

Tumorigenesis and Neoplastic Progression

Plexin-B1 and Semaphorin 4D Cooperate to Promote Perineural Invasion in a RhoA/ROK-Dependent Manner

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AQ: 16 **Perineural invasion (PNI) is a tropism of tumor cells for nerve bundles located in the surrounding stroma. It is a pathological feature observed in certain tumors, referred to as neurotropic malignancies, that severely limits the ability to establish local control of disease and results in pain, recurrent growth, and distant metastases. Despite the importance of PNI as a prognostic indicator, its biological mechanisms are poorly understood. The semaphorins and their receptors, the plexins, compose a family of proteins originally shown to be important in nerve cell adhesion, axon migration, and proper central nervous system development. Emerging evidence has demonstrated that these factors are expressed in tissues outside of the nervous system and represent a widespread signal transduction system that is involved in the regulation of motility and adhesion in different cell types. We believe that the plexins and semaphorins, which are strongly expressed in both axons and many carcinomas, play a role in PNI. In this study, we show that plexin-B1 is overexpressed in tissues and cell lines**

from neurotropic malignancies and is attracted to nerves that express its ligand, semaphorin 4D, in a Rho/Rho kinase-dependent manner. We also demonstrate that nerves are attracted to tumors through this same system of proteins, suggesting that both plexin-B1 and semaphorin 4D are important in the promotion of PNI. (Am J Pathol 2012, 180:xxx; DOI: 10.1016/j.ajpath.2011.12.009)

Many carcinomas display perineural invasion (PNI), a tropism of tumor cells for surrounding nerve bundles. PNI promotes cancer cell survival and severely limits the ability to establish local control of disease. As a result, these tumors can exhibit pain and persistent growth, with a long clinical course and late onset of metastases, a pattern that has been observed in neurotropic tumor types, such as prostate and pancreatic cancers,¹ head and neck squamous cell carcinoma (HNSCC),² and the salivary gland malignancies adenoid cystic carcinoma (ACC)³ and polymorphous low-grade adenocarcinoma (PLGA).⁴ PNI is an independent predictor of poor outcome in neurotropic malignancies.^{4–6}

The semaphorins are a large family of proteins characterized by cysteine-rich semaphorin domains originally identified based on their ability to provide attractive and repulsive axon guidance cues during development.⁷ Recently, semaphorins have been identified in a variety of adult and embryonic tissues, in which they regulate development of the lungs,⁸ the heart and vasculature,⁹ branching morphogenesis in epithelium,¹⁰ angiogenesis,^{11,12} and proliferation and activation of lymphocytes,^{13,14} suggesting that they are important proteins that compose a system controlling migratory events in numerous tissues and cell types. The main functional

Supported by grants from King Abdulaziz University, Jeddah, Saudi Arabia (N.O.B.), and the National Cancer Institute (R01 CA133162 to J.R.B.).

Accepted for publication December 2, 2011.

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receptors for semaphorins are a family of proteins known as the plexins.¹⁵ The nature of the signals generated by semaphorin-plexin binding is still being deciphered, but there is a great deal of evidence that it impinges on the cytoskeleton and affects cell motility by acting through G-protein–signaling pathways.^{11,16–18}

Herein, we demonstrate that cell lines and tissues derived from neurotropic tumors express high levels of plexin-B1 compared with nontransformed controls or tumors that are not known for PNI, whereas nerves express its ligand, semaphorin 4D (Sema4D). The prostate cancer cell lines PC3 and Du-145 migrate toward nerve cell lines expressing Sema4D, a response abrogated when plexin-B1 or Sema4D is knocked down through RNA interference (RNAi) or when signaling of RhoA and its downstream effector Rho kinase (ROK) is inhibited. PC3 and Du-145 also exhibit a robust response in an invasion assay toward dorsal root ganglia (DRG) when using wild-type, but not Sema4D knockout, nerve tissue as the chemoattractant. LnCAPs, which are poor expressers of plexin-B1, fail to migrate toward nerve lines or tissues regardless of Sema4D status. Finally, we noted greater PNI in an *in vivo* tumor xenograft model by neurotropic malignant cells with functional plexin-B1 compared with that seen in cells in which plexin-B1 was silenced through RNAi; this could enhance neural spread in LnCAPs by overexpressing plexin-B1 in the grafted cells.

Emerging models of PNI strongly suggest that interactions between tumor cells and nerves induce tumor cell migration and stimulate nerve growth or axonogenesis. Herein, we show that nerve cell lines migrate toward PC3 cells and exhibit extended nerve processes in a Sema4D/plexin-B1–dependent manner, a response also blocked by Rho/ROK inhibition. We observed neurite outgrowth in a co-culture of PC3 cells with wild-type DRG, but not from DRG harvested from plexin-B1 knockout mice or in which Sema4D was silenced in the PC3 cells. Confirming these findings, we noted greater nerve density in biopsy specimens of HNSCC xenografts expressing Sema4D compared with tumors from cells in which Sema4D was silenced. In addition, we noted higher nerve densities in neurotropic tumors in general compared with nonneurotropic tumors or normal control tissues, indicating that the process of PNI involves enhanced nerve growth. Taken together, these results show that the plexins and semaphorins, which are strongly expressed in both axons and many carcinomas, play a significant role in PNI.

Materials and Methods

Cell Culture

All cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 U/mL penicillin/streptomycin/amphotericin B (Sigma, St Louis, MO), with the following exceptions: immortalized normal oral keratinocytes were cultured in defined keratinocyte media (Gibco, Invitrogen, Carlsbad, CA). The prostate cancer epithelial cell lines PC3, Du-145, and LnCAP were cultured in RPMI

1640 media (Cellgro, Manassas, VA). The nontransformed human pancreatic cell line hTERT-HPNE was cultured in 75% DMEM and 25% Medium M3 Base (Incell Corp, San Antonio, TX), supplemented with 5% FBS, 10 ng/mL human recombinant epidermal growth factor, 5.5 mmol/L D-glucose, and 750 ng/mL puromycin. Human pancreatic carcinoma cells were cultured in a 1:1 mixture of DMEM and Ham's F12 medium (ATCC, Manassas, VA), supplemented with 5% FBS, 0.002 mg/mL insulin, 0.005 mg/mL transferrin, 40 ng/mL hydrocortisone, and 10 ng/mL epidermal growth factor. Capan-1 cells were cultured in Iscove's modified Dulbecco's medium (ATCC), supplemented with 20% FBS and 100 U/mL penicillin/streptomycin/amphotericin B (Sigma).

Immunoblot Analysis

Cells were lysed in buffer (50 mmol/L Tris-HCl, pH 7.4; 150 mmol/L NaCl; and 1% Nonidet P-40), supplemented with protease inhibitors (0.5 mmol/L phenylmethylsulfonyl fluoride and 1 μ L/mL aprotinin and leupeptin; Sigma) and phosphatase inhibitors (2 mmol/L NaF and 0.5 mmol/L sodium orthovanadate, Sigma) for 15 minutes at 4°C. After centrifugation, protein concentrations were measured using the Bio-Rad assay (Bio-Rad, Hercules, CA), subjected to SDS-PAGE, and transferred onto a polyvinylidene fluoride membrane (Immobilon P; Millipore, Billerica, MA). The membranes were then incubated with the following antibodies: Sema4D (BD Transduction Labs, BD Biosciences, Palo Alto, CA), plexin-B1 (Santa Cruz A8, Santa Cruz, CA), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Sigma). Proteins were detected using the enhanced chemiluminescence system (Pierce, Thermo Fisher Scientific, Rockford, IL).

