *SEMA3E*, a recent study showed that this semaphorin induces EMT,¹⁸⁹ an

effect that depends on PI3K and ERK/MAPK-mediated Snail1 translocation to the nucleus. SEMA3E also promotes tumor cell survival. In the absence of Sema3E, plexin-D1 interacts with the nuclear receptor NR4A1 and triggers a mitochondrial-dependent death pathway leading to Caspase 9 activation.¹⁸⁸ Conversely, in the presence of Sema3E, the plexin-D1-NR4A1 complex is disrupted, which prevents tumor cell apoptosis. Supporting these results, a peptide consisting of the Sema domain of plexin-D1 was shown to promote plexin-D1-mediated apoptosis in vitro, and inhibit tumor growth and metastasis in vivo, by trapping Sema3E.

Tumor-suppressive functions of class 3 semaphorins

SEMA3A expression correlates positively with increased sensitivity to radiation therapy in cancers, and SEMA3A levels decrease in breast cancer tumors with the transition from in situ to invasive carcinoma.^{192,193} SEMA3A is also down-regulated in cancers of the tongue, and levels correlate positively with patient survival and negatively with lymph node metastases.¹⁹⁴ In several cancer cell lines, SEMA3A is down-regulated by the chromatin-associated and pro-tumoral factor, high mobility group box 1 (HMGB1), which promotes heterochromatin formation and decreased occupancy of acetylated histones at the *SEMA3A* genetic locus.¹⁹⁵ Functionally, SEMA3A inhibits the migration and invasion of breast and prostate cancer cells, as well as the ability of multiple myeloma cells to induce neo-angiogenesis in vitro.^{179,182,196–198} In normal mesothelial cells, Sema3A inhibits VEGF-mediated upregulation of cyclin D1 and induction of cell proliferation.¹⁹⁹ Intriguingly, Sema3A is itself upregulated by VEGF in mesothelial and endothelial cells.^{197,199} However, in malignant mesothelioma and multiple myeloma, this pathway is disrupted, leading to increased cell proliferation. Therefore, it has been suggested that, in normal cells, SEMA3A signaling serves as a negative feedback loop to prevent excessive proliferative effects induced by growth factors. In breast cancer models, SEMA3A inhibits adhesion and migration of tumor cells by increasing integrin $\alpha 2\beta 1$ levels and promoting RhoA translation through a mechanism involving eIF4E.^{198,200} SEMA3A also sensitizes tumor cells to anti-tumor agents such as curcumin and dacarbazine, while curcumin was shown to promote apoptosis and poly ADP ribose polymerase (PARP) cleavage induced by SEMA3A.²⁰¹ In models of drug resistance induced by either chronic exposure to sunitinib, a prototypical small-molecule TK inhibitor, or DC101, a VEGFR-2-blocking antibody, Sema3A prevented tumor invasion and metastasis.²⁰² This effect correlated with

increased tumor perfusion and oxygenation, together with a prolonged vascular normalization window. As a consequence, Sema3A inhibited sunitinib-induced hypoxia and NF- κ B activity, reducing HIF-1 α levels to baseline.

SEMA3B was originally cloned from 3p21.3, a chromosomal region affected by homozygous deletions and frequent loss of heterozygosity (LOH) in lung, ovarian, and gallbladder cancers.^{203–205} Reduced SEMA3B mRNA levels correlate with frequent promoter hypermethylation in neuroblastoma, lung, liver, breast, gastric, and gallbladder cancers.^{193,205–209} In vitro, SEMA3B expression is up-regulated by direct binding of p53 to its promoter region and is downregulated by promoter methylation.^{210–212} SEMA3B inhibits tumor cell growth and induces apoptosis in vitro and in vivo.^{213–215} The anti-proliferative and pro-apoptotic effects of SEMA3B involve inhibition of the Akt signaling pathway in lung and breast cancer cells.²¹⁶ Although the mechanism of AKT inhibition by SEMA3B has not been established, it may involve PTEN binding to NRP1.¹⁷² In ovarian cancers, SEMA3B expression is restored by follicle-stimulating hormone and estrogens, and its expression inhibits colony formation, cell adhesion, invasion, and cell viability.²¹⁷

SEMA3D function in cancer is largely unknown, but data support some anti-tumor activity. To date, SEMA3D has been shown to inhibit breast and glioblastoma-multiforme (GBM) tumor growth, to reduce tumor-angiogenesis, and to prolong the survival of mice bearing orthotopic GBM xenografts.^{218,219} However, the signaling pathways mediating these effects of Sema3D have not been identified. In addition, further work must be done to establish whether SEMA3D differentially affects the several molecular subtypes that characterize both breast cancers and GBMs.

