

# The Plexin-A1 Receptor Activates Vascular Endothelial Growth Factor-Receptor 2 and Nuclear Factor- $\kappa$ B to Mediate Survival and Anchorage-Independent Growth of Malignant Mesothelioma Cells

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## Abstract

**The semaphorins and their receptors, the neuropilins and the plexins, are constituents of a complex regulatory system that controls axonal guidance. Moreover, many types of tumor cells express various members of semaphorins and receptors, but the biological activities within tumor mass and the signal transduction mechanism(s) they use are largely unknown. Here, we show that in asbestos-related malignant pleural mesothelioma (MPM), Semaphorin-6D (Sema6D) and its receptor plexin-A1 are frequently expressed and trigger a prosurvival program that promotes anchorage-independent growth of MPM cells. Interestingly, the same response is also controlled by the tyrosine kinase receptors of vascular endothelial growth factor (VEGF) through a nuclear factor- $\kappa$ B (NF- $\kappa$ B)-dependent pathway. We found that in MPM cells, plexin-A1 and VEGF-receptor 2 (VEGF-R2) are associated in a complex. Moreover, the presence of Sema6D promotes the tyrosine phosphorylation of VEGF-R2 in a plexin-A1-dependent manner. This is necessary for basal and Sema6D-induced NF- $\kappa$ B transcriptional activity, and NF- $\kappa$ B mediates tumor cell survival. Expression of Sema6D and plexin-A1 is induced by asbestos fibers and overexpression of plexin-A1 in nonmalignant mesothelial cells inhibits cell death after asbestos exposure. This work identifies a new biological function of semaphorins in cancer cells and suggests the involvement of an undescribed survival pathway during MPM tumorigenesis. [Cancer Res 2009;69(4):1485–93]**

## Introduction

Malignant pleural mesothelioma (MPM) is an asbestos-related malignancy that afflicts about 3,000 Americans per year. Currently, the overall survival of MPM patients ranges between 3% and 6% despite aggressive multimodality intervention (1). These data highlight the need for a more comprehensive understanding of the molecular mechanisms that cause MPM and contribute to the development of innovative therapies for the disease.

MPM is characterized by vascular endothelial growth factor (VEGF) overexpression that is implicated not only in angiogenesis but also in growth factor stimulation (2). VEGF-receptor 1 (VEGF-R1), VEGF-R2, and VEGF-R3 were expressed in MPM and

selective inhibitors of the tyrosine kinase VEGF-R2 have been introduced in the clinical use (3). More recently, an additional nontyrosine kinase receptor for VEGF, called neuropilin-1 (NP-1), has been identified, which is able to significantly enhance the affinity of some VEGF isoforms to VEGF-R2 (4, 5). MPM cells express NP-1 (6), but there are no studies that directly examine the role of neuropilins in MPM biology. Several data indicate that NP-1 expression is instrumental in tumor formation. Indeed, NP-1 regulates hepatocyte growth factor-induced invasiveness (7). Moreover, overexpression of NP-1 induced rapid tumor growth and progression in tumors, developing from either prostate cancer-derived cells or colon carcinoma cells (8, 9). However, because neuropilins lack intracellular kinase domains, it has been postulated that unknown additional membrane-bound proteins are required for their tumorigenic properties (10).

NP-1 was also observed to form complexes with additional cell surface receptors (i.e., plexins; ref. 11). They are transmembrane receptors distinguished by the presence of a split GTPase-activating cytoplasmic domain (12). Plexins are the signal-transducing elements of semaphorins, which are a large family of evolutionary conserved molecules implicated in axon guidance, organogenesis, angiogenesis, immune responses, and oncogenesis (13, 14). In particular, the type A plexins (Plxn-A1, Plxn-A2, Plxn-A3, and Plxn-A4), together with ligand-binding neuropilins, are the signaling moiety of the receptor complex for class 3 semaphorins (11). Moreover, Plxn-As are also the primary receptors for class 6 transmembrane semaphorins [e.g., Semaphorin-6A (Sema6A) and Sema6D] that do not bind neuropilins but activate VEGF-R2-mediated signal transduction (15). Despite a growing body of evidence indicating that Plxn-As are expressed in cancer cells (14, 16–18), the underlying mechanisms and the *in vivo* function of these receptors still remain poorly understood.