Immunohistochemistry Findings

Slides were hydrated through graded alcohols and incubated in 3% hydrogen peroxide for 10 minutes to quench the endogenous peroxidase. The sections were then incubated in blocking solution [2% bovine serum albumin (BSA)] for 1 hour at room temperature, followed by treatment with anti-Sema4D antibody (1:50 dilution; BD Transduction Labs), anti-plexin-B1 antibody (Santa Cruz A8, 1:10 dilution), or neurofilament protein (1:100 dilution; Cell Signaling, Danvers, MA), where indicated, overnight at 4°C. After washing with PBS, the slides were incubated with the biotinylated secondary antibody (1:400 dilution; Vector Laboratories, Burlingame, CA) for 1 hour, followed by the ABC complex (Vector Stain Elite, ABC kit; Vector Laboratories) for 30 minutes or with streptavidin peroxidase (LSAB+HRP kit; Dako, Carpinteria, CA) at room temperature. The slides were developed in 3,3'-diaminobenzidine (DAB Substrate kit for peroxidase; Vector Laboratories) and counterstained with hematoxylin. Images were taken using an Aperio Scanscope (Aperio Technologies, Vista, CA). To confirm staining, lymphocytes, which express high levels of Sema4D, were used as internal controls for this protein, whereas nerves were used as internal controls for expression of plexin-B1. For measuring protein expression, a scale of 0 to 3 was used

AQ: 2

AQ: 3

AQ: 4

AQ: 1

to assess the number of positively stained cells and the staining intensity, with the results added together to yield a final staining score.

shRNA and Lentivirus Infections

The short-hairpin RNA (shRNA) sequences for human Sema4D and plexin-B1 were obtained from Cold Spring Harbor Laboratory's RNAi library (RNAi Central, http://cancan.cshl.edu/RNAi_central/RNAi.cgi?type=shRNA, last accessed December 15, 2011).^{19,20} The sequences used as PCR templates for Sema4D shRNA have been previously reported.²¹ The sequence used for plexin-B1 shRNA was as follows: 5'-TGCTGTTGACAGTGAGC-GCGCCAGTATGTGGCCAAGAAGACTAGTGAAGCCACAGATGTAGTTCTTGCCACATACTGGGCATGCCT-ACTGCCTCGGA-3'. Oligos were synthesized (Invitrogen), and the resulting PCR products were cloned into pWPI GW, a Gateway-compatible CSCG-based lentiviral destination vector, as previously described.²¹⁻²³ Viral stocks were prepared and infections were performed as previously reported.^{21,24,25}

Migration Assays

Serum-free medium containing the indicated cell type or chemoattractant was placed in the bottom well of a Boyden chamber, whereas serum-free medium containing the migrating cells, with or without 10 $\mu\text{mol/L}$ of the ROK inhibitor fasudil (Calbiochem, Darmstadt, Germany), where indicated, was added to the top chamber. The two chambers were separated by a polyvinylpyrrolidone membrane (8 μm pore size, Osmonics; GE Water Technologies, Trevose, PA), and the migration assay was performed as described.¹¹ Cell migration was expressed as staining intensity of scanned migration membranes relative to the negative control wells. Each experiment was performed in triplicate, and the average and SD were calculated.

Invasion Assays

DRG from the cervical, thoracic, and lumbar areas of 4- to 7-week-old control and Sema4D knockout mice were dissected from donor animals, as previously reported.²⁶ Cultrex basement membrane material (100 μL ; Trevigen, Gaithersburg, MD) was placed into the upper chamber of 24-well transwell inserts (Millipore) and allowed to polymerize. DRG were placed in the lower chamber, and 1 million PC3, Du-145, or LnCAP cells in DMEM containing 1% FBS were added to the coated insert and incubated for 6 hours at 37°C in a humidified atmosphere of 5% CO₂. Inserts were stained and processed for cell content, according to the manufacturer's instructions.

Neurite Outgrowth Assay

DRG were isolated from wild-type and plexin-B1 knockout mice, as previously reported,²⁶ placed on a coverslip under sterile conditions in 20 μL of Cultrex (Trevigen),²⁷ and co-cultured with control-infected PC3 cells or PC3

infected with lentivirus coding for Sema4D shRNA. DRG and PC3 cells were maintained at 37°C in a humidified atmosphere of 5% CO₂. The number of neurites was counted along the entire DRG circumference, and images were captured using a digital SPOT camera (SPOT Imaging Solutions, Sterling Heights, MI) attached to an inverted Nikon phase-contrast microscope (Nikon Instruments, Melville, NY) on days 1, 3, 5, 7, 9, and 11. All experiments were performed in triplicate.

Tumor Tissues

Prostate cancer tissue arrays containing carcinoma samples and normal controls were obtained from Biomax (Rockville, MD). Individual paraffin blocks of formalin-fixed tissues from normal mucosa/acanthosis, basal cell carcinoma (BCC), squamous cell carcinoma, mucocèles, low- and high-grade mucoepidermoid carcinomas (MECs), ACCs, and PLGAs were obtained from the Department of Oncology and Diagnostic Sciences, University of Maryland (Baltimore, MD).

In Vivo PNI and Nerve Density Assays

A total of 2×10^6 PC3, LnCAP, HN6, or HN12 cells infected *ex vivo* with control virus or virus coding for plexin-B1 shRNA, plexin-B1, or Sema4D shRNA, where indicated, were resuspended in 250 μL of serum-free DMEM with an equal volume of Cultrex basement membrane extract (BME; Trevigen) and injected s.c. into the flanks of nude mice.²⁸ The mice were sacrificed, and the tumor masses were removed, fixed with formalin, and processed for immunohistochemistry (IHC) for neurofilament protein (Cell Signaling).

Immunofluorescence for Neurite Outgrowth on sNF96.2 Cells

sNF96.2 cells (ATCC) transfected with TrkA-PB1, TrkA-PB1 Δ PDZ, and TrkA-PB1 RasGAP mutant were seeded at 40% confluence on fibronectin (Santa Cruz Biotechnology, Santa Cruz, CA) coated coverslips. Cells were grown overnight in DMEM supplemented with 5% FBS and 100 $\mu\text{g/mL}$ nerve growth factor (NGF). At the conclusion of the growth period, cells were stained for immunofluorescence. Neurons were fixed in 4% paraformaldehyde, permeabilized with 0.25% Triton X-100, blocked with 2% (w/v) BSA, and incubated overnight at 4°C with anti-neurofilament antibody (Cell Signaling) diluted 1:100 in 1% BSA. For the secondary antibody, cells were incubated for 1 hour at room temperature with fluorescein isothiocyanate-conjugated donkey anti-rabbit antibody (Jackson ImmunoResearch Laboratories, Inc., Baltimore, MD), diluted 1:200 in PBS with 1% BSA, and 10 $\mu\text{g/mL}$ Hoechst 33342 (Sigma). Image acquisition was performed on a Nikon Eclipse E800 Microscope (Nikon, Melville, NY) using a $\times 40$ objective lens. Image analysis was performed using the Neuron J module of ImageJ version 1.46c (NIH, Bethesda, MD), measuring the num-

