

# Semaphorin 3B Inhibits the Phosphatidylinositol 3-Kinase/Akt Pathway through Neuropilin-1 in Lung and Breast Cancer Cells

Emely Castro-Rivera,<sup>1</sup> Sophia Ran,<sup>5</sup> Rolf A. Brekken,<sup>1,3,4</sup> and John D. Minna<sup>1,2,3</sup>

<sup>1</sup>Hamon Center for Therapeutic Oncology Research-Simmons Cancer Center and Departments of <sup>2</sup>Internal Medicine, <sup>3</sup>Pharmacology, and <sup>4</sup>Surgery, University of Texas Southwestern Medical Center, Dallas, Texas; and <sup>5</sup>Department of Medical Microbiology, Immunology and Cell Biology, Southern Illinois University, School of Medicine, Springfield, Illinois

## Abstract

**Semaphorin 3B (SEMA3B), located at 3p21.3, is a secreted member of the semaphorin family important in axonal guidance. SEMA3B undergoes allele and expression loss in lung and breast cancer and can function as a tumor suppressor. Previously, we found that SEMA3B induces apoptosis in tumor cells either by reexpression or when applied as a soluble ligand. SEMA3B-induced apoptosis was mediated, in part, by blocking vascular endothelial growth factor autocrine activity in tumor cells. In the current study, treatment of lung and breast cancer cells with picomolar concentrations of soluble SEMA3B inhibited their growth; induced apoptosis; and was associated with decreased Akt phosphorylation, increase in cytochrome *c* release and caspase-3 cleavage, as well as increased phosphorylation of several proapoptotic proteins, including glycogen synthase kinase-3 $\beta$ , FKHR, and MDM-2. Lung and breast cancer lines resistant to SEMA3B did not show these signaling changes and a tumor-derived missense SEMA3B mutant was inactive in this regard, providing specificity. SEMA3B-mediated inhibition of proliferation and induction of apoptosis in cancer cells were blocked by expressing a constitutively active Akt mutant and are linked to tumor cell expression of neuropilin-1 (Np-1). SEMA3B-insensitive Np-1-negative tumor cells acquired sensitivity to SEMA3B after forced expression of Np-1, whereas SEMA3B-sensitive Np-1-positive tumor cells lost sensitivity to SEMA3B after knockdown of Np-1 by small interfering RNA. We conclude that SEMA3B is a potential tumor suppressor that induces apoptosis in SEMA3B-inactivated tumor cells through the Np-1 receptor by inactivating the Akt signaling pathway. [Cancer Res 2008;68(20):8295–303]**

## Introduction

Semaphorin 3B (SEMA3B) encodes a secreted protein with tumor suppressor activity that is located at chromosome region 3p21.3, a site of frequent allele loss in the early pathogenesis of lung and breast cancers (1–4). SEMA3B belongs to the secreted class 3 semaphorins (SEMA3), which form a complex with neuropilins (Np-1 and Np-2) and plexins on the cell surface. The literature reports that neuropilins provide binding sites for SEMA3B molecules, whereas plexins are responsible for signal transduction

(5, 6). Neuropilins also serve as coreceptors for vascular endothelial growth factor-A (VEGF-A). In neural cells, neuropilins regulate axon guidance, whereas in endothelial cells they regulated angiogenesis and endothelial cell migration (7, 8). Thus, neuropilins and VEGF-A play an important role in tumorigenesis and, along with semaphorins, may serve as important therapeutic targets. Previously, we showed that exogenously added soluble SEMA3B or forced expression of SEMA3B in breast and non-small cell lung cancer (NSCLC) cells induced apoptosis and dramatically decreased their ability to form colonies (1, 3). Independent studies showed that SEMA3B also inhibited ovarian tumor formation in a xenograft model (4). Recently, we established that SEMA3B and VEGF<sub>165</sub> play antagonistic roles in regulation of apoptosis and survival of lung and breast carcinoma cell lines. We found that externally added VEGF<sub>165</sub> rescues cancer cells from SEMA3B-induced apoptosis. These results suggest that tumor suppressor effects of SEMA3B might be mediated by blocking an autocrine VEGF-induced signaling, possibly through competition for binding of Np-1 receptors (1).

Although the proapoptotic and growth-inhibiting activities of SEMA3B in epithelial cancer cells have been shown in several laboratories, the signal transduction pathway(s) underlying these actions has not yet been established. The functional relationship between VEGF-A and some of the members of SEMA3 has been shown in cancer and noncancer systems (1, 9, 10). Furthermore, SEMA3A and SEMA3F are linked with an antagonistic effect in the presence of VEGF-A that leads to down-regulation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway (10). Akt, also known as protein kinase B, modulates the function of numerous substrates involved in the regulation of cell survival, cell cycle progression, and cellular growth (11, 12). Akt is activated downstream of PI3K in response to receptor stimulation (11). Full activation of Akt requires phosphorylation at T308 by pyruvate dehydrogenase kinase (PDK1) and at S473 in the hydrophobic motif, which can be mediated by several kinases, including PDK1 (13), integrin-linked kinase (ILK; ref. 14), Akt itself (15), DNA-dependent protein kinase (16), and mammalian target of rapamycin (17). Akt-mediated phosphorylation leads to inactivation of apoptotic regulators Bad, procaspase-9, several members of the forkhead family that induce expression of Fas, and other proapoptotic factors. Phospho-Akt also activates MDM-2, which targets p53 for degradation (18–21). Akt activation also promotes cell cycle progression and cell growth by preventing nuclear localization of the cyclin-dependent kinase inhibitors p21 and p27, by maintaining levels of the antiapoptotic protein survivin, and by inhibiting glycogen synthase kinase-3 (GSK-3), leading to the stabilized expression of cyclin D1 (22–24). Akt seems to play a critical role in the pathogenesis of various human cancers, such as lung cancer, in which constitutive activation or overexpression of Akt correlates with poor prognosis and drug resistance (25, 26).

**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

**Requests for reprints:** John D. Minna, Hamon Center for Therapeutic Oncology Research, University of Texas Southwestern Medical Center, 6000 Harry Hines Boulevard, Dallas, TX 75390-8593. Phone: 214-648-4921; Fax: 214-648-4940; E-mail: john.minna@utsouthwestern.edu.

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Finally, transgenic mice overexpressing Akt in combination with other genetic alterations show significantly increased tumor formation (27).

To better understand both the role and the potential for SEMA3B as a novel therapeutic for cancer, we studied the signaling pathways altered by SEMA3B in human lung and breast cancer cells. Our results indicate that the antiproliferative effect of SEMA3B is mediated by Np-1-dependent down-regulation of Akt phosphorylation and kinase activity, leading to expected changes in signaling molecules downstream of Akt. These data further support the suppressive role of SEMA3B in tumorigenesis and its potential as a therapeutic agent by linking the loss of SEMA3B to increased activation of Akt mediated through the Np-1 receptor important for both SEMA3B and VEGF-A signaling.