Here, we have characterized the role of Plxn-A1 in MPM cells. We show that Plxn-A1 and its ligand Sema6D are expressed and active in MPM cells and that inhibition of Plxn-A1 perturbs survival and anchorage-independent growth of MPM cells in a VEGF-R2-dependent manner. These observations may be important in therapeutic and diagnostic approaches to MPM.

## Materials and Methods

**Materials.** The HuSH 29-mer short hairpin RNA against NP-1, Plxn-A1, Sema6D, VEGF-R2, and control plasmids were purchased from Origene (Eppendorf). The constructs were transfected into cells using Lipofectamine (Invitrogen) according to the manufacturer's protocols followed by selection with 1 ng/mL puromycin (Sigma). Antibiotic-resistant pools and individual clones were isolated and maintained in the presence of puromycin. Human recombinant VEGF and other growth factors were purchased from Calbiochem. Anti-human VEGF neutralizing antibody and irrelevant, control isotype antibody (from Abcam) were added

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

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at the serum-free medium at dose of 40 ng/mL for 48 h. Anti-Plxn-A1, anti-Ser<sup>563</sup>-phosphorylated p65, and anti-total NF- $\kappa$ B p65 antibodies were purchased from Cell Signaling. Anti-Sema6D antibody was from R&D Systems. Anti-NP-1 was from Santa Cruz Biotechnology. Any other reagents—if not differently specified—were from Sigma and all concentrations used *in vitro* were previously determined (6, 19, 20).

**Cell culture.** Human MPM cells and nonmalignant met-5A cells were available in our laboratory (21, 22). Three primary mesothelial (HMC) cultures were established from patients and identified morphologically and by extensive phenotypic analysis as described previously (6, 21). After 2 wk in culture, >99% of HMC cells stained positive for calretinin. They were then expanded and used for the experiments. Primary cells were used between the third and the seventh passage. All cells were screened periodically for *Mycoplasma* contamination (Celbio).

**Plxn-A1 transfectants.** Plxn-A1 expression lentiviral vectors and the infection of MPM cells have been described previously (6). The plexin constructs included a vesicular stomatitis virus (VSV) tag, detectable with an anti-VSV-G monoclonal antibody (V-5507; Sigma) by immunostaining. This method ensured stable gene transfer with very high efficiency (over 95% transgene-positive cells, as determined by standard immunofluorescence protocol), without need to select individual cell clones.

**Generation of Sema6D concentrated conditioned medium.** The plasmid coding wild-type (WT) Sema6D (transcript variant 4) was obtained from Origene (Eppendorf). Although Sema6D is a transmembrane-type semaphorin, it has been shown that a soluble form of Sema6D is cleaved from the cell surface (15). Thus, it is possible to generate a conditioned medium (CM) containing high levels of soluble Sema6D, as previously described for other semaphorins (19, 23, 24). To this end, COS-7 cells were cultured at a subconfluence density and transfected using Lipofectamine for 4 h. Transfected COS-7 cells were grown for 48 h and selected, by limiting dilution, with an appropriate drug. More than 10 clones were generated. After stable transfection, clones were washed and cultured in serum-free containing medium for 48 and 72 h. The CM obtained from these clones was then concentrated 5-fold by means of a Centricon-10 concentrator (Millipore). Cell lysates and CM of transfectants were screened for Sema6D expression by immunoblotting. Representative CM with high Sema6D levels (Sema6D-CM) was selected for the experiments. CM of COS-7 cells transfected with vector alone (Control-CM) was used as control.