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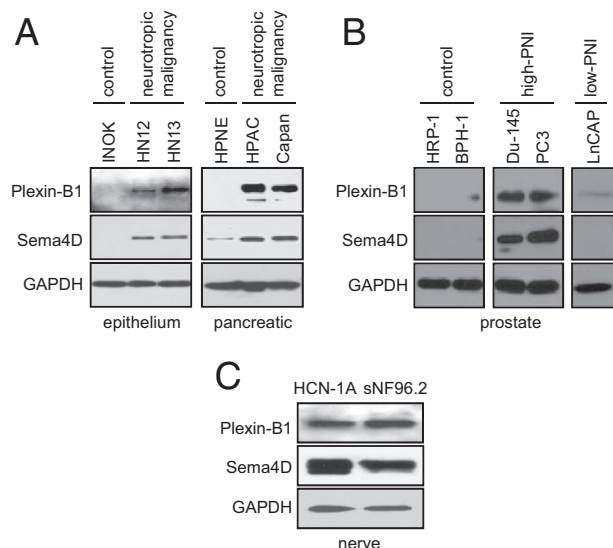


Figure 1. Plexin-B1 and Sema4D are highly expressed in neurotropic tumor and nerve cell lines but not in normal or nonneurotropic controls. **A:** SDS-PAGE immunoblot for plexin-B1 and Sema4D in the HNSCC lines HN12 and HN13 and the corresponding nontransformed control line, immortalized normal oral keratinocytes (INOKs), and the pancreatic cancer cell lines, HPAC and Capan-1, compared with nontransformed control HPNE cells. **B:** Du-145 and PC3, prostate cancer cell lines that exhibit PNI, express high levels of plexin-B1 and Sema4D compared with the nontransformed controls HPR-1 and BPH-1 (from benign prostatic hypertrophy), or LnCAP cells, which exhibit low PNI in xenografts. **C:** Plexin-B1 and Sema4D expression in the nerve cell lines HCN-1A and sNF96.2. GAPDH was used as a loading control for all immunoblots.

ber of cells exhibiting neurites and summing the total neurite length in pixels.

Results

Neurotropic Tumor and Nerve Cell Lines and Tissues Express Plexin-B1 and Sema4D

Immunoblots of cell lines derived from different neurotropic malignancies revealed high levels of expression of plexin-B1 and Sema4D in the HNSCC lines HN12 (isolated from a lymph node metastasis) and HN13 (isolated from a base of tongue cancer) but not in their corresponding nontransformed controls (Figure 1A). Similar results were observed in the human pancreatic cancer cell lines (a primary pancreatic adenocarcinoma epithelial cell line) and Capan-1 (derived from transformed pancreatic duct cells), when compared with HPNE (a nontransformed human pancreatic cell immortalized with h-TERT) (Figure 1A). We then identified the prostate cell lines PC3 (a bone metastasis from a stage IV prostatic adenocarcinoma) and Du-145 (derived from a brain metastatic deposit), which exhibit PNI *in vivo*, and compared levels of Sema4D and plexin-B1 in an immunoblot with their corresponding nontransformed control lines HPR-1 (a prostate cell line immortalized from normal human prostate epithelial cells) and BPH-1 (a benign prostatic hypertrophy cell line) and also with LnCAP, a line not known for PNI.²⁹ The results of this immunoblot demonstrate that, as expected, PC3 and Du-145 express

higher levels of Sema4D and plexin-B1 compared with nontransformed controls but also when compared with the low-PNI cell line, LnCAP (Figure 1B), suggesting a function for these proteins in PNI. The nerve cell lines HCN-1A and sNF96.2 also express Sema4D and plexin-B1 (Figure 1C).

We looked to confirm these findings in control and tumor tissues. We found plexin-B1 was expressed in most tissues we studied from tumors that are known to exhibit PNI, including HNSCC, high-grade MEC, ACC, PLGA (another salivary gland tumor that exhibits PNI), and prostate carcinoma (Figure 2A). Plexin-B1 was not expressed in their corresponding nontransformed control tissues or in tumors derived from the same sites in the body that are not known to exhibit robust PNI, including basal carcinoma, low-grade MEC, acinic cell adenocarcinoma, and benign prostatic hypertrophy (BPH) (Figure 2A). Nerves express Sema4D, as expected (Figure 2B). Quantification of tumor tissue staining for plexin-B1 in high-PNI tumors, determined by the number of positive cells and staining intensity, is shown in Figure 2C by site of origin and demonstrates that most tissues from neurotropic malignancies exhibit moderate to strong staining for all sections examined.

Sema4D/Plexin-B1-Mediated Attraction of Neurotropic Tumor Cells toward Nerves Is Dependent on Activation of RhoA

To determine the effects of expression of Sema4D and plexin-B1 on tumor cell migration toward nerves, we examined chemotaxis of PC3, infected with control lentivirus or lentivirus expressing plexin-B1 shRNA, toward the nerve cell line sNF96.2, infected with control lentivirus or lentivirus expressing Sema4D shRNA, in a Boyden chamber migration assay. PC3 cells infected with plexin-B1 shRNA-expressing lentivirus exhibited reduced levels of plexin-B1 protein (Figure 3A), whereas sNF96.2 infected with Sema4D shRNA expressing lentivirus exhibited knockdown of this protein in an immunoblot (Figure 3A), when compared with control-infected cells. PC3 migrated toward sNF96.2 cells, except when they were expressing lower levels of plexin-B1, when the sNF96.2 cells failed to express Sema4D, or both (Figure 3B).

To determine the effects of expression of Sema4D and plexin-B1 on the ability of neurotropic tumor cells to invade the extracellular matrix in an attempt to reach nearby nerves, we cultured DRG from C57BL/6 control or Sema4D knockout mice³⁰ in reconstituted BME and examined the response of the high-PNI prostate cell lines PC3 and Du-145 and the low-PNI line LnCAP in an invasion assay. PC3 and Du-145, which express high levels of endogenous plexin-B1 (Figure 1B), exhibited an approximately twofold greater invasion through the BME toward DRG harvested from wild-type mice compared with DRG from Sema4D knockouts, which exhibited invasion rates similar to those of the negative control (Figure 3C). LnCAPs, which express low levels of plexin-B1 (Figure 1B), do not exhibit statistically significant differences in invasion toward either wild-type or knockout DRG (Fig-