SEMA3E is the only class 3 semaphorin known to interact with a plexin independently of NRPs. Thus, expression of the SEMA3E receptor, plexinD1, is crucial for responses to this ligand. In melanoma, SEMA3E overexpression has anti-metastatic effects, while plexinD1 levels correlate positively with tumor progression and metastasis. Moreover, SEMA3E levels are inversely correlated with plexinD1.^{219,220} SEMA3E inhibits prostate cancer cell adhesion/migration, as well as glioblastoma tumor growth in an orthotopic model.¹⁸² In endothelial cells, SEMA3E binding to plexinD1 exerts an anti-angiogenic effect by inhibiting R-Ras and activating a pathway involving PIP5KI β , GEP100/Brag2, and Arf6, leading to the inactivation of integrins and disassembly of adhesive structures.^{108,221} In contrast to the pro-tumoral effects of furin-processed p61 SEMA3E, this anti-angiogenic effect is mediated by a furin-resistant mutant that also inhibits

tumor growth and prevents metastases in several tumor models.^{190,219,222}

SEMA3F, like *SEMA3B*, was cloned from the 3p21.3 homozygous deletion region identified in some small cell lung cancer (SCLC) cell lines.^{223–225} This region is also affected by frequent LOH in renal, lung, cervical, ovarian, and breast cancers.^{204,226,227} In prostate cancer, polymorphisms in the *SEMA3F* gene correlate with a poor prognosis in Hispanic and non-Hispanic White men.²²⁸ In normal lung, we showed that SEMA3F localizes at the cell surface, while in tumors, the presence of SEMA3F in the cytoplasm correlates with the presence of VEGF at the membrane of tumor cells, consistent with an antagonistic relationship between these ligands.^{229,230} In addition, SEMA3F levels are frequently reduced and inversely correlated with tumor stage in lung, endometrial, and ductal breast cancers.^{193,229–231} SEMA3F has potent tumor suppressor activity that inhibits adhesion, migration, colony formation, and occasionally proliferation in vitro. In several in vivo cancer models, SEMA3F inhibits tumor growth and neo-angiogenesis, as well as metastatic spread.^{83,213,217–219,231–244} Expression of SEMA3F is induced by p53,²⁴⁰ the transcription factor E47,²⁴¹ and the transcriptional regulator, retinoid orphan nuclear receptor (ROR)- α .²⁴³ Conversely, SEMA3F expression can be inhibited by multiple mechanisms including methylation of the promoter,²⁴⁵ by the EMT-induced transcription factor, ZEB-1,²⁴⁶ and the inhibitor of E47, Id2.²⁴¹ SEMA3F induces retraction of lamellipodia, loss of cell–cell contacts accompanied by a delocalization of E-cadherin and β -catenin, and delocalization of Rac1-GTP at the base of the receding lamellipodia.^{233,237} Mechanistically, SEMA3F inhibits kinase pathways involving integrin-linked kinase (ILK), ERK1/2, AKT, signal transducer and activator of transcription (STAT)-3, and focal adhesion kinase (FAK), as well as integrin β 1 and α V β 3 activation and expression. It also reduces levels of matrix metalloproteinase (MMP)-9, MMP2, HIF-1 α , and VEGF.^{217,234,236,239,242} In a glioma model, SEMA3F was further shown to induce cell collapse by preventing stress fiber formation through a mechanism involving NRP2-plexinA1, along with the inactivation of ABL2/ARG, p190RhoGAP and RhoA and cofilin (Figure 2B).⁸³