## Materials and Methods

**Cell lines.** NSCLC lines A549, NCI-H1299, NCI-H441, NCI-H23, NCI-H1792, NCI-H2009, and NCI-H1993 were obtained from the Hamon Center Repository. Additional cell lines included Cos7 monkey kidney cells, and breast cancer cells MDA-MB-231 and ZR-75-A were purchased from the American Type Culture Collection. Lung carcinoma cells and Cos7 were grown in RPMI 1640 (Invitrogen) supplemented with 5% fetal bovine serum. MDA-MB-231 and ZR-75-A cells were grown in RPMI 1640 supplemented with L-glutamine and 10% fetal bovine serum. Cell lines tested free of *Mycoplasma* and were routinely DNA fingerprinted to assure their identity.

**Expression plasmids.** All Akt expression plasmids were kindly provided by Dr. James R. Woodgett (Ontario Cancer Institute, Toronto, Ontario, Canada). For transfection of mammalian cells, genes encoding human wild-type *SEMA3B*, wild-type *Akt* (pAkt), mutant *Akt* (pAktMUT), and constitutive active *Akt* (pAktDD) were inserted into *neo* containing pcDNA3 expression vector (Promega). *Np-1* inserted into pcDNA3 expression vector was provided by Dr. Gary Gallick (M. D. Anderson Cancer Center, Houston, TX). Genes encoding human wild-type *SEMA3B*, mutant *SEMA3B* (SEMA3B-MUT) containing a lung cancer-derived single missense mutation D397H (SEMA3Bmut), and *VEGF<sub>165</sub>* were inserted into pcDNA3 expression vector (Promega). Cells were transfected using Lipofectamine and Lipofectamine Plus reagent (Invitrogen) according to the manufacturer's instructions and analyzed 48 h after transfection.

**Reverse transcription-PCR.** Total RNA was extracted using RNeasy Mini kit (Qiagen). Reverse transcription-PCR (RT-PCR) was performed using the SuperScript One-Step RT-PCR Systems (Invitrogen), and amplification products were resolved on 2% agarose gels. A schedule for typical RT-PCR consisted of 1 h of reverse transcription at 42°C followed by 35 cycles of 1 min of denaturation at 95°C, 1 min of annealing, and 1 min of extension at 72°C. All samples analyzed by RT-PCR were also tested for  $\beta$ -actin expression to confirm the integrity of the RNA. Primer sequences for neuropilins and plexins have been previously described (28).

**Antibodies and Western blot analysis.** Antibodies used for Akt kinase assay and Western blot analysis were obtained from Cell Signaling Technology with the exception of the anti-p53 monoclonal antibody that was purchased from Oncogene Science. Our mouse monoclonal anti-SEMA3B antibody has been previously described (1). For Western blot analysis, cells were lysed in NP40 extraction buffer [40 mmol/L HEPES-NaOH (pH 7.4), 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mmol/L NaCl, proteinase inhibitors]. Total protein (30–50  $\mu$ g) was separated on 10% SDS-PAGE gels and transferred to Hybond-P membrane (Schleicher & Schuell). Membranes were blocked for 30 min with 5% dry milk in 0.1% Tween 20 in TBS and incubated with primary antibodies as recommended by Cell Signaling Technology at 4°C overnight. Primary antibody excess was removed and membranes were incubated with horseradish peroxidase-labeled secondary antibodies from Amersham Pharmacia for 40 min before development using SuperSignal Chemiluminescence substrate (Pierce). For detection of cytochrome *c* on Western blot, we used a BD ApoAlert Cell Fractionation kit (BD Biosciences).

**Cell growth assays and soluble SEMA3B conditioned medium preparation.** Cos7 cells were transfected with the vector pcDNA3 (referred to as vector control) or plasmids encoding wild-type or a missense mutant SEMA3B and medium was collected 48 h after transfection to produce control-CM, SEMA3B-CM, or SEMA3Bmut-CM. An average of 15 to 40 ng/mL of SEMA3B was detected in SEMA3B-CM as determined by semiquantitative ELISA using our monoclonal anti-SEMA3B antibody (1). Cells were seeded in six-well (35 mm) plates at a density of 10,000 per well in the presence of control-CM or SEMA3B-CM diluted 1:2 with medium, and cells were enumerated 5 d later. Assays were performed in triplicate and repeated at least twice. For proliferation assays, cells were transfected with Akt constructs (pAkt, pAktMUT, and pAktDD) and selected using G418 before reseeding and treatment with SEMA3B-CM.

**Cell cycle analysis.** Cells were harvested 72 h after transfection, fixed with 70% ethanol, treated with 5 mg/mL RNase A (Sigma), stained with 50  $\mu$ g/mL propidium iodide, and analyzed by flow cytometry for DNA content and cell cycle status (FACSCalibur instrument, Becton Dickinson, equipped with CellQuest software).

**Akt kinase assay.** Akt kinase assay of cells treated with SEMA3B-CM was performed according to the manufacturer's instructions (Cell Signaling Technology). Cells ( $0.5\text{--}1 \times 10^6$ ) were plated, treated with SEMA3B-CM for 2 h, washed once with ice-cold PBS, and then placed for 15 min in 500  $\mu$ L of ice-cold lysis buffer [20 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L  $\beta$ -glycerophosphate, 1 mmol/L  $\text{Na}_3\text{VO}_4$ , 1  $\mu$ g/mL leupeptin, 1 mmol/L phenylmethylsulfonyl fluoride]. Lysates were cleared by centrifugation and allowed to immunoprecipitate overnight at 4°C with anti-Akt antibody. Immunoprecipitates on beads were washed twice with lysis buffer and kinase buffer [25 mmol/L Tris (pH 7.5), 5 mmol/L  $\beta$ -glycerophosphate, 2 mmol/L DTT, 0.1 mmol/L  $\text{Na}_3\text{VO}_4$ , 10 mmol/L  $\text{MgCl}_2$ ]. The kinase reaction was performed for 30 min at 30°C in kinase buffer supplemented with 200  $\mu$ mol/L ATP and 1  $\mu$ g of GSK-3 $\alpha/\beta$  fusion protein. The reaction was terminated with addition of 3 $\times$  SDS buffer. Samples were heated at 100°C for 3 min and separated on a 10% SDS-polyacrylamide gel. Phosphorylated protein bands were visualized as described under Western blot analysis.

**Small interfering RNA.** The small interfering RNA (siRNA) directed against Np-1, control siRNA, and Np-1-detecting antibody was from Santa Cruz Biotechnology. *In vitro* transfection was performed using Oligofectamine reagent from Invitrogen, and 50% confluent cells in six-well plates were processed according to the manufacturer's instructions and then treated with SEMA3B-CM 72 h after transfection.

**Statistical analysis.** The results are expressed as mean  $\pm$  SD for at least three separate (replicate) experiments for each treatment. Statistically significant differences among groups were assessed by a two-tailed Student's *t* test with values of  $P < 0.05$  sufficient to reject the null hypothesis for all analyses. All experiments were designed with matched control conditions within each experiment to enable statistical comparison as paired samples. All experiments were repeated thrice.