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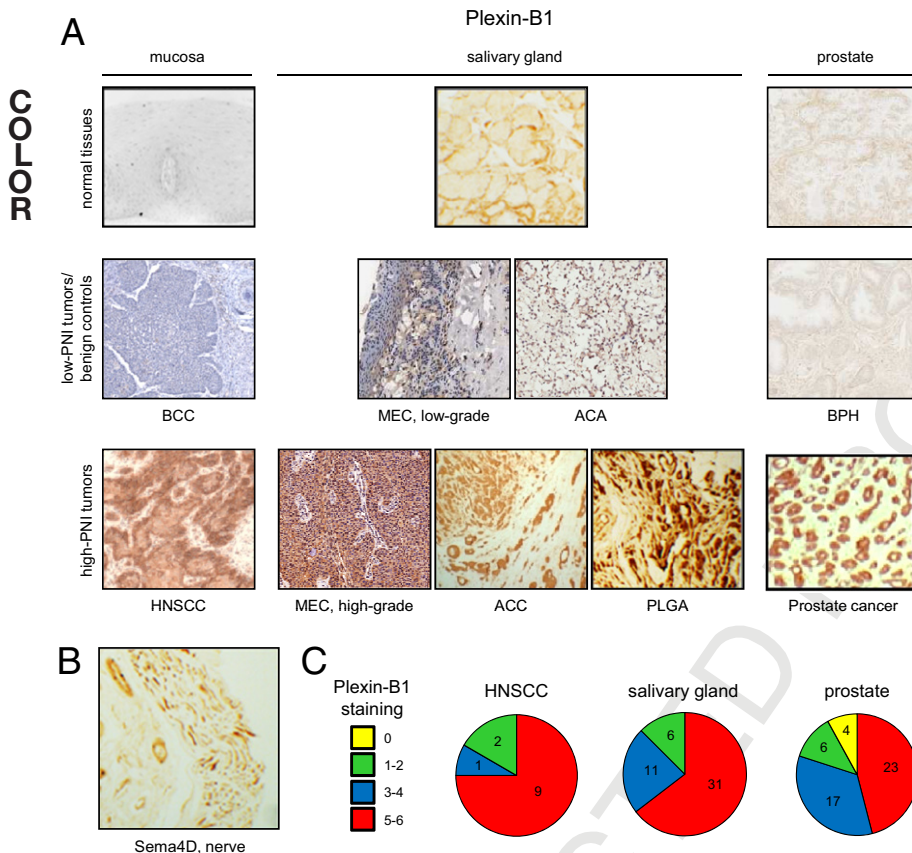


Figure 2. Plexin-B1 is expressed in neurotropic tumors, whereas nerves express Sema4D. **A:** IHC analysis for plexin-B1 in normal tissues, tumors not known to exhibit PNI, or benign controls (low-PNI tumors/benign controls), and tumors known for PNI (high-PNI tumors), grouped by site of origin, either mucosa, salivary gland, or prostate. HNSCC, high-grade MEC, ACC, PLGA, and prostate cancers demonstrate abundant plexin-B1 staining. Original magnification, $\times 20$. **B:** Cross section of a nerve showing expression of Sema4D. Original magnification, $\times 20$. **C:** A scale of 0 to 3 was used to assess the number of positively stained cells and the staining intensity for plexin-B1 in the high-PNI tumor groups shown. These two scores were added together to yield the ranges shown in the pie charts.

ure 3C). Taken together, these results suggest that Sema4D expression by nerves is important as a chemotactic compound for cells from neurotropic tumors expressing plexin-B1.

Plexin-B1 acts as a GTPase activating protein (GAP), inactivating R-Ras, while simultaneously binding the Rho GTP/GDP exchange factors (GEF) PDZ RhoGEF and leukemia-associated Rho GEF and activating RhoA and its downstream effector ROK.^{17,31} We have previously shown that activation of Rho by plexin-B1 was necessary for chemotaxis of endothelial cells toward Sema4D.¹¹ To determine which of these properties of plexin-B1 was necessary for migration of neurotropic tumor cells toward nerves, we transfected PC3 cells with myc-tagged chimeric receptors consisting of the extracellular portion of the rat NGF receptor, Trk-A, fused to the transmembrane and intracellular portion of plexin-B1,¹¹ both wild type and mutated, to inactivate the RasGAP function.³² We then examined their migration in the presence of NGF, with or without the ROK inhibitor fasudil. PC3 cells expressed both Trk-A receptors, those fused to wild-type plexin-B1 and the RasGAP mutant, in an immunoblot for myc (Figure 3D). PC3 cells migrated toward NGF when expressing the wild-type or RasGAP mutant plexin-B1 segment, compared with negative control populations, but failed to do so in the presence of fasudil (Figure 3E). To further examine the biological significance as it relates to PNI, we examined chemotaxis of PC3 toward the nerve cell line sNF96.2 in a migration assay, in the presence or absence of fasudil.

PC3 cells migrated toward sNF96.2 cells, except when treated with fasudil (Figure 3F). These results indicate that Sema4D/plexin-B1-mediated cell migration is dependent on the ability of plexin-B1 to activate RhoA and ROK and not on its ability to inactivate R-Ras.

Neurotropic Tumor Cells Are Attracted to Nerves *In Vivo* in a Plexin-B1-Dependent Manner

To study the contribution of plexin-B1 in tumor cells to PNI *in vivo*, we injected PC3 cells infected with control lentivirus or lentivirus-expressing plexin-B1 shRNA into nude mice. After 4 weeks, tumors were removed and processed into slides to look for invasion into nerves. We observed frequent examples of PNI by control-infected tumor cells but fewer instances of this phenotype in PC3 with silenced plexin-B1 (Figure 4A). Quantification of these results is shown in Figure 4A. We then grafted the low-PNI cell line LnCAP, control infected or infected with virus coding for full-length plexin-B1, and looked for differences in PNI. We observed little PNI in control-infected cells, as expected, but could induce this response in cells that overexpressed plexin-B1 (Figure 4B). Taken together, these results suggest that plexin-B1 expression is necessary for the ability of tumor cells to exhibit PNI in the stroma.