SEMA3G is the most recently identified class 3 semaphorin, and its function and mechanism of action in cancer remains largely obscure. Data currently available indicate that SEMA3G has primarily a tumor suppressive function. The *SEMA3G* gene is located in 3p21.1, a region like 3p21.3 that is affected by frequent LOH in malignant mesothelioma.²⁴⁷ Its expression correlates with increased overall survival in patients with glioma.²⁴⁸ In addition, SEMA3G

is expressed by endothelial cells and has a destabilizing effect on endothelial cell–smooth muscle cell interactions.²⁴⁹ SEMA3G also prevents migration and invasion in an autocrine and paracrine fashion by inhibiting the activity of MMP2 in vitro, and inhibits tumor growth in an orthotopic model of GBM.^{219,250}

Role of NRPs and interacting pro-tumorigenic growth factors in cancer

NRPs are frequently overexpressed in cancer, and their levels have been correlated with more aggressive disease in several tumor types, including, for example, melanomas and carcinomas of the breast and lung.²⁵¹ NRPs are expressed by endothelial cells lining blood and lymphatic vessels, where they enhance VEGFR signaling during tumor angiogenesis.^{234,235} Moreover, several studies have shown that both NRPs are expressed on the plasma membrane of malignant cells. In this context, NRPs usually enhance tumor cell survival and growth while promoting migration and invasion into local and distant tissues. In lung cancers, we showed that levels of NRP1 and NRP2 increase in the progression from dysplasia to micro-invasive carcinoma and are correlated with VEGF levels.²³⁰ NRPs are expressed in a high proportion of resected lung tumors, and their levels correlate with advanced stages, mesenchymal transformation, invasion, and poor prognosis.^{129,130,252,253} NRPs promote tumor growth and invasion in cancers derived from other tissues, including colon,³⁶ gastrointestinal tract,²⁵⁴ kidney,²⁵⁵ skin,²⁵⁶ prostate,²⁵⁷ breast,²⁵⁸ pancreas,^{25,46} and brain,^{259,260} among others. Mechanistically, reduced levels (or loss) of tumor-suppressive class 3 semaphorins combined with increased growth factor receptor levels contribute to the pro-tumoral function of NRPs. However, the exact molecular pathways affected by NRPs in each case are difficult to define because 1) NRPs interact with multiple pro-tumoral ligands and their cognate receptors, and 2) NRPs are expressed by many cell types, both in the tumor and in the tumor microenvironment. The role of NRPs in response to growth factors during tumor progression is summarized and discussed below. It should be noted that most studies have focused on NRP1, with relatively few addressing the role of NRP2. While their strong sequence homology supports the concept that NRP2 functions are redundant with NRP1, experimental data supporting this are rather thin, and there are several examples of clearly distinct roles for the two NRPs.

Of all the non-semaphorin ligands for NRPs, VEGF and the VEGF-signaling pathway is the most thoroughly

explored and the best understood (Figure 3). Indeed, NRPs are expressed at the surface of endothelial cells in both the arterial/venous (NRP1) and lymphatic (NRP2) systems, and their expression contributes to tumor-angiogenesis and lymphangiogenesis, respectively. Initial studies showed that VEGF-A binding to VEGFR-2/NRP1 or NRP2 receptor complexes promoted endothelial cell proliferation, migration, and tube formation in vitro as well as angiogenesis in vivo.^{234,235,261,262} In endothelial cells, NRPs enhance the binding of VEGF-A₁₆₅ to its receptor and increase ERK1/2 MAPK activation.²⁶³ The VEGF-A/NRP1/proline-rich TK2 (PYK2/PTK2B)/p130Cas (BCAR1) axis is also required for endothelial cell chemotaxis.³² NRP2 promotes lymphangiogenesis through a mechanism involving VEGF-C and VEGFR-3.²⁰ Blocking the VEGF-C binding site with an NRP2-blocking antibody inhibits tumor lymphangiogenesis and metastatic spread to local lymph nodes and distant sites.²⁶⁴ In addition to its effects on angiogenesis, VEGF-A₁₆₅ promotes tumor cell survival through an NRP- and PI3K/AKT-dependent mechanism.^{265–267} VEGF-A₁₆₅ also promotes physical interaction between NRP1 and c-MET, facilitates c-MET, Src kinase, and STAT3 activation, and leads to the upregulation of the pro-survival factor, MLC-1.²⁶⁸ VEGF-C binding to NRP2