**Immunoprecipitation and immunoblot analysis.** Immunoprecipitation and immunoblots were performed from whole-cell lysates as previously described (20–22). Briefly, cells were lysed with EB buffer [20 mmol/L Tris-HCl (pH 7.4), 5 mmol/L EDTA, 150 mmol/L sodium chloride, 10% glycerol, 1% Triton X-100] in the presence of 1  $\mu$ g/mL leupeptin, 3  $\mu$ g/mL aprotinin, 1  $\mu$ g/mL pepstatin, 2 mmol/L phenylmethylsulfonyl fluoride, and 1 mmol/L sodium orthovanadate. After immunoprecipitation with antibodies against Plxn-A1 and VEGF-R2, high-stringency washes were performed (EB buffer containing 1 mol/L lithium chloride). Western blots were then performed, and appropriate antibodies were detected using a chemiluminescence-based system (Promega). In some experiments, blots were reprobated with an anti-actin monoclonal antibody (AC15, 1  $\mu$ g/mL; Sigma) as loading control. For each protein tested, Western blot analyses were repeated at least thrice.

**RNA isolation and real-time reverse transcription-PCR.** Total RNA from tumor cell lines was isolated by using RNeasy Protect Mini kit (Qiagen) according to the manufacturer's instructions. cDNA preparation was performed according to standard procedures using Moloney murine leukemia virus reverse transcriptase (Promega) and oligo(dT) primers (Sigma). Real-time reverse transcription-PCR analysis was done in a Chromo4 sequence detector (Bio-Rad) as previously described (19). The primers and probes of semaphorins, neuropilins, and plexins were determined by Laboratory Tools software analysis of Stratagene. Details of sequences and thermal cycle conditions are available on request. Data were acquired and analyzed with the sequence detector Chromo4 software.

**MPM biopsies and immunohistochemistry.** A total of 38 specimens, diagnosed between 1999 and 2006, were analyzed in this study: 29 MPM (19 epithelioid, 3 sarcomatous, and 7 biphasic phenotypes) and 9 non-neoplastic pleural tissues. With regard to MPM, asbestos exposure was known in 23 cases, 2 had no history of asbestos exposure, and there were

no data available for 4 cases. The material was fixed in buffered formalin and embedded in paraffin wax. For all nonneoplastic tissues and 11 MPM, the sections came from the material of pneumectomy or pleuropneumectomy. For the 18 MPM, the sections came from the biopsies obtained by thoracoscopy. Histologic diagnosis of MPM was established according to the WHO criteria (25). Immunohistochemical analysis was performed using standard techniques. Briefly, 4- $\mu$ m paraffin-embedded sections were heated; deparaffinized; heated in citrate buffer; blocked for endogenous peroxidase, avidin, and biotin; incubated with specific anti-Sema6D and anti-Plxn-A1 antibodies; washed; and developed using the LSAB kit (DAKO). Gastric and lung tumors were used as positive controls for Sema6D and Plxn-A1, respectively. All sections were reviewed and diagnosed by two independent examiners. Staining was interpreted as positive if >5% of tumor cells showed specific staining.

**Colony formation assays.** Anchorage-independent growth was quantified by soft agar assays (22). Cells ( $10^4$ ) were suspended in 1.5 mL of 0.4% (w/v) noble agar (Sigma) and overlaid on 0.6% (w/v) agar. After 2 wk of incubation at 37°C, colonies were visualized by staining with nitro-tetrazolium blue chloride (1 mg/mL), and those >0.5  $\mu$ m in diameter were counted. All experiments were done in triplicate and repeated at least thrice.

**Apoptosis assay.** Apoptosis was assayed by the Live/Dead Assay (Invitrogen) or by detection of internucleosomal DNA fragmentation in fixed cells using an *in situ* terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay (Boehringer) or via immunodetection of activated caspase-3 as previously described (20). Percent death was calculated as cells positive for ethidium bromide expressed as a percentage of the total number of live cells scored positive by calcein staining (FITC). The apoptotic labeling index was calculated as the percentage of total cells positive for FITC-labeled 3'OH DNA ends, and percent apoptosis was determined as the percentage of total cells positive for activated caspase-3. The minimum number of cells scored was 200 to 400 per experimental condition. Cell death by apoptosis was also confirmed by Annexin and propidium iodide fluorescent staining.