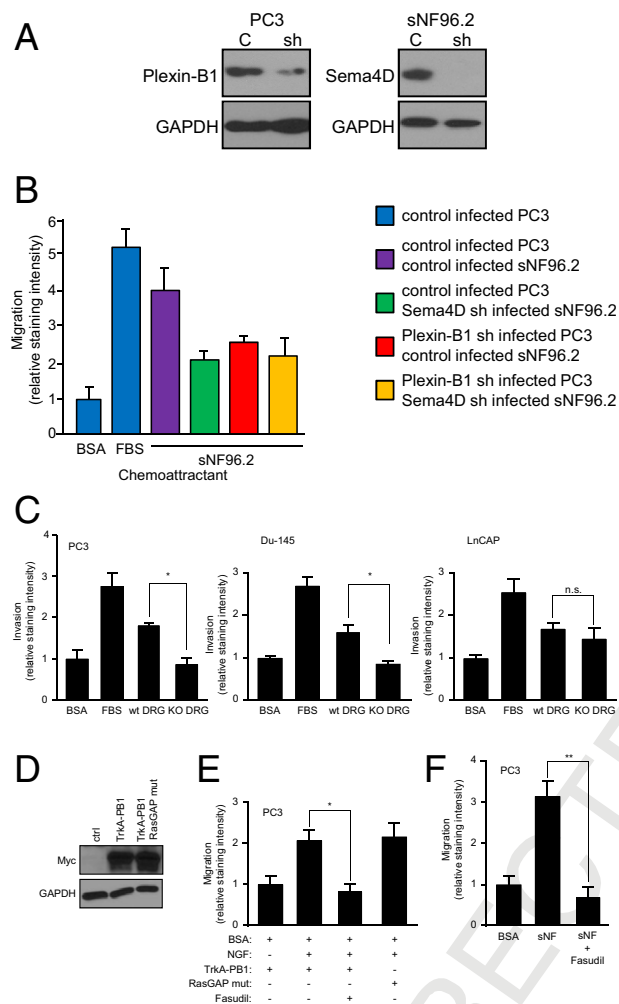


Figure 3. Sema4D/plexin-B1-mediated attraction of neurotropic tumor cells toward nerves is dependent on activation of RhoA. **A:** Immunoblot analysis for plexin-B1 and Sema4D on lysates from PC3 and sNF96.2, respectively, infected with empty vector control lentivirus (C) or virus coding for the appropriate shRNA (sh). **B:** The 0.1% BSA (negative control), 10% FBS (positive control), and sNF96.2 cells were used as the chemoattractants for PC3 in a migration assay. The PC3 and sNF96.2 cells were used with control lentivirus or lentivirus coding for Sema4D shRNA or plexin-B1 shRNA, where indicated. **C:** The 0.1% BSA (negative control), 10% FBS (positive control), and either wild-type (wt) or Sema4D knockout (KO) DRG were used as the chemoattractants for PC3, Du-145, or LnCAP cells in an invasion assay. * $P < 0.05$. n.s., not significant. **D:** PC3 cells were transfected with empty vector control (ctrl) or the myc-tagged chimeric constructs Trk-A/plexin-B1 wild-type (TrkA-PB1) and Trk-A/plexin-B1 RasGAP mutant (TrkA-PB1 RasGAP mut) and expression checked in an immunoblot. GAPDH was used as a loading control in all immunoblots. **E:** The 0.1% BSA (BSA, negative control) and NGF, with and without the ROK inhibitor fasudil, were used as the chemoattractants for PC3 cells expressing Trk-A/wild-type plexin-B1 chimeric receptors (TrkA-PB1) or chimeras in which the RasGAP function was lost due to mutation (RasGAP mut), in a migration assay. * $P < 0.05$. **F:** The 0.1% BSA (negative control) and sNF96.2 cells were used as the chemoattractants for PC3 cells, control treated or treated with fasudil, in a migration assay. ** $P < 0.01$.

Tumors Producing Sema4D Induce Axonogenesis through Plexin-B1

Emerging models of PNI strongly suggest that interactions between tumor cells and nerves induce tumor cell migration and stimulate nerve growth or axonogenesis. We hypothesized that if Sema4D, produced by nerves, could act as a chemoattractant for tumor cells expressing plexin-B1, then production of Sema4D by tumor cells

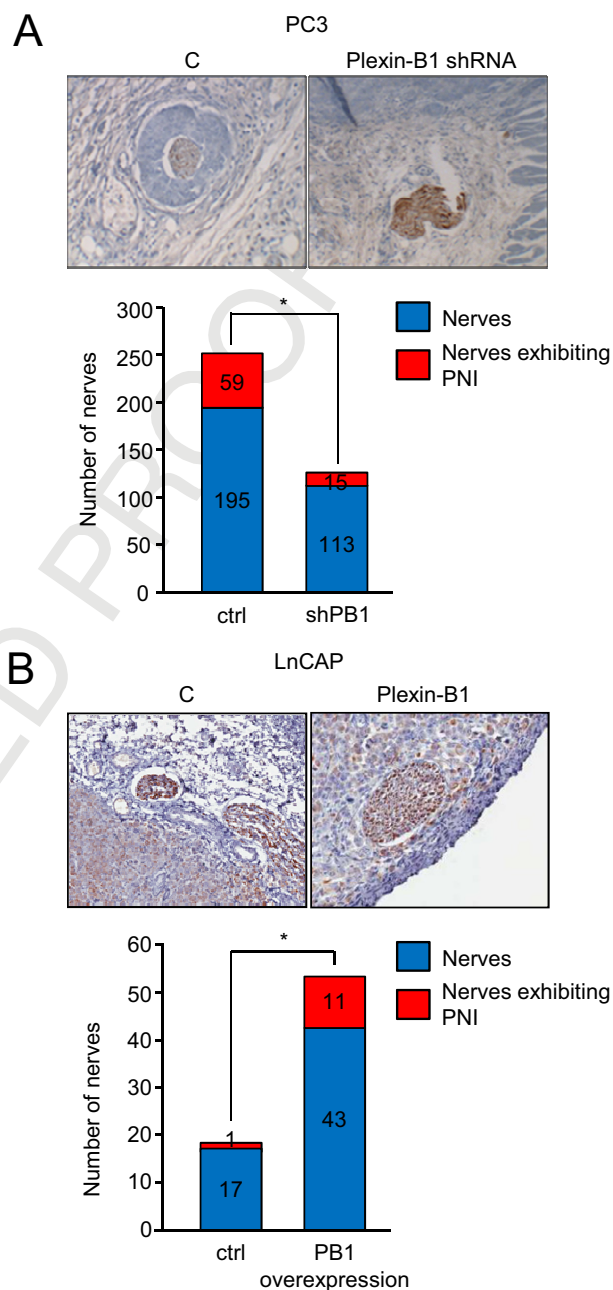


Figure 4. Tumor cells expressing plexin-B1 are attracted to nerves *in vivo*. **A:** Tumors derived from PC3 cells infected with a control lentivirus (C) and tumors from plexin-B1 shRNA-infected cells are shown in hematoxylin-stained sections. More PNI is seen in tumors derived from control PC3 cells compared with tumors derived from plexin-B1 shRNA-infected cells. The number of nerves exhibiting PNI per total nerves counted in xenografts derived from control and plexin-B1 shRNA-infected PC3 cells is shown in the bar graph. * $P < 0.05$. **B:** Tumors derived from LnCAP cells infected with a control lentivirus (C) or a virus expressing wild-type plexin-B1 are shown in hematoxylin-stained sections. More PNI is seen in tumors derived from cells overexpressing plexin-B1 compared with controls. The number of nerves exhibiting PNI per total nerves counted in xenografts derived from control and plexin-B1-infected LnCAP are shown in the bar graph. * $P < 0.05$. Nerves are highlighted by neurofilament staining in IHC analysis of tumor tissues. Original magnification, $\times 40$ (all panels).

might attract nerves expressing plexin-B1. All of the neurotropic tumor cell lines we tested express Sema4D in an immunoblot (Figure 1A), whereas low-PNI LnCAP failed to do so (Figure 1B). Tissues from neurotropic tumors