## Results

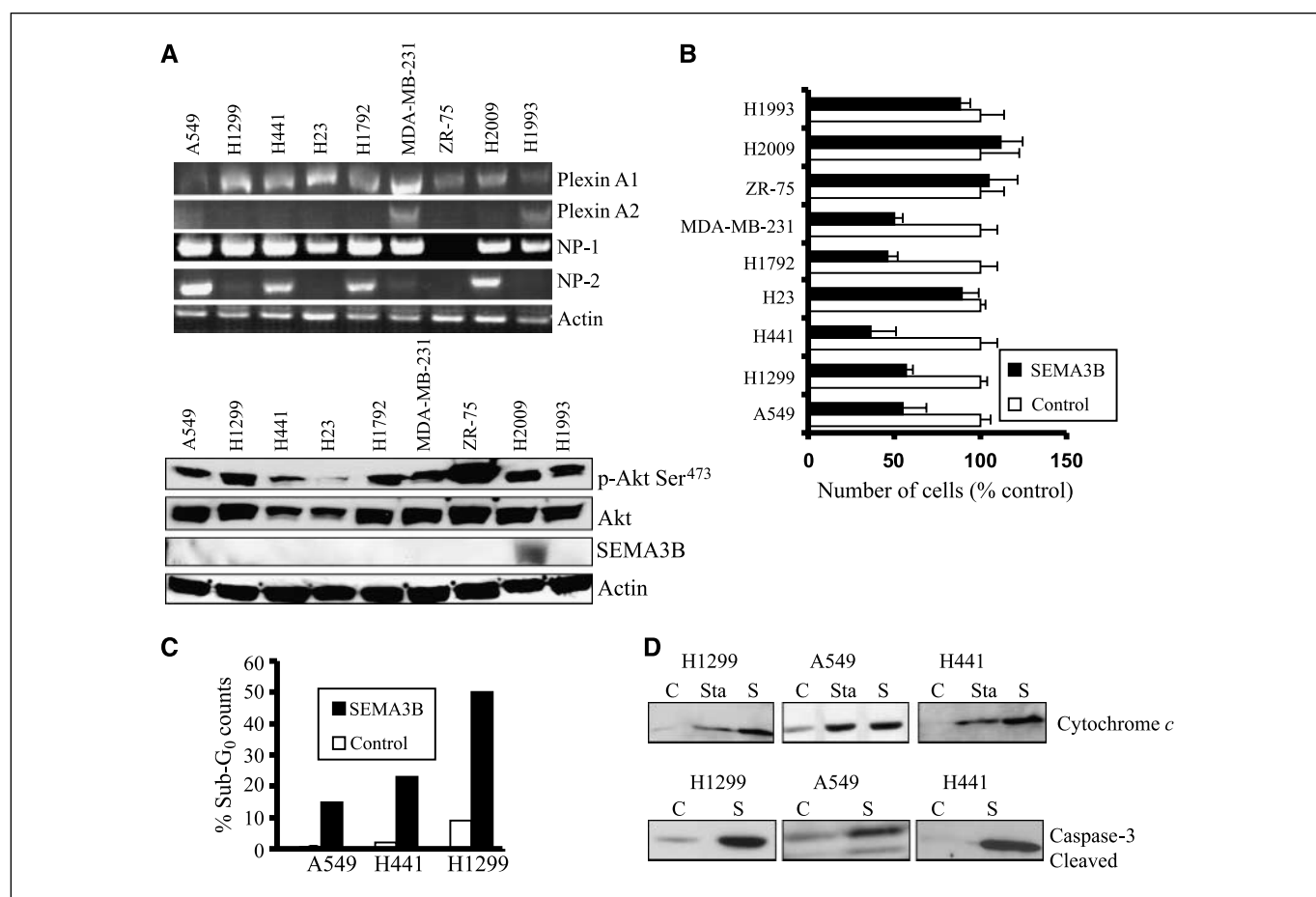
**Expression of Np-1, Np-2, plexin A1, plexin A2, Akt, and SEMA3B in lung and breast cancer cells.** In preparation for the functional studies described below, we first determined the mRNA expression of receptors for SEMA3B (Np-1, Np-2, plexin A1, and plexin A2) in seven lung and two breast cancer cell lines (Fig. 1A). Plexin A receptors are reportedly used by SEMA3 for activation (29, 30). Plexin A1 was detected in most tumor lines, whereas plexin A2 was detected only in A549, H1993, and MDA-MB-231 cells (Fig. 1A). Np-1 was expressed in all the cell lines with the exception of ZR-75 breast cancer cells, whereas Np-2 was expressed only in NSCLC cells A549, H441, H1792, and H2009 with trace expression detected in NSCLC H1299 and breast cancer MDA-MB-231 cells (Fig. 1A, top). We also determined under standard culture conditions total Akt and phospho-Akt proteins as well as SEMA3B protein (Fig. 1A,

bottom). Both phospho-Akt (Ser<sup>473</sup>) and total Akt proteins were detected in all cells examined albeit at different levels. Np-1-negative ZR-75-A cells showed the highest levels of phospho-Akt. SEMA3B protein was detected in only one cell line (NSCLC H2009) out of the nine lines examined in this study, supporting the proposed role for SEMA3B as a tumor suppressor. This is consistent with reports showing loss of SEMA3B expression in the majority of lung carcinomas.

**SEMA3B inhibits proliferation and induces apoptosis in lung and breast carcinoma lines.** Previously, we have shown that transfection with plasmid encoding SEMA3B inhibits the growth of many, but not all, lung and breast cancer cell lines (1, 3). In the present study, we examined the effect of SEMA3B-CM or control-CM on tumor cell line proliferation. Treatment with SEMA3B-CM for 4 days decreased proliferation of breast cancer line MDA-MB-231 and lung cancer lines A549, H441, H1792, and H1299 by 40% to 70% (Fig. 1B). SEMA3B-CM had no effect on SEMA3B-resistant