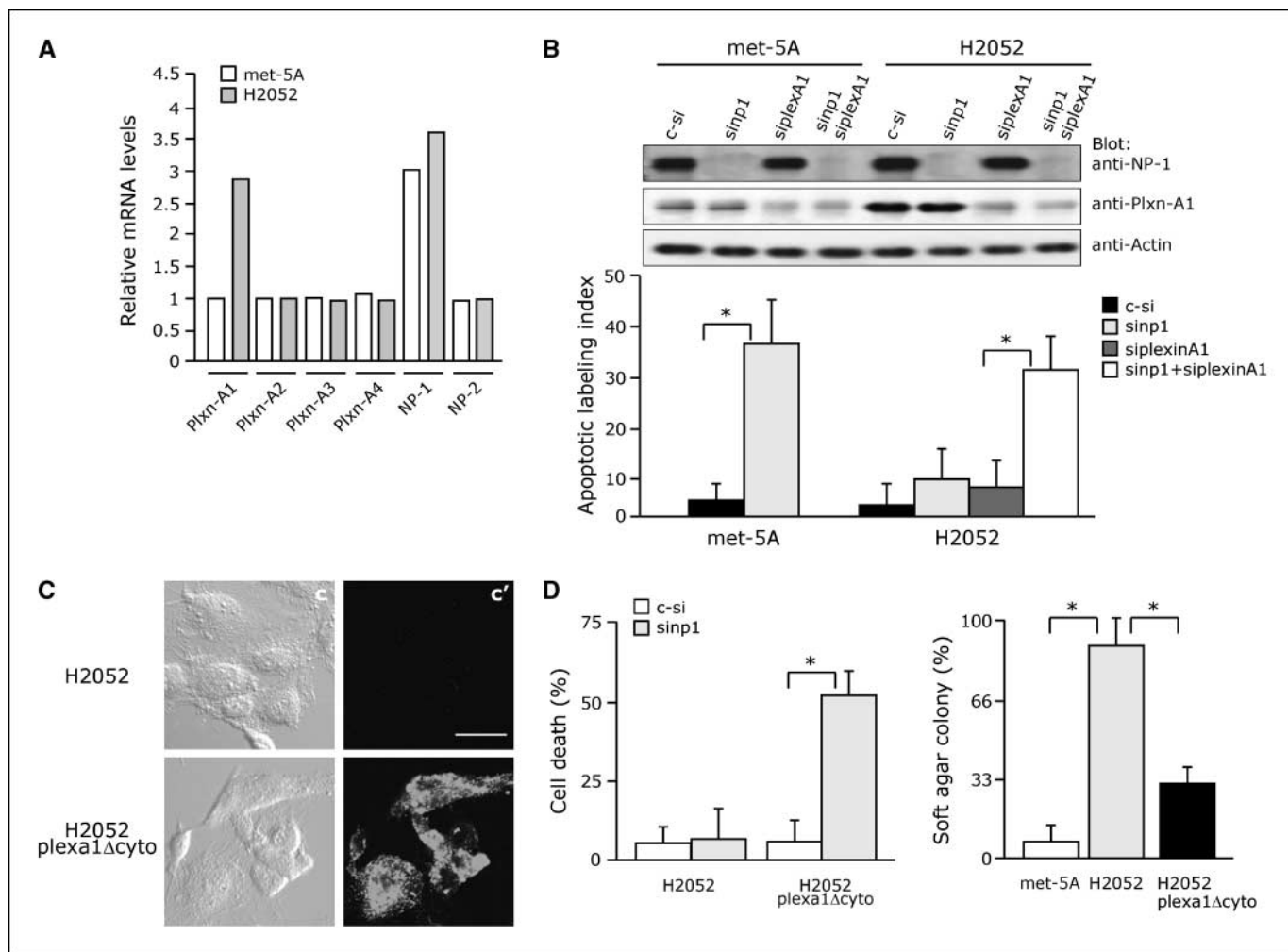
**NF- $\kappa$ B reporter assays.** The NF- $\kappa$ B reporter plasmid has been described (19). We cotransfected 0.1 to 0.2  $\mu$ g of reporter constructs with 2 ng of pRL-CMV (Promega) in the indicated cells and measured luciferase activity with the Dual Luciferase Reporter Assay System (Promega). We calculated the relative luciferase activity as the ratio of firefly/*Renilla* luciferase activity. Data are reported as the ratio between the relative luciferase activity in transfectants to that in control cells.