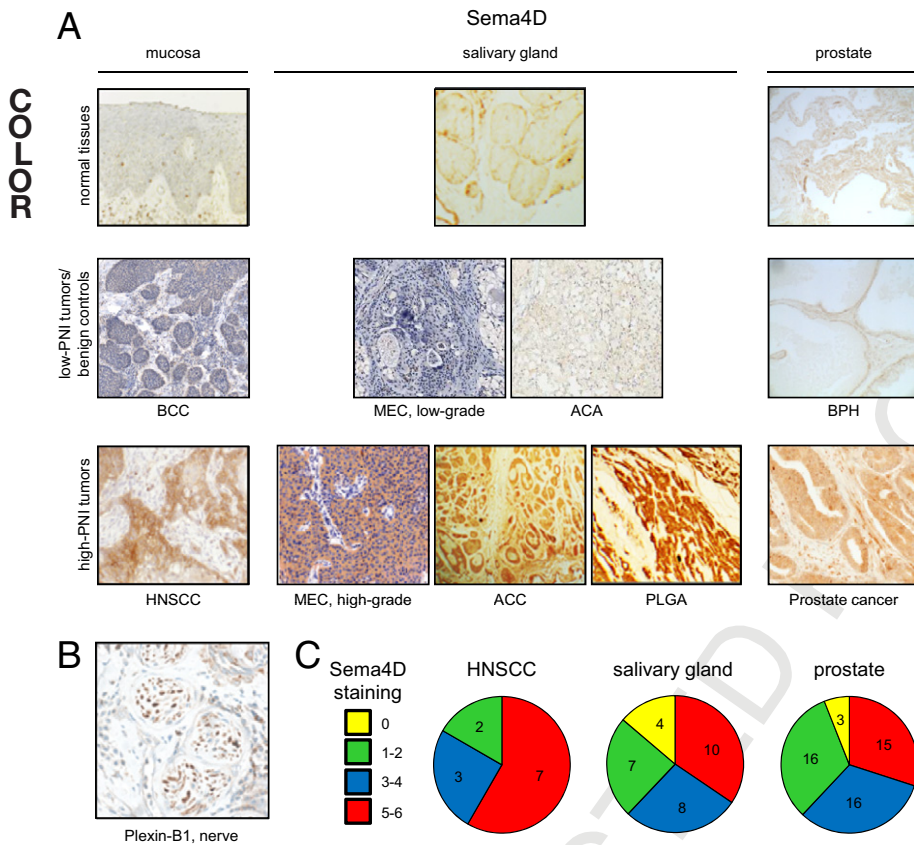


Figure 5. Neurotropic tumors express Sema4D, whereas nerves express plexin-B1. **A:** IHC analysis for Sema4D in normal tissues, tumors not known to exhibit PNI, or benign controls (low-PNI tumors/benign controls), and tumors known for PNI (high-PNI tumors), grouped by site of origin (mucosa, salivary gland, or prostate). HNSCC, high-grade MEC, ACC, PLGA, and prostate cancers demonstrate abundant Sema4D staining. Original magnification, $\times 20$. **B:** Cross section of a nerve showing expression of plexin-B1. Original magnification, $\times 20$. **C:** A scale of 0 to 3 was used to assess the number of positively stained cells; the staining intensity for Sema4D in the high-PNI tumor groups is shown. These two scores were added together to yield the ranges shown in the pie charts.

F5 also show high levels of Sema4D expression (Figure 5A) when compared with their nontransformed counterparts (Figure 5A) or tissues from tumors that are not known to exhibit PNI (Figure 5A), whereas nerve cell lines (Figure 1B) and nerves in tissues (Figure 5B) express plexin-B1. Quantification of Sema4D expression in neurotropic tumors is shown in Figure 5C and demonstrates that most HNSCC high-PNI salivary gland tumors (high-grade MEC, ACC, and PLGA) and prostate cancers studied exhibited moderate to strong Sema4D expression. To determine the roles that Sema4D and plexin-B1 play in the ability of neurotropic tumor cells to induce axonogenesis, we examined chemotaxis of the nerve cell line sNF96.2, infected with control lentivirus or lentivirus-expressing plexin-B1 shRNA, toward soluble Sema4D and PC3 cells infected with control lentivirus or lentivirus-expressing Sema4D shRNA. sNF96.2 cells infected with plexin-B1 shRNA-expressing lentivirus exhibited reduced levels of plexin-B1 protein, whereas PC3 infected with Sema4D shRNA-expressing lentivirus exhibited knock-down of this protein in an immunoblot, when compared with control infected cells (Figure 6A). sNF96.2 migrated toward soluble Sema4D and PC3 cells in an *in vitro* migration assay, except when they were expressing lower levels of plexin-B1, when the PC3 failed to express Sema4D, or both (Figure 6B). To determine whether plexin-B1-mediated RhoA activation or R-Ras inactivation was necessary for formation of dendrites and axonal processes from nerve cells, we examined sNF96.2 cells. These cells were transfected with empty vector control or

F6

vectors coding for the chimeric receptors Trk-A, fused to wild-type plexin-B1 (TrkA-PB1), the RasGAP mutant (TrkA-PB1 RasGAP mut), or a Trk-A/plexin-B1 construct lacking the PDZ binding motif necessary for recruiting PDZ-RhoGEF and leukemia-associated Rho GEF and activating Rho (TrkA-PB1 Δ PDZ). Then, we treated with NGF while recording the subsequent morphological changes and measuring the nerve processes (Figure 6C). Under these conditions, cells expressing the wild-type construct exhibited formation of elongated neural processes compared with untreated controls, as did cells expressing the RasGAP mutant (Figure 6C). Cells expressing the Δ PDZ mutant did not show this morphological characteristic (Figure 6C). The number of cells exhibiting nerve extensions and the total length of those extensions are shown in the bar graphs (Figure 6C). These results suggest that formation of nerve processes is dependent on the ability of plexin-B1 to activate RhoA and ROK and not on its ability to inactivate R-Ras.

To further establish biological significance, we cultured DRG from control or plexin-B1 knockout mice³³ in BME, along with PC3 cells, control infected or infected with lentivirus-expressing Sema4D shRNA, and looked for outgrowth of neurites. DRG harvested from wild-type mice exhibited neurite outgrowth when co-cultured with control-infected PC3 cells, a response greatly attenuated in DRG from plexin-B1 knockout mice or when using PC3 in which Sema4D levels were reduced by shRNA (Figure 6D). These results are shown graphically in Figure 6E and