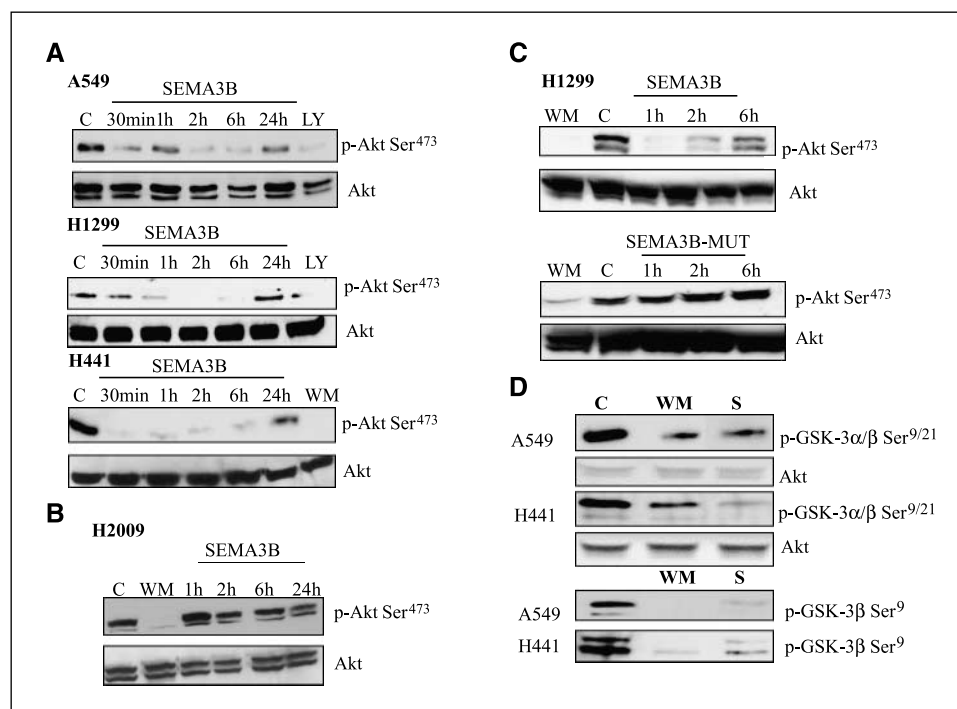
lines that included breast carcinoma ZR-75 (3) and NSCLC lines H23, H2009, and H1993 (Fig. 1B).

Three SEMA3B-sensitive cell lines were chosen to study the effect of SEMA3B on apoptosis using fluorescence-activated cell sorting analysis, cytochrome *c* release, and caspase-3 cleavage assays (Fig. 1C and D; ref. 3). A549, H441, and H1299 cells were transfected with empty vector or a SEMA3B expression plasmid, and cell viability was measured 48 h after transfection. At this time, >90% of the cells transfected with control vector were alive, with only 0.5% to 9% of cells displaying signs of apoptotic death (Fig. 1C). By contrast, up to 50% of tumor cells in SEMA3B-transfected cultures were apoptotic (Fig. 1C). Similar induction of apoptosis was observed after treatment of tumor cells with SEMA3B-CM and confirmed by measuring release of cytochrome *c* from the mitochondria to the cytosol (Fig. 1D). This event is crucial for formation of the apoptosome and caspase-9 complex as well as caspase-3 cleavage, which eventually leads to cell death.



**Figure 1.** SEMA3B induces apoptosis in lung and breast cancer cells. **A**, semaphorin receptors, Akt, and SEMA3B expression in lung and breast cancer cells. *Top*, RNA was collected from dividing cells and the mRNA expression of semaphorin receptors (plexin A1, plexin A2, Np-1, and Np-2) was determined by RT-PCR. RT-PCR for actin was used as a loading control and for assessment of RNA integrity. *Bottom*, the expression of endogenous phospho-Akt (p-Akt; Ser<sup>473</sup>), Akt, and SEMA3B using whole-cell extracts from dividing cells cultured in serum-containing medium was determined by Western blot. The level of  $\beta$ -actin protein was used as a loading control. **B**, identification of tumor cells whose growth is sensitive or resistant to treatment with soluble SEMA3B-CM. Cells were seeded into six-well plates, treated 24 h later with SEMA3B-CM (15–40 ng/mL; *black columns*) or vector control-CM (*white columns*), and counted 5 d later. *Columns*, mean; *bars*, SD. SEMA3B-sensitive tumor cells are A549, H1299, H441, H1792, and MDA-MB-231, whereas SEMA3B-resistant cells are H1993, H2009, ZR-75 (ZR-75-A), and H23. **C**, induction of apoptosis by SEMA3B in lung cancer cells. Cells were transfected with SEMA3B expression plasmid or control vector, cell cycle analysis was performed by flow cytometry 72 h after transfection, and tumor cells undergoing apoptosis were quantified by sub-G<sub>0</sub> peak counts. *White columns*, treatment with control vector; *black columns*, transfection with SEMA3B expression vector. **D**, cytochrome *c* release and caspase-3 cleavage after treatment with SEMA3B. Treatment of lung cancer cells indicated with control (C), SEMA3B-CM (S), or staurosporine (Sta; positive control) followed by cell fractionation and Western blot analysis for cytochrome *c* (*top*) or caspase-3 cleavage (*bottom*).





**Figure 2.** SEMA3B reduces Akt phosphorylation and inhibits Akt kinase activity in SEMA3B-sensitive NSCLC cells. Extracts of A549, H441, H1299, and H2009 cells were collected after treatment with control-CM (C), SEMA3B-CM, or SEMA3Bmut-CM for different times. PI3K inhibitors wortmannin (WM) and LY294002 (LY) were used as positive controls for inhibition of Akt phosphorylation at Ser<sup>473</sup>. **A**, inhibition of lung cancer p-Akt levels by treatment with SEMA3B. Cell extracts from the lung carcinoma cell lines were collected after treatment with control-CM (C), LY294002, or SEMA3B-CM (SEMA3B) at the indicated time points after treatment and evaluated for the levels of p-Akt and total Akt by Western blotting. **B**, SEMA3B-resistant H2009 lung cancer cells show no change in p-Akt after SEMA3B treatment but do respond to wortmannin. Western blot analysis for p-Akt in H2009 lung carcinoma cells treated with SEMA3B-CM, control-CM (C), or wortmannin at different time points. **C**, SEMA3B-sensitive H1299 lung cancer cells showed decreased pAkt level in response to wild-type (SEMA3B-CM, top) but not to mutant SEMA3B-CM (SEMA3Bmut-CM, bottom), determined by Western blot analysis. **D**, SEMA3B inhibition of Akt kinase activity in phosphorylating GSK in lung cancer cells A549 and H441. Cells were treated with SEMA3B-CM (S), control-CM (C), or wortmannin for 6 h. Top, endogenous Akt was immunoprecipitated after treatment and tested for *in vitro* ability to phosphorylate GSK-3 $\alpha/\beta$ ; bottom, Western blot analysis detecting phospho-GSK-3 $\beta$  (p-GSK-3 $\beta$ ) in the whole-cell extracts of H441 and A549 before immunoprecipitation by anti-Akt antibody.

Similar release of cytochrome *c* was detected in cells treated by staurosporine (Fig. 1D), a nonselective protein kinase inhibitor frequently used as an inducer of apoptosis. Treatment with SEMA3B-CM also induced cleavage of caspase-3 in A549 and H441 NSCLC cells (Fig. 1D), as previously reported for H1299 cells (1). Cleavage of procaspase-3, a hallmark of induced apoptosis, is considered a nonreversible event in the apoptotic cascade. Thus, treatment of three different tumor lines with SEMA3B resulted in identical proapoptotic outcome, including decreased cell number, release of cytochrome *c*, and cleavage of caspase-3. These data are consistent with our previous studies and collectively implicate SEMA3B in tumor growth inhibition and as a potent apoptotic inducer in multiple lung and breast cancer cell lines.