prevents oxidative stress and promotes cancer cell survival and autophagy.^{269,270} While the mechanism is unknown, the VEGF-C/NRP2 axis inhibits mammalian target of rapamycin complex (mTORC)-1 activity, relieving its suppression of autophagy and thus contributing to tumor cell survival under stress. Tumor cell proliferation is induced by VEGF-A binding to NRP1 through a Ras-dependent mechanism.²⁷¹ Adhesion to extracellular matrix and therefore motility and invasive capabilities are also affected by VEGF/NRP pathways. For example, VEGF-A/NRP2 activates PKC to promote integrin $\alpha 6 \beta 1$ -dependent adhesion to laminin, a mechanism that also involves integrin $\alpha 6 \beta 1$ -mediated activation of FAK and Src.²⁷² Tumor cell invasion is induced by VEGF-A/NRP1-dependent induction of chemokine receptor (CXCR)-4.²⁵⁸ VEGF-A/NRP1 also stimulates the EMT using a mechanism that involves GSK-3 β inhibition and Snail translocation to the nucleus.^{273,274} Osteopontin, a ligand for integrins and CD44 that has pro-metastatic functions, was shown to promote tumor growth and angiogenesis by inducing VEGF-A expression.²⁷⁵ Mechanistically, osteopontin promotes Brk/NF- κ B-inducing kinase (NIK)-dependent NF- κ B activation, which translocates ATF4 into the nucleus, inducing the expression of VEGF-A. Consequently, VEGF binding to NRP1 at the surface of tumor

Figure 3 Signal transduction pathways activated by VEGF binding to neuropilins.

Notes: Among the growth factors that neuropilins can bind, VEGF is the most described. VEGF has autocrine and paracrine functions and influences tumor cells as well as cells from the tumor microenvironment. VEGF/NRP signaling increases adhesion, migration/invasion, proliferation, and survival, and inhibits differentiation of cells during angiogenesis, lymphangiogenesis, tumor growth, and metastasis. While neuropilins can bind VEGF-A, -B, and -C, most mechanisms that have been described involve VEGF-A and -C. The receptor complexes are either NRP-VEGFR-2 or NRP-cMET for VEGF-A. For VEGF-C, the receptor complex contains NRP and VEGFR-3. Green arrows: activation; red bars: inhibition.

Abbreviations: EMT, epithelial to mesenchymal transition; ERK, extracellular signal-related kinase; FAK, focal adhesion kinase; HGF, hepatocyte growth factor; INT, intracellular environment; mTORC1, mammalian target of rapamycin complex I; NRP, neuropilin; PI3K, phosphoinositide 3 kinase; STAT, signal transducer and activator of transcription; VEGF, vascular endothelial growth factor.

and endothelial cells increases their motility. In two studies, VEGF-A was shown to directly increase, through NRP1, the pool of cancer stem cells (CSC) in skin cancer and GBM, and to promote CSC proliferation and tumor growth.^{256,276} Recently, in a breast cancer initiation model, VEGF/NRP2 was shown to activate the $\alpha 6 \beta 1$ integrin signaling pathway, leading to FAK and downstream RAS/MEK activation. This led to activated Gli1 through a non-canonical pathway that did not involve the hedgehog components, SMO and suppressor of fused (SUFU). Gli1 was shown to induce BMI-1, a key stem cell factor and component of the polycomb complex 1. Interestingly, this pathway involves an autocrine loop, since Gli1 also induces VEGF and NRP2.²⁷⁷ However, most of these studies have not clearly established whether the tumor cell function of NRP was completely independent of VEGFR-1, -2, or -3 or required interaction with these canonical receptors.

PlGF-2, a VEGF family member, was the second growth factor identified to physically interact with NRPs.^{262,278} However, while the expression of PlGF and NRP has been correlated with poor prognosis in cancer,^{279,280} very little is known about either the pathways activated by PlGF/NRP interaction or their cellular function(s). Nevertheless, a recent study showed that inhibition of the PlGF/NRP1 pathway has

anti-tumor and anti-metastatic effects on medulloblastoma (Figure 4).²⁸¹ Interestingly, PlGF expression is induced by sonic hedgehog (Shh) in the stroma and regulates the survival of NRP1-expressing tumor cells (Figure 4). Of note, in another model, NRP1 transcription was induced by Shh, and NRP1 overexpression stimulated Shh signaling, supporting the hypothesis of a positive feedback loop.²⁸² In renal cancer, NRP1-driven Shh signaling activation promotes an undifferentiated phenotype.²⁵⁵ However, whether these pathways depend upon or modulate the response to PlGF is unknown.