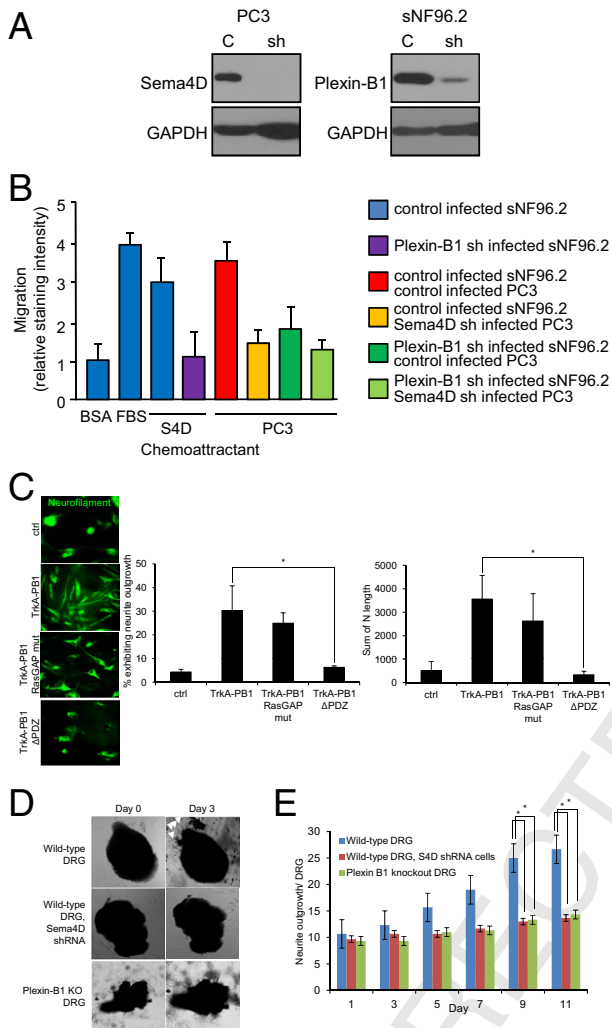


Figure 6. Sema4D production by neurotropic malignancies mediates nerve cell chemotaxis and neurite outgrowth. **A:** Immunoblot analysis for Sema4D and plexin-B1 in lysates from PC3 and sNF96.2, respectively, infected with empty vector control lentivirus (C) or virus coding for the appropriate shRNA (sh). GAPDH was used as a loading control. **B:** The 0.1% BSA (negative control), 10% FBS (positive control), soluble Sema4D, and PC3 cells were used as the chemoattractants for sNF96.2 cells in a migration assay. sNF96.2 and PC3 cells were infected with control lentivirus or lentivirus coding for plexin-B1 shRNA or Sema4D shRNA, where indicated. **C:** sNF96.2 cells were transfected with empty vector control DNA (ctrl) or the chimeric receptors coding for Trk-A/plexin-B1 wild-type (TrkA-PB1), a RasGAP mutant (TrkA-PB1 RasGAP mut), or a construct lacking the PDZ binding motif (TrkA-PB1ΔPDZ), treated with NGF and examined for nerve processes (neurofilament staining). The number of cells exhibiting nerve extensions and total length of axons is shown in the bar graphs. **P* < 0.05. **D:** DRG from wild-type control or plexin-B1 knockout mice were cultured in BME along with PC3 cells, control infected or infected with lentivirus expressing Sema4D shRNA. The white arrowheads indicate neurite outgrowth at day 3. **E:** Quantification of neurite outgrowth to day 11. **P* < 0.05.

strongly suggest that Sema4D produced by tumor cells would attract nerves in the tumor stroma *in vivo*.

Sema4D-Producing Tumors Exhibit Enhanced Nerve Density

If Sema4D, produced by neurotropic tumor cells, enhances axonogenesis, then it might be expected that

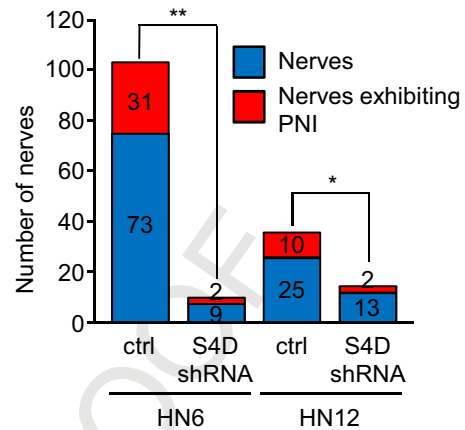


Figure 7. Neurotropic tumors exhibit enhanced nerve density in a Sema4D-dependent manner. Analysis of neurofilament-stained sections of tumor xenografts composed of control-infected HN6 or HN12 cells (ctrl) or those infected with Sema4D shRNA-expressing lentivirus (Sema4D shRNA). Sema4D shRNA tumors exhibit less PNI and far fewer nerves. **P* < 0.05, ***P* < 0.01.

increased nerve density would be observed in the stroma surrounding these tumors compared with tumors not expressing Sema4D. Indeed, we observed more PNI in two different control HNSCC xenografts versus xenografts of the same lines with silenced Sema4D; we also observed more total nerves in controls (Figure 7). These results are quantified in Table 1, which demonstrates significantly higher nerve density, as determined by the number of nerves per square millimeter, in control HN6 and HN12 xenografts compared with lines in which Sema4D was silenced by shRNA. We confirmed these findings in tumor tissues by staining for the nerve marker neurofilament. We observed more nerves in the stroma of high-PNI neurotropic malignancies, such as ACC, compared with normal control tissues (Figure 8). An analysis of normal/reactive salivary gland tissue and low-grade MEC revealed many fewer nerves per square millimeter when compared with ACC and PLGA (Table 2). A grouping of epithelial tissues showed that reactive acanthotic epithelium and basal cell carcinoma, a low-PNI malignancy, also exhibited a lower density of nerves compared with HNSCC biopsy specimens (Table 2). Taken together, these results indicate the phenomenon of cancer-related neurogenesis, as other groups have shown,³⁴ and suggest that Sema4D production by neurotropic tumor cells acts through

Table 1. Parameters of Tumor Xenografts

Tumor xenograft	<i>n</i>	Total area (mm) ²	Nerves counted	Nerves (No./mm) ²
HN6				
Control	4	852	104	0.12
Sema4D shRNA	7	202	11	0.06
HN12				
Control	4	148	35	0.24
Sema4D shRNA	5	138	15	0.11

The total number of samples, tumor area, nerves, and nerves per square millimeter in tumor xenografts composed of control-infected HN6 and HN12 cells or cells infected with lentiviruses expressing Sema4D shRNA from Figure 7.

ROF0C

ROF0C

F7
T1

F8

T2

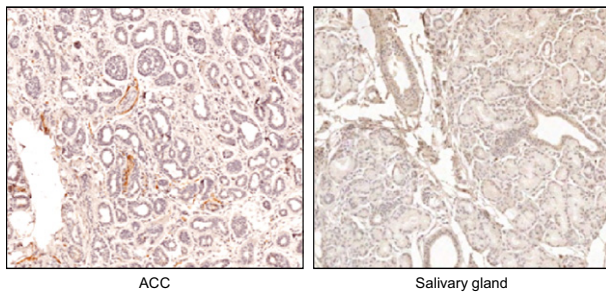


Figure 8. IHC stain for neurofilament in ACC compared with normal salivary gland tissue. Original magnification, $\times 20$. ACC shows higher nerve density.

plexin-B1 on nerves to mediate this effect, which then plays a role in promoting PNI.

Discussion

PNI is a tropism of malignant tumor cells for nerve bundles in the surrounding tissues, defined as the presence of malignant cells in the perineural space with total or near-total circumferential involvement of the nerve in tangential histopathological sections.³⁵ PNI is a form of tumor spread that hinders the ability to establish local control of a malignancy because neoplastic cells can spread along nerve tracts far from the primary lesion and are often missed during surgery. As a result, PNI is an independent prognostic factor for many human carcinomas, such as prostate,²⁷ pancreatic,³⁶ colorectal,³⁷ salivary gland,⁴ and HNSCC.³⁸ cDNA microarrays used to profile gene expression in ACC have identified dysregulation of numerous genes associated with the cell cycle, the cytoskeleton, and extracellular matrix binding, which influence the production of neurotropic factors and adhesion molecules that could contribute to PNI.³⁹ We have previously shown that production of Sema4D by some tumors attracts plexin-B1-expressing endothelial cells in a RhoA and ROK-dependent manner, a process co-opted by malignancies to induce blood vessel growth into a tumor.²¹ Therefore, we postulated that, because some malignancies also express high levels of plexin-B1, perhaps the tumor cells might be attracted to nerves, which are known sources of Sema4D. Indeed, prostate tumors, which invade along local peripheral nerves, have demonstrated overexpression of plexin-B1 in cancer development and progression.⁴⁰ We observed that plexin-B1 is highly expressed in neurotropic malignancies and cell lines but less so in healthy controls or in cell lines and tissues from malignancies that are not known to exhibit much PNI, whereas Sema4D is expressed in nerves. Through the use of RNAi, chimeric receptor constructs, and inhibitory compounds, our studies illustrate a model in which Sema4D acts as a chemoattractant for malignancies that promote PNI.