**SEMA3B inhibits Akt kinase activity in NSCLC.** Because Akt kinase activity can inhibit apoptosis and Akt is expressed prominently in most lung and breast cancer cells, we examined the effect of SEMA3B-CM on the phosphorylation status of Akt in these cells. Treatment with SEMA3B significantly (>95%) reduced phospho-Akt in A549, H441, and H1299 lung carcinoma cells (Fig. 2A; Supplementary Table S1 for quantification). The reduction in phospho-Akt was observed 30 min after exposure to SEMA3B-CM, persisted for at least 6 h, and returned to near baseline levels 24 h after treatment. Two PI3K inhibitors, wortmannin (500 nmol/L) and LY294002 (50  $\mu$ mol/L), were used as controls to show functionality and responsiveness of the Akt pathway to inhibitors in the cancer cells at the time of the treatment. Both SEMA3B-sensitive and

SEMA3B-resistant tumor cells responded to wortmannin and LY294002 inhibitors by down-regulating phospho-Akt. However, only tumor lines sensitive to SEMA3B growth inhibition, but not the resistant line H2009, down-regulated phospho-Akt in response to SEMA3B treatment (Fig. 2A and B). These findings provide specificity of the SEMA3B effects and support the notion that antiproliferative effect of SEMA3B in tumor cells sensitive to its action is mediated, in part, by suppressing Akt activity. This conclusion is also supported by the failure of a soluble SEMA3B missense mutant (SEMA3Bmut-CM) to decrease phosphorylation of Akt at Ser<sup>473</sup> (Fig. 2C). SEMA3Bmut-CM was previously shown to be inactive in cell proliferation and colony formation assays (3) and thus adds to the case for correlating the antiproliferative effects of SEMA3B with attenuation of the Akt pathway.

To further show the functional significance of the effect of SEMA3B on Akt, we measured Akt kinase activity extracted from control-treated and SEMA3B-CM-treated cells. This was done by precipitating Akt from cell lysates using an anti-Akt-specific antibody and analyzing the capacity of precipitates to phosphorylate a known Akt substrate, GSK-3 $\beta$ , *in vitro*. The relative kinase activities of Akt immunoprecipitates were compared with those in cells treated by wortmannin (Fig. 2D). In A549 and H441 cell lines, SEMA3B-CM and wortmannin inhibited Akt kinase activity to similar levels and both also decreased phosphorylation of endogenous GSK-3 $\beta$  as determined by the Akt kinase assay in whole-cell extracts (Fig. 2D). Collectively, these data indicate that SEMA3B

can decrease Akt kinase activity in cells that are sensitive to SEMA3B.

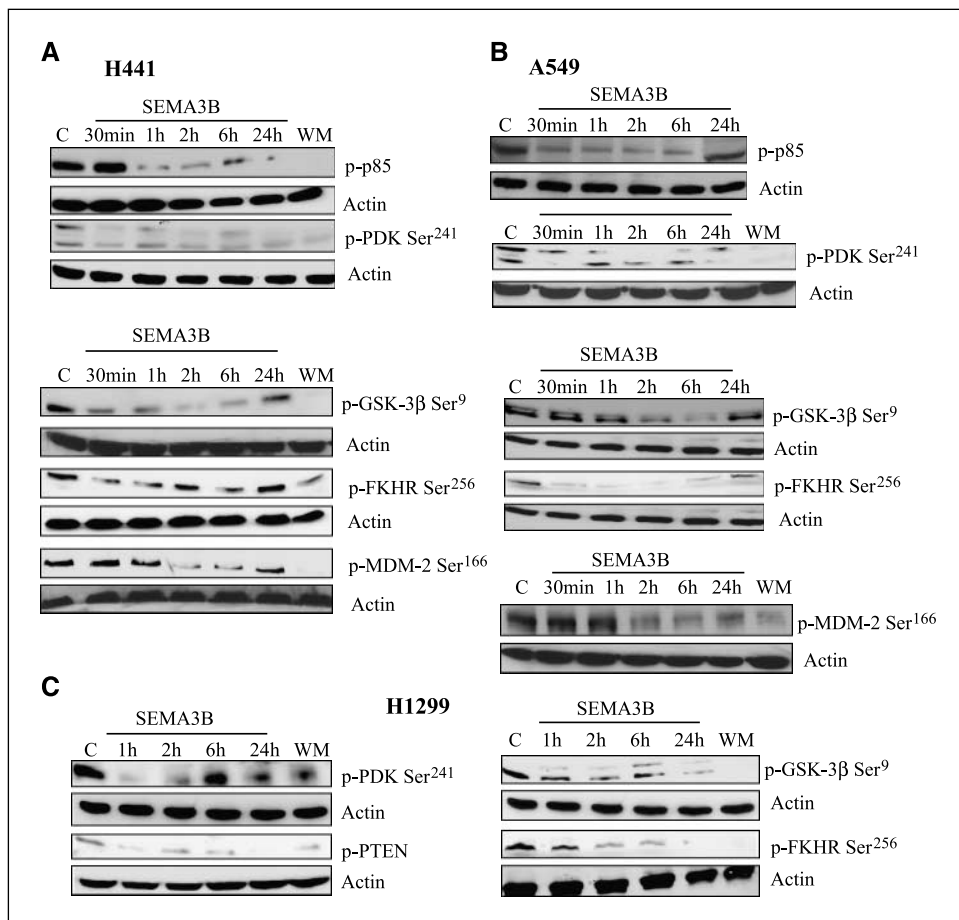
**SEMA3B inhibits signaling molecules both upstream and downstream of Akt.** Akt kinase is a critical regulator of the PI3K pathway, which promotes cell survival (18). Therefore, the specificity of the inhibitory effect of SEMA3B on Akt kinase was assessed in comparison with other mediators involved in the PI3K/Akt pathway. Western blotting was used to analyze several upstream and downstream proteins in this pathway in control-treated and SEMA3B-treated A549, H441, and H1299 lung cancer cells (Fig. 3; Supplementary Table S1 for quantification). The list of analyzed proteins included the activated p85, the regulatory subunit of the PI3K complex, PDK1, and PTEN that are functionally located upstream of Akt and involved directly in its activation. We also analyzed several downstream proteins, including FKHR, GSK-3 $\beta$ , and MDM-2, whose phosphorylation is increased in response to Akt activation.

Treatment of A549 and H441 cells with SEMA3B-CM decreased the phosphorylation of Tyr<sup>458</sup> in p85 and subsequently Ser<sup>241</sup> in PDK1 up to 65% (Fig. 3A and B). In H1299 cells, a 60% decrease in phosphorylation of PTEN was also observed (Fig. 3C). Dephosphorylated PTEN can counteract Akt activation by reducing phosphorylation in PIP3, a direct product of PI3K activation (31). Thus, multiple downstream Akt substrates showed decreased phosphorylation after SEMA3B treatment in several tumor lines (Fig. 3). For example, phosphorylation of S256 in FKHR, S9 in GSK-3 $\beta$ , and S166 in MDM-2 showed up to an 85% decrease after

SEMA3B-CM treatment in A549, H441, and H1299 cell lines. Collectively, these data show the inhibitory effect of SEMA3B-CM on multiple upstream and downstream proteins in the PI3K/Akt pathway in several SEMA3B-sensitive tumor lines.