Galectin-1 has recently been identified as an NRP ligand. Galectins comprise a large family of β -galactoside-binding proteins characterized by one or two carbohydrate-binding domains.²⁸³ Galectins are found in both the cytoplasm and the extracellular milieu, where they can link glycoproteins with N- or O-linked glycan moieties by dimerization. Galectins have been linked to angiogenesis, tumor cell migration, and adhesion. Galectin-1 is highly expressed in tumor-associated endothelial cells, and its binding to NRP1 induces VEGFR-2 phosphorylation, stress-activated protein kinase (SAPK1)/Jun amino-terminal kinase (JNK) activation, and promotes cell proliferation and adhesion (Figure 4).²³ Interestingly, galectin-1 is highly expressed by mesenchymal stem cells

Figure 4 Role of neuropilins in signaling pathways activated by growth factors other than VEGF.

Notes: It is important to note that, while neuropilins bind several growth factors and improve their function, the molecular mechanisms affected by neuropilins remain often unclear. Also, for some growth factors, like TGFβ, the role of neuropilins could be tissue/context dependent. Indeed, in some models, neuropilins bind TGFβ directly and increase Smad activation. In other models, TGFβ promotes the expression of neuropilins or neuropilin ligands, such as VEGF, to increase invasion and metastatic spread.

Abbreviations: EMT, epithelial to mesenchymal transition; ER, estrogen receptor; ERK, extracellular signal-related kinase; HGF, hepatocyte growth factor; HIF, hypoxia-inducible factor; JNK, Jun amino-terminal kinase; MSC, mesenchymal stem cells; NRP, neuropilin; PDGF, platelet-derived growth factor; PDGFR, PDGF receptor; PlGF, placental growth factor; SAP, stress-activated protein; SMC, smooth muscle cell; TGF, transforming growth factor; VEGF, vascular endothelial growth factor.

(MSCs) and inhibits the proliferation of NRP1-expressing T-cells.²⁸⁴ This latter activity suggests that galectin-1 could have a suppressive effect on the immune system and create an environment favorable for tumor development.

While it is not proven that NRPs directly bind HGF, several studies indicate that NRPs interact with c-Met, leading to increased HGF/c-Met signaling. NRP1 promotes glioma progression by increasing tumor cell survival and proliferation in an HGF/c-Met-dependent manner (Figure 4).²⁴ In prostate cancer cells, VEGF increases physical interaction between NRP1 and c-MET and stimulates c-MET phosphorylation (Figure 3). This leads to Src and STAT3 activation, and results in increased tumor cell survival.²⁶⁸ Whether this mechanism involves HGF is unknown. However, in pancreatic cancer cells, invasion is mediated by HGF and is also increased by NRP1 expression.²⁸⁵ This response is mediated through the activation of p38, Src, and AKT.

PDGF has been shown to physically interact with NRP1.²⁷ In breast cancer cell models, PDGF secreted by tumor cells promotes the migration of NRP1-expressing vascular smooth muscle cells (SMCs) (Figure 4). In addition, PDGF-B interacts with NRP1 and promotes the differentiation of MSCs into pericytes.³⁰ However, the pathways regulating these two functions have not been defined.

Very recently, both NRP1 and 2 have been identified as co-receptors for the latent and active forms of TGF β , a key factor that drives the EMT.^{33,34} TGF β exerts its effects by interacting with a receptor complex that transduces the signal through TGF β RI. In canonical signaling, TGF β RI phosphorylates R-Smads (Smad2 and 3), which interact with Smad 4 and translocate to the nucleus.²⁸⁶ Alternatively, TGF β can also signal through non-canonical pathways, such as ERK1/2, PI3K/Akt, JNK/p38, and Rho-like GTPases.²⁸⁷ NRPs interact with TGF β RI and RII, affecting TGF β canonical signaling and EMT induction, as well as cell phenotype, migration, and invasion (Figure 4).^{34–36} However, in a prostate cancer model, NRP1 was shown to be indirectly targeted by TGF β .²⁷⁴ Indeed, Mak et al²⁷⁴ showed that TGF β induces hypoxia and HIF-1 α expression by inhibiting estrogen receptor (ER)- β . As a consequence, levels of VEGF-A increased and, through an NRP1-dependent mechanism, induced GSK-3 β inhibition and Snail translocation to the nucleus, leading to the loss of E-cadherin, and increased migration/invasion (Figure 4). Of note, none of these previously described studies reported an effect of TGF β stimulation on NRP expression. In contrast, we recently observed that TGF β 1 up-regulates NRP2 expression in lung cancer models (Figure 4).²⁵² A similar induction of NRP2 had been