Interestingly, we also observed that nerve cells are stimulated to migrate and form dendritic projections toward neurotropic tumors using the same system of signaling proteins, findings that support the theory that PNI also may result from tumor-mediated stimulation of axonogenesis or neurogenesis. Although semaphorins and

plexins have mostly been associated with axonal guidance as repulsive signals, some semaphorin-plexin interactions actually enhance nerve growth. Prostate cancers have exhibited increased nerve density as a result of production of Sema4F by the tumors,³⁴ whereas there are also reports that, depending on the context, Sema4D can act as an attractive signal for neurite and axon growth.^{41,42} In one study⁴³ focusing on how Sema4D and plexin-B1 modulate axonal trajectories and dendritic morphological characteristics in neurons over time, the authors show that Sema4D increases the complexity and arborization of developing neurites in hippocampal neurons by activating plexin-B1. Such a phenomenon may be at work in our system because this effect required activation of the RhoA-ROK pathway, which we demonstrated herein, and the receptor tyrosine kinase phosphatidylinositol 3-kinase pathway, identical to the mechanism of plexin-B1 signaling we have previously observed in endothelial cells exhibiting a pro-angiogenic phenotype in the presence of Sema4D.^{28,32}

Sema4D is expressed on the surface of cells as a homodimer, but it has also been shed into the surrounding environment through proteolytic cleavage, allowing it to work at a distance.^{44,45} This is important in availability of Sema4D and also for its function, because membrane-bound and cleaved forms of different semaphorins have exhibited somewhat opposing effects on their target tissues. For example, invertebrate transmembrane sema1a is a chemoattractant in axon pathfinding *in vivo*, whereas its soluble form acts as a repulsive factor.⁴⁶ Sema4D also has exhibited a dual role. The transmembrane form is known to repel growing axons,⁴⁷ but the soluble, extracellular portion promotes neurite outgrowth in PC12 cells and axon outgrowth in DRG.⁴⁸ In general, bifunctionality of the semaphorins is not unusual, with Sema3A, E, and C attracting or repelling axons and dendrites, depending on the form, experimental system, and cell type used.^{41,48,49}

Because we show that both Sema4D and plexin-B1 are expressed in neurotropic malignancies, it is plausible that autocrine or paracrine signaling involving these proteins exists in neurotropic tumor cells that influence PNI. However, we believe the data do not support such a mecha-

Table 2. Parameters of Tissue Biopsy Specimens

Tissue	<i>n</i>	Total area (mm ²)	Nerves counted	Nerves (No./mm ²)
Salivary gland				
Mucocele	9	1050.5	49	0.05
LG MEC	12	865.5	52	0.06
ACC	7	1053.1	158	0.15
PLGA	5	1298.2	182	0.14
Epithelial				
Acanthosis	8	276.4	36	0.13
BCC	8	335.1	33	0.10
HNSCC	6	333.7	103	0.31

Quantification of nerve density in neurofilament-stained biopsy specimens of a salivary gland group, consisting of the high-PNI malignancies, ACC and PLGA, compared with the low-PNI malignancy, LG MEC, and normal/reactive gland (mucocele) and an epithelial group, composed of HNSCC compared with BCC and normal/reactive mucosa (acanthosis). LG, low grade.

nism. We used RNAi for *Sema4D* and *plexin-B1* and tissues from knockout mice to show that high-PNI tumor cells exhibit chemotaxis to nerve cells and whole nerve tissues but fail to do so when those nerve cell lines or tissues do not express *Sema4D*. This happens even when the tumor cells themselves still express both *Sema4D* and *plexin-B1* and the autocrine or paracrine mechanism would presumably be intact. There is also the possibility that *Sema4D*–*plexin-B1*–mediated promotion of PNI represents just one facet of tumor aggressiveness, and that tumors expressing both of these proteins might exhibit enhanced invasive behavior in general, as suggested by Casazza et al⁵⁰ for *Sema3E* and *plexin-D1*. There would seem to be evidence in the literature to support the idea that *Sema4D* and *plexin-B1* promote metastasis and aggressive growth, but it is not consistent and there are even studies that demonstrate the opposite. Rody et al^{51,52} have shown that the presence of *plexin-B1* in estrogen receptor–positive breast tumors correlates with a favorable prognosis, and other groups⁵³ have shown that *plexin-B1* behaves as a tumor suppressor in melanoma. There is a great deal of evidence that PNI is a specific pathological phenomenon distinct from other aspects of tumor aggressiveness. Some tumor types exhibit characteristic neural invasion, whereas other, more aggressive, tumors fail to do so even at advanced stages. Therefore, it is likely that the particular set of conditions or genetic background necessary for a tumor cell to acquire the ability to invade nerves is different from other aspects of tumor invasion, such as the ability to metastasize. For example, the microcystic adnexal carcinoma, a rare tumor of the midface in middle-aged women, is a locally aggressive malignancy known to infiltrate muscle and show PNI, but it hardly ever metastasizes.⁵⁴ High-grade MEC, on the other hand, behaves locally in a similar manner (infiltrative growth and PNI) but does exhibit metastasis. Even for well-studied semaphorin-plexin pairs, local tumor growth and distant spread are separable events.⁵⁰

PNI likely involves a complex interaction between nerve and cancer cells, similar to the signals exchanged between nerves, stromal cells, and epithelium during development and regeneration in response to injury, with the semaphorins, plexins, and other factors, both membrane bound and secreted, essentially generating a microenvironment in which tumor and nerve cells grow toward each other.²⁷ Combined with the role they play in tumor-induced angiogenesis, it could explain why up-regulation of *Sema4D* and *plexin-B1* in prostate cancer,⁴⁰ breast cancer,⁵⁵ and some sarcomas⁵⁶ contributes to such a poor prognosis. Deciphering the mechanisms of PNI will lend insight into the biological characteristics of tumor-stroma interactions and have wide-ranging effects on the ability to control cancers, such as prostate and pancreatic cancers, HNSCC, and salivary gland tumors, helping to alleviate the pain and significant morbidity associated with these neurotropic malignancies.

Acknowledgments

We thank Drs. Stefan Offermanns and Thomas Wozfeld (Max-Planck-Institute, Bad Nauheim, Germany) for *plexin-B1* knockout mice, Dr. Hitoshi Kikutani (Osaka University) for *Sema4D* knockout mice, Dr. Daniel Martin (NIH, National Institute of Dental and Craniofacial Research, Bethesda, MD) for design and production of lentiviral shRNAs, and Jeffrey Kaufman (Adenoid Cystic Carcinoma Research Foundation) for support and encouragement.

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