**Constitutively activated Akt negates SEMA3B effect on cell proliferation and apoptosis.** To further examine the effect of SEMA3B on the Akt pathway, we transfected and selected A549, H441, and H1299 lung carcinoma cells with Akt expression vectors, pAkt, pAktMUT, and pAktDD. The pAkt vector encodes wild-type cDNA, whereas pAktMUT contains the K179A, T308A, and S473A mutations, which render Akt unable to bind ATP or be activated. The pAktDD vector encodes a constitutively active Akt due to T308D and S473D mutations, thereby rendering it permanently "activated" and no longer under phosphorylation control. A549, H441, and H1299 lung carcinoma cells stably expressing the Akt vectors were treated with SEMA3B-CM for 4 days and cell number was determined. Tumor lines stably transfected with wild-type Akt, inactive Akt, or an empty vector control had 50% to 70% reduction in cell number after SEMA3B-CM treatment compared with control-CM or untreated cells (Table 1). Lines transfected with wild-type pAkt and inactive pAktMUT showed an increase in caspase-3 cleavage after 48 h of treatment with SEMA3B-CM compared with control-CM. By contrast, SEMA3B-CM did not significantly decrease cell proliferation in tumor cells stably expressing constitutively active pAktDD. In fact, in some cases, pAktDD combined with SEMA3B increased proliferation (Table 1). Ectopic expression of Akt vectors in H1299 clearly showed a ~5-fold

**Figure 3.** SEMA3B affects upstream and downstream Akt pathway proteins in lung cancer cells. Cells extracts were collected after treatment with control-CM (C) or SEMA3B-CM (SEMA3B) at different time points or wortmannin for H441 (A), A549 (B), and H1299 (C). Western blot analysis for phospho-PDK1 (p-PDK), phospho-p85 (p-p85), and phospho-PTEN (p-PTEN; Akt pathway upstream proteins) and phospho-GSK-3 $\beta$ , phospho-MDM-2 (p-MDM-2), and phospho-FKHR (p-FKHR; Akt pathway downstream proteins). Actin was used as an internal loading control.



**Table 1.** Effect of SEMA3B-CM on NSCLC cells stably transfected with wild-type, inactive, or constitutively active Akt expression vectors

Vector*	Treatment <sup>†</sup>	Cell line		
		H1299	H441	A549
		No. cells <sup>‡</sup> (% control)		
Vector Control	Control-CM <sup>§</sup>	100 ± 9	100 ± 10	100 ± 10
	SEMA3B-CM <sup>§</sup>	44 ± 7	23 ± 4	100 ± 10
	<i>P</i> <sup>‡</sup>	0.001 <sup>  </sup>	0.001 <sup>  </sup>	0.001 <sup>  </sup>
pAkt Wild-type	Control-CM	100 ± 4	100 ± 17	100 ± 10.9
	SEMA3B-CM	49 ± 12	35 ± 11	31.2 ± 5
	<i>P</i>	0.002 <sup>  </sup>	0.001 <sup>  </sup>	0.001 <sup>  </sup>
pAktMUT Inactive	Control-CM	100 ± 5.16	100 ± 17	100 ± 10.1
	SEMA3B-CM	32 ± 8	45 ± 10	29 ± 3
	<i>P</i>	0.002 <sup>  </sup>	0.001 <sup>  </sup>	0.004 <sup>  </sup>
pAktDD Constitutively active	Control-CM	100 ± 8.6	100 ± 0.1	100 ± 11
	SEMA3B-CM	86 ± 3	141 ± 21	114 ± 10
	<i>P</i>	0.050 <sup>  </sup>	0.363	0.013

\*Expression vectors for pAkt (wild-type), pAktMUT (inactive), pAktDD (active), or empty vector (control) were transfected into H1299, H441, and A549 lung carcinoma cells and stable transfectants selected with G418.

<sup>†</sup> Cos7 SEMA3B or control-CM was added to the H1299, H441, and A549 transfectants and cell numbers were determined after 4 d of treatment.

<sup>‡</sup> Values represent mean ± SD of triplicate wells (cells in control wells were taken as 100%) and *P* values were calculated using two-tailed Student's *t* test.

<sup>§</sup> Cos7 cells were transfected with vector control (control-CM) or with SEMA3B expression vector (SEMA3B-CM) and conditioned medium was collected 48 h later.

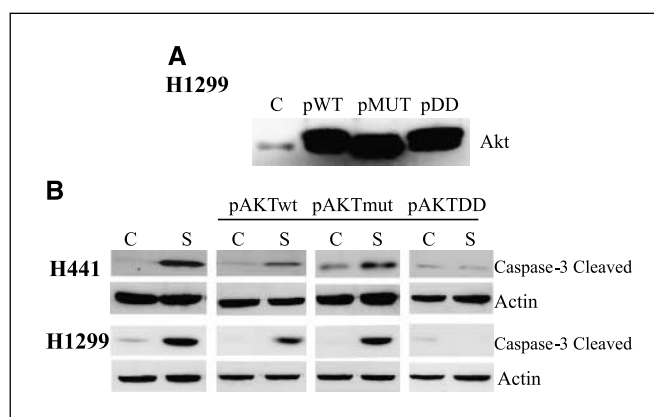
<sup>||</sup> Statistically significant decrease compared with control-CM for each set.

overexpression of Akt when compared with endogenous Akt (Fig. 4A). These results are consistent with reduced caspase-3 cleavage after SEMA3B-CM treatment in pAktDD-transfected tumor cells as observed on Western blots (Fig. 4B). Notably, H441 NSCLC cells expressing the inactive pAktMUT vector showed higher basal levels of activated caspase-3 (Fig. 4B). These findings show that constitutively active pAktDD in tumor cells overcomes the proapoptotic effect of SEMA3B.