found in a model of renal fibrogenesis.²⁸⁸ In fact, TGF β stimulates NRP2 translation but has at most a moderate effect on its transcription. This mechanism involves TGF β non-canonical pathways, including ERK1/2 and AKT, and, to some extent, ZEB-1, a transcription factor involved in EMT. In turn, NRP2 expression also stimulates ERK1/2, inhibits epithelial gene expression, promotes mesenchymal gene expression, and increases migration and invasion in vitro and in vivo.

Strategies developed to target semaphorin signaling

Three main avenues have been pursued to develop anti-tumor therapeutic strategies targeting semaphorin signaling: 1) restoring the tumor-suppressive effects of SEMA3s; 2) inhibiting the pro-tumoral effects of other SEMA3s; 3) blocking the pro-tumoral effects of growth factors that also bind NRP by inhibiting NRP function.

Restoring Sema3-mediated tumor-suppressive effects

Strategies used to restore the expression of anti-tumoral class 3 semaphorins either involved compounds that restore expression in tumor cells or vectors and genetically modified delivery systems that specifically target tumor cells. An alternative approach does not restore anti-tumor SEMA3 expression, but exploits peptides derived from the semaphorin sequence as a system to deliver toxic agents to cancer cells and prevent tumor progression.

Compounds that can restore SEMA3 expression in tumors include steroid hormones. For example, in endometrial cancer, low levels of SEMA3B and SEMA3F increase in response to progesterone (P4) and 1,25-dihydroxyvitamin D(3) (1,25(OH)(2)D(3)), two molecules that reduce tumor growth by increasing apoptosis.²³¹ Conversely, the down-regulation of these two semaphorins attenuates the growth inhibition mediated by these two drugs, highlighting the important role of both semaphorins in this response. In pancreatic cancer, (-)-epigallocatechin-3-gallate (EGCG), a bioactive agent found in green tea, inhibits tumor growth, in part by up-regulating SEMA3F and down-regulating VEGF and NRP expression.²⁸⁹ EGCG blocks the EMT process by inhibiting the ERK1/2 and PI3K/AKT pathways, and by increasing E-cadherin and decreasing N-cadherin, as well as ZEB1 expression. Interestingly, we recently showed that NRP2 expression is induced by TGF β 1-mediated activation of ERK1/2, AKT, and, at least in part, ZEB1, in lung cancer.²⁵² This suggests that EMT and SEMA3F/NRP2 pathways could antagonize each other in a broad

spectrum of cancers and that EGCG could have a potential therapeutic effect in SEMA3F-negative tumor cells that express NRP2.

The second strategy consists of increasing the concentration of SEMA3s in the tumor using cell-based delivery systems. This has recently been achieved using transformed tumor-infiltrating Tie2 monocytes to deliver SEMA3A.²⁹⁰ SEMA3A released from these monocytes affects tumor vasculature and vessel functionality, reducing the growth of primary tumors and the amount of metastases found in the lungs.

A third strategy uses a peptide sequence matching a portion of a SEMA3 as a 'drug-delivery system' to carry a linked toxin into cancer cells in order to inhibit tumor growth. For example, a peptide derived from the SEMA domain of SEMA3A was fused to a cytotoxic lytic peptide containing D- and L-cationic-rich amino acids.²⁹¹ This amino acid sequence forms amphipathic partial α -helices that specifically disrupt the cancer cell membrane. The 'SEMA-lytic' hybrid proved to have a potent apoptotic effect on cancer cells expressing NRP1. Importantly, no cytotoxic effect was observed on normal cells, *in vitro*. However, the effects of this 'SEMA-lytic' hybrid peptide have not yet been reported *in vivo*.