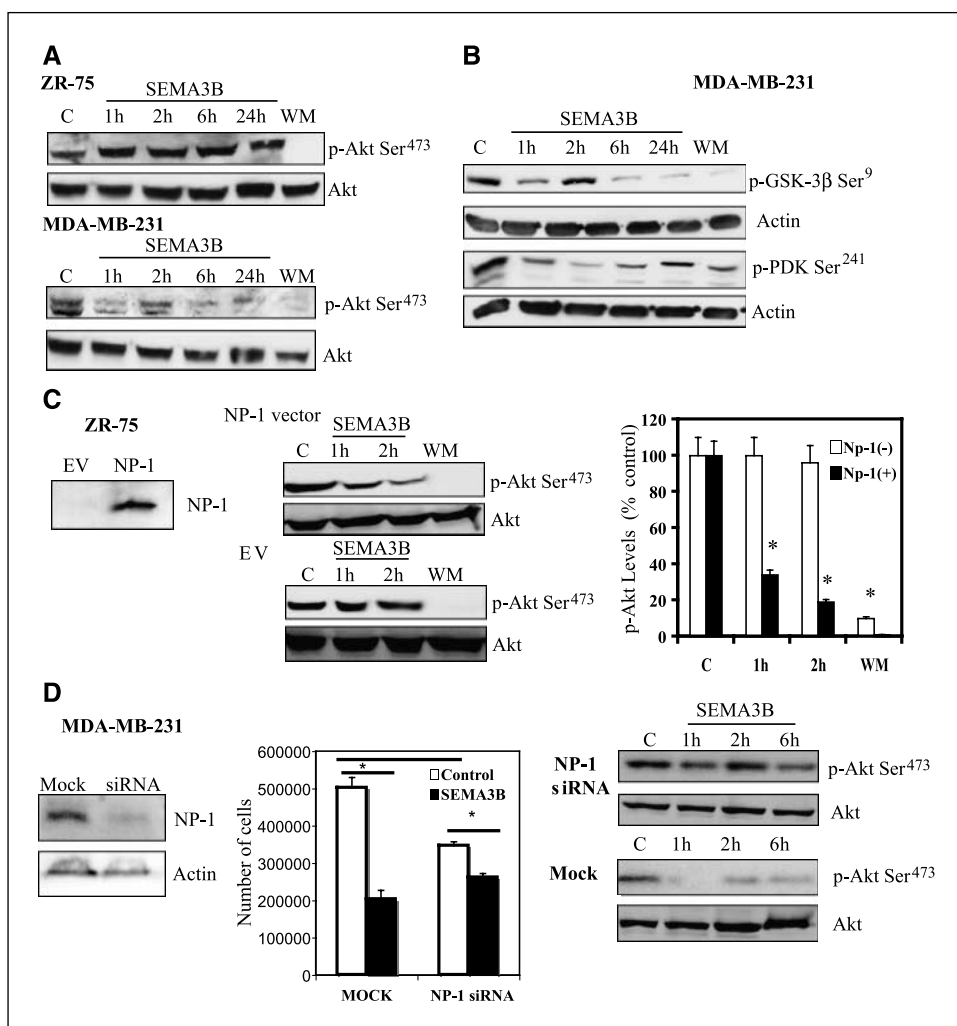
**Np-1 is required for SEMA3B to inhibit Akt activity in cancer cells.** MDA-MB-231 breast cancer cells express Np-1 and are sensitive to SEMA3B. ZR-75 cells do not express Np-1 and are resistant to SEMA3B treatment. The properties of these two lines provide an opportunity to compare the effect of SEMA3B on Akt activity in representative SEMA3B-sensitive and SEMA3B-resistant breast carcinoma lines. Both lines were sensitive to wortmannin treatments; however, treatment with SEMA3B significantly reduced phospho-Akt in the Np-1-positive MDA-MB-231 but not in the Np-1-negative ZR-75 cells. This effect was observed 1 to 24 h after exposure to SEMA3B-CM (Fig. 5A). SEMA3B treatment also reduced activation of PDK1 and GSK-3β in MDA-MB-231 cells, similarly to the effect observed in NSCLC lines (Fig. 5B). Moreover, when ZR-75 cells were engineered to stably express Np-1, these cells acquired responsiveness to SEMA3B shown, in part, by down-regulation of phospho-Akt in the presence of SEMA3B (Fig. 5C).

To gain better understanding for the requirement for Np-1 to mediate the effects of SEMA3B, MDA-MB-231 cells were treated with Np-1-specific siRNA or siRNA of irrelevant target specificity. As shown in Fig. 5D (*top left*), Np-1 protein was reduced by 40% after specific siRNA treatment. Down-regulation of Np-1 signifi-

cantly reduced response to SEMA3B-CM (26% versus 60% in control knockdown) and decreased tumor cell proliferation in the absence of SEMA3B treatment (Fig. 5D, *bottom*). MDA-MB-231 cells with decreased Np-1 expression also lost the ability to down-regulate phospho-Akt in response to SEMA3B, whereas this effect was preserved in the cells treated with siRNA control (Fig. 5D,



**Figure 4.** SEMA3B induces the cleavage of caspase-3-dependent apoptosis and constitutively active pAktDD abolishes this effect in lung cancer cells. H441 and H1299 cells were transfected with pAkt, pAktMUT, and pAktDD and selected with G418 for 1 wk. *A*, Western blot for Akt expression levels in H1299 cells after transfection with pAkt, pAktMUT, and pAktDD. *B*, stably transfected tumor cells were seeded and treated with control-CM (C) and/or SEMA3B-CM (S) for 72 h. Cell extracts were analyzed by Western blot for caspase-3 cleavage. Actin was used as a control for loading.



**Figure 5.** Expression of Np-1 is required for SEMA3B inhibition of p-Akt. Cells extracts were analyzed by Western blot after different time exposures to control-CM (C) and SEMA3B-CM (SEMA3B) or treatment with wortmannin that was used as a positive control for inhibition of p-Akt at Ser<sup>473</sup>. **A**, Western blot for p-Akt after treatment with SEMA3B-CM in sensitive MDA-MB-231 and resistant ZR-75 breast cancer cells shows inhibition of p-Akt in sensitive but not resistant tumor cells. **B**, Western blot for phospho-PDK and GSK (upstream and downstream targets of the Akt pathway) in MDA-MB-231 cells after SEMA3B-CM treatment. **C**, reexpression of Np-1 in SEMA3B-resistant ZR-75 cells leads to reactivation of SEMA3B response in down-regulation of p-Akt. *Left*, expression of Np-1 in the stably transfected ZR-75 is confirmed by Western blot and compared with empty vector (EV) control. *Middle*, Western blot for p-Akt after introduction of Np-1 by stable transfection in ZR-75 followed by treatment with SEMA3B. Cells stably transfected with an empty vector were used as a control. *Right*, quantitative representations of p-Akt levels after SEMA3B-CM treatment normalized to total Akt in ZR-75 with (black columns) or without (white columns) Np-1. **D**, *Left*, Western blot shows >40% reduction of Np-1 (determined by densitometric analysis of Western blots) after Np-1 siRNA but not control siRNA (Mock) treatment in MDA-MB-231 cells. *Right*, Western blot for p-Akt in MDA-MB-231 cells after Np-1 siRNA or control siRNA knockdown followed by treatment with SEMA3B-CM. *Middle*, proliferation studies after knockdown of Np-1 in MDA-MB-231 cells and treatment with SEMA3B-CM (15–40 ng/mL of SEMA3B) or vector control-CM, with cell numbers determined 4 d later. Columns, mean; bars, SD. The reduction of 60% in cell numbers in the presence of Np-1 with SEMA3B treatment is significantly ( $P < 0.05$ ) greater than the 26% reduction seen with Np-1 knockdown. Also note that Np-1 knockdown with no SEMA3B treatment resulted in a decreased number of cells. \*,  $P < 0.05$ , significant comparisons between the different treatments shown.

*right*). Together, these studies indicate that treatment with soluble SEMA3B inhibits both the phosphorylation and the activity of Akt in SEMA3B-sensitive lung and breast cancer cells in a Np-1-dependent manner.

## Discussion

SEMA3B is a candidate tumor suppressor because it frequently undergoes allele loss and reduced expression or function in lung and breast cancers (usually through epigenetic mechanisms), whereas reexpression decreases cell proliferation and induces apoptosis in lung, breast, and ovarian cancer cells (3, 4). Recently, our laboratory showed that SEMA3B exerts its tumor suppressor

effects through competition with VEGF<sub>165</sub> (1). This presumably occurs because SEMA3B and VEGF<sub>165</sub> compete for neuropilin receptors on the tumor cell surface (1). VEGF-A has been shown to promote tumor cell proliferation and survival in an autocrine manner through binding Np-1. Thus, a reasonable hypothesis is that SEMA3B mediates its tumor-suppressing effects, in part, by blocking VEGF-A autocrine activity in tumor cells. VEGF-A signaling includes activation of the PI3K/Akt pathway (32). The link between the PI3K/Akt pathway and semaphorin-induced signaling in tumor cells has been previously suggested by the studies with SEMA3F, another member of the semaphorin family. SEMA3F, a protein with similar tumor suppressor properties as those of SEMA3B, is located in the 3p21.3 region that is ~75 kb



away from SEMA3B (33, 34). SEMA3F has been shown to antagonize neurotrophin-induced PI3K and mitogen-activated protein kinase signaling in sympathetic neurons leading to growth cone collapse (9). Reintroduction of SEMA3F in H157 NSCLC cells suppressed tumor cell density and growth as well as ILK-extracellular signal-regulated kinase 1/2 and Akt/signal transducer and activator of transcription 3 signaling (35). In the present report, we tested the hypothesis that SEMA3B, a member of the same semaphorin family, exerts similar tumor-inhibitory effects by binding Np-1 receptor that induces signals suppressing the PI3K/Akt pathway activation. The results of the studies reported here showed that exogenously added SEMA3B inhibits the PI3K/Akt pathway acting through Np-1 receptors in cancer cells, leading to decreased cell survival and increased apoptosis. Our studies showed that SEMA3B decreased phosphorylation of Akt and multiple other proteins related to the Akt pathway, including p85 (PI3K complex), PDK, PTEN, GSK-3 $\beta$ , FKHR, and MDM-2. These Akt pathway effects were observed in NSCLC and breast cancer cells that are sensitive to the antiproliferative effect of SEMA3B. By contrast, SEMA3B-driven changes in Akt phosphorylation or related pathway changes did not occur in SEMA3B-resistant tumor cells or in cells treated with a SEMA3B missense mutant protein, indicating the specificity of the effect of SEMA3B. The reduced level of Akt phosphorylation in sensitive cells after SEMA3B-CM treatment is associated with diminished Akt capacity to phosphorylate GSK-3 $\alpha/\beta$  substrate. We confirmed these observations by the introduction of pAktDD, which overcame the SEMA3B effects on NSCLC cells. Furthermore, exogenous expression of Np-1 in ZR-75 cells (Np-1 negative) and knockdown of Np-1 in MDA-MB-231 cells (Np-1 positive) show that in these cells Np-1 mediates SEMA3B effects on the Akt pathway. However, the fact that SEMA3B-resistant lines H2009, H23, and H1993 express Np-1 suggests that Np-1 is necessary but not sufficient for SEMA3B to exert its effect on cell survival, inhibition of Akt kinase activity, and subsequently reduction of phosphorylation of survival-promoting proteins. The mechanism of SEMA3B resistance will be of great interest to unravel but, as described in Results, these "SEMA3B-resistant" tumor lines provide important specificity controls for all of our experiments. Thus, despite these exceptions between Np-1 expression and response to SEMA3B, in a major subset of NSCLC and breast cancer cells, the addition of SEMA3B, at picomolar concentrations, functions through Np-1 to inactivate Akt signaling activity, consequently decreasing proliferation and inducing apoptosis.

Np-1 is expressed at a high level in tumors of epithelial origin (carcinomas), whereas tumors from different origin such as melanomas strongly express Np-2 (36–38). Increased expression of Np-1 and Np-2 correlates with tumor aggressiveness, advanced disease stage, and poor prognosis (39–41). In contrast, several studies have reported that Np-1 overexpression in tumor cells reduces motility and tumorigenicity or prolonged patient survival (42–45). Possible reasons for discrepancies between various studies may include the expression of functional receptor/ligand repertoire (e.g., plexins)

and/or the ratio of VEGF-A to SEMA3 within the particular tumor microenvironment. Np-1 internalizes on ligand binding at different rates depending on a ligand type (46). Preferential receptor binding and ligand-mediated internalization suggest a mechanism by which a common receptor, Np-1, can function in prioritizing conflicting signals (46). These effects of neuropilins suggest their potential as targets for antitumor therapy.

The Akt pathway is activated in a variety of carcinomas of lung, breast, colon, and pancreatic and other tumor types through overexpression of Akt protein. Additionally, *KRAS*, *EGFR*, and *PI3K* mutations activate this pathway as well as loss of negative regulatory proteins such as PTEN that shuts down the PI3K pathway (47). In the clinical setting, aberrations in the Akt pathway are associated with poor prognosis and a decreased patient survival rate after combined radiotherapy and chemotherapy (25). Recent reports have shown that radiation activates the Akt pathway, which may promote endothelial cell survival (48). Moreover, radiation-induced Akt activation also increased cell migration and subsequently metastasis in animal models of breast cancer and in cultured pancreatic carcinoma cells (49, 50). These studies collectively show the effect that the Akt pathway exerts on tumorigenesis, tumor angiogenesis, tumor progression, and metastasis as well as tumor responses to anticancer therapies. Drugs that negatively regulate the Akt pathway in either tumor cells or angiogenic endothelial cells could be of therapeutic value. In our experimental model, exogenously added SEMA3B induces apoptosis, in part, by inhibiting the Akt pathway in lung and breast cancer cells. SEMA3B effect on phosphorylation starts with the upstream protein, PI3K, and continues down the Akt pathway also affecting downstream proteins, such as GSK-3 $\beta$ , FKHR, and MDM-2. The inhibitory effect of SEMA3B on the Akt pathway in tumor cells with numerous genetic and epigenetic changes and the role of Np-1 in this effect suggest SEMA3B as a potential novel systemic anticancer therapeutic agent. Moreover, given the important role of Akt in cell survival, SEMA3B down-regulation of Akt activity might also sensitize tumor cells to chemotherapy, radiotherapy, or other types of therapy. Based on the data reported here, further assessment of antitumor and antimetastatic effects of soluble SEMA3B is warranted.

## Disclosure of Potential Conflicts of Interest

J.D. Minna: commercial research grant, AstraZeneca. J.D. Minna has applied for a patent for SEMA3B as a cancer therapeutic. The other authors disclosed no potential conflicts of interest.

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## Semaphorin 3B Inhibits the Phosphatidylinositol 3-Kinase/Akt Pathway through Neuropilin-1 in Lung and Breast Cancer Cells

Emely Castro-Rivera, Sophia Ran, Rolf A. Brekken, et al.

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