Blocking the pro-tumoral effects of SEMA3s

A preclinical study was recently reported in which a plexin-D1-derived polypeptide was used as a ligand trap to inhibit SEMA3E tumor-promoting effects.¹⁸⁸ The polypeptide contained the SEMA domain and two flanking PSI domains of plexin-D1. The authors showed that repeated intra-peritoneal injections of this polypeptide were able to inhibit tumor growth and reduce metastatic spread in two breast cancer models. Indeed, in the absence of the ligand, plexin-D1 binds to the nuclear receptor NR4A1 and mediates apoptosis.

SEMA3A immuno-suppressive and pro-tumoral functions in glioblastoma and colon cancers could be inhibited by a selective inhibitor, SM-216289, that interferes with SEMA3A binding to NRP1.²⁹² Similar results were obtained with an antibody, YW107.4.87, directed specifically against the semaphorin-binding domain of NRP1.²⁹³ Indeed, the inhibitor counteracted SEMA3A negative effects on axon regeneration and neuron survival in a spinal cord injury model and the antibody blocked SEMA3A-induced neuron collapse.

Blocking the pro-tumoral function of NRP

Administration of natural soluble variants of Nrpl (sNRP1) can inhibit the tumor-promoting effects of NRP by acting

as a trap for multiple ligands. While it is known that NRP1 interacts with several growth factors, the strategy was initially developed to trap VEGF and this showed some tumor-inhibiting effects.^{51,294} Other strategies consist of inhibiting NRP expression by short hairpin RNA (shRNA) or small interfering RNA (siRNA) or blocking their function with peptides or antibodies. Several studies describe peptides and small-molecule inhibitors that have been developed to inhibit NRP function.^{38,253,267,295–308} Most of these studies were originally designed to identify peptides that would prevent VEGF binding to NRP1 and, therefore, would inhibit downstream VEGF signaling and function. From these studies, a minimal consensus amino acid sequence, R/KXXR/K, present in the C-terminal sequence of VEGF-A, has been identified that is crucial for VEGF binding to the 'b1' domain of NRP1. Peptides derived from this so-called C-end Rule (CendR) sequence prevented NRP1/VEGFR-2 complex formation, inhibited VEGF signaling, and promoted receptor internalization. A recent report showed that NRP2 can also internalize CendR peptides.³⁰⁶ In addition, dimeric and tetrameric forms of these peptides have even higher affinity for NRPs than does the monomeric form.²⁹⁶ CendR peptides induce apoptosis and inhibit migration of tumor and endothelial cells, *in vitro*. Furthermore, they inhibit tumor growth, metastases, and tumor-associated angiogenesis, *in vivo*, in several cancer models including breast, lung, leukemia, and lymphoma. In some studies, CendR peptides have been modified and attached to therapeutic peptides^{305,307} or co-injected with other drugs such as abraxane, doxorubicin, paclitaxel, cisplatin, and trastuzumab.^{299,300,302,304} In all these studies, the combination with CendR peptides increased the efficacy of the drugs by improving their internalization in the cells as well as the depth of their penetration in the tumor tissue. While these results are encouraging, the specificity, efficacy, and safety of CendR peptides in cancer therapy remains to be established. Another strategy consisted of targeting the transmembrane domain of NRP1 to prevent homo- and hetero-dimer formation.^{38,301} A peptide derived from a GXXXG motif present in the transmembrane segment of NRP1 inhibited human and murine glioma cell proliferation and migration *in vitro* as well as tumor-associated angiogenesis and tumor growth *in vivo*.³⁰¹ Geretti et al²⁶⁷ generated a mutated and soluble peptide similar in sequence to the B-domain of NRP2 (mutB-NRP2). Using it as an alternative ligand trap for VEGF, the authors showed that, compared with Avastin (bevacizumab), an anti-VEGF antibody, mutB-NRP2 prevented the binding

of VEGF to NRP1 and NRP2. As a consequence, the combination of MutB-NRP2 and Avastin improved the efficacy of each treatment and inhibited tumor growth in a melanoma model. Following the same idea, a small-molecule inhibitor, EG00229, designed to target the 'b1' domain appeared to affect VEGF-A binding to NRP1 and the viability of A549 lung carcinoma cells.³⁰² Interestingly, it also increased the potency of paclitaxel and 5-fluorouracil (5-FU).

Antibodies against NRP1 (anti-Nrp1^A and anti-Nrp1^B) and NRP2 (anti-NRP2^B) have been developed by Genentech.^{264,293} The 'A' antibodies were designed to target the semaphorin-binding domains, while the 'B' antibodies were designed to target the VEGF-binding domains of NRP1.³⁰⁹ Surprisingly, both anti-NRP1 antibodies had a negative effect on primary tumor growth by reducing angiogenesis and vascular remodeling. However, while it was speculated that the binding of the antibodies could result in the internalization of the receptor complex, a more recent study revealed that sNRP1 plasma levels increased with the dose of anti-Nrp1^B antibody injected.³¹⁰ Therefore, one could speculate that soluble ecto-domains resulting from the shedding of transmembranous NRP1 from the cell surface could act as a trap for growth factors in the tumor microenvironment. The antibody generated to block NRP2 offers some different possibilities compared with the anti-NRP1 antibodies in terms of therapeutic potential. Indeed, while treatment with the anti-NRP2 antibody had no effect on primary tumor growth, it inhibited tumor-associated lymphangiogenesis and lung metastases.²⁶⁴ This suggests that the two antibodies could have additive effects or could be used sequentially to prevent tumor growth and invasion in some cancers.

Recent advances indicate that cancer stem cells are the leading cause of drug resistance in cancer treatment. In breast cancer, tranilast, a drug that inhibits cancer stem cells, suppressed NRP1 and NF- κ B expression.³¹¹ Moreover, NRP1 down-regulation prevented mammosphere formation and inhibited constitutive NF- κ B. Therefore, while the role of NRPs and the pathways involving NRPs during the development of drug resistance remain obscure, therapies aimed at targeting NRPs could, in theory, delay the development of resistance mechanisms in several cancers.

Conclusion and future perspectives

During the past decade, an impressive effort has been devoted to understanding the role of class 3 semaphorins and their receptors during tumor growth and metastatic spread. It is now evident that these molecules control

various cellular functions, including viability, apoptosis, proliferation, adhesion, migration, and invasion. More importantly, SEMA3s and NRPs influence both the tumor compartment and its micro-environment and can either promote or inhibit tumor growth. This duality of response raises the question as to which effect will be dominant in any given context. In addition, several studies show that different class 3 semaphorins can affect similar cancer models. This raises the possibility of an overlap in their function. Accordingly, NRPs have often been shown to be expressed in the same cell types and frequently described as having similar functions. Yet, in some specific contexts it has been proven that the expression of only one or two semaphorins is lost in cancer and that NRP expression can vary in an opposite manner. Therefore, while class 3 semaphorins and NRPs share common functions, it is clear that each of them may have a specific role during key steps of tumor progression and metastatic spread. However, this question remains poorly addressed in current cancer models and will need further investigation to determine whether a universal 'semaphorin-NRP-based therapy' is possible or if therapies targeting these molecules need to be more specific. All in all, the complexity inherent in the SEMA/NRP signaling axis suggests that it will be difficult to predict *a priori* the outcome of any therapeutic strategy and that only careful testing can establish circumstances that maximize efficacy. Because SEMA3s and NRPs are expressed in several organs, an important future challenge will be to target their function specifically in the tumors to limit important side effects. Also, while important aspects of SEMA3 and NRP function are understood, others remain obscure. For example, the role of some SEMA3s remains elusive. In several cancer models, it is also frequently unclear whether NRP1 and NRP2 share overlapping or separate functions and whether they are differentially expressed during important phases of tumor progression. Both NRPs are glycosylated, and a few studies have shown that their level of glycosylation can influence the ability to bind certain ligands or the ability of cells to migrate. Whether changes in the glycosylation status of NRP occur during tumor progression is therefore an important question to address. Another important aspect is the role of each NRP isoform, which is virtually unknown and has been almost completely overlooked in cancer studies. Future work will hopefully address these questions in order to develop therapies that will specifically target tumor cells and improve patient outcomes.

Disclosure

The authors declare no conflicts of interest relevant to this work.

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