**Human NF- $\kappa$ B signaling pathway gene array.** The expression levels of NF- $\kappa$ B signaling pathway genes were analyzed in total RNA using the human NF- $\kappa$ B signaling gene array (SuperArray Biosciences Corp.) according to the protocol of the manufacturer.

**Statistical analysis.** All experiments were done at least in triplicate and were expressed as mean  $\pm$  SE. The Bonferroni multiple comparison test and *t* test were used for the statistical analysis of comparative data using StatView version 5.0 (NET Engineering). Values of  $P \leq 0.05$  were considered significant and are indicated by asterisks in the figures.

## Results

**Plxn-A1 mediates anchorage-independent survival of MPM cells.** We firstly used met-5A (nonmalignant) and H2052 (tumorigenic) human mesothelial cells to investigate whether neuropilins and Plxn-As govern growth and survival of MPM cells. We found that Plxn-A1 is the only member of the A-subfamily expressed at higher mRNA levels in H2052 cells comparable with met-5A (Fig. 1A). NP-1 is expressed at remarkably higher levels than NP-2 in H2052 and met-5A. We then performed RNA interference (RNAi) of endogenous NP-1 and Plxn-A1 by treatment with specific small interfering RNAs (siRNA; Fig. 1B, top). Knockdown of NP-1 by siRNA induced apoptosis in met-5A cells but not in H2052 cells (Fig. 1B, bottom). Conversely, loss of function of both NP-1 and Plxn-A1 resulted in H2052 cell death (Fig. 1B, bottom). This effect was not unique to H2052 cells. Knockdown of both NP-1 and



**Figure 1.** Plxn-A1 mediates anchorage-independent survival of MPM cells. *A*, the type A plexin (Plxn-A1, Plxn-A2, Plxn-A3, and Plxn-A4) and neuropilin (NP-1 and NP-2) expression levels were measured by real-time PCR in both met-5A (nonmalignant) and H2052 (tumorigenic) mesothelial cells. Taqman probe for human  $\beta$ -actin was used as internal control for each sample. The mRNA expression levels in mesothelial cells were then normalized to the average (Y axis = 1) of values obtained in three different nontumor cell lines (mammary epithelial cells MCF-10A, HUVEC, and resting T cells). *B*, met-5A and H2052 cells were transfected with control siRNA (*c-si*), NP-1 siRNA (*sinp1*), Plxn-A1 siRNA (*siplexA1*), or NP-1 siRNA plus Plxn-A1 siRNA for 72 h and immunoblotted with the indicated antibodies. Apoptotic labeling indices were calculated as indicated in Materials and Methods. *C*, phase-contrast (*c*) and conventional immunofluorescence (anti-VSV; *c'*) showing uniform expression of tailless Plxn-A1 (*plexa1Δcyto*) in H2052 cells. Scale bar, 50  $\mu$ m. *D*, left, cell viability was calculated using the Live/Dead Assay for H2052 or H2052 *plexa1Δcyto* cells grown for 72 h with control siRNA or NP-1 siRNA. Right, colony formation was counted using an inverted microscope, and for each cell line (H2052 *plexa1Δcyto* or H2052 expressing Plxn-A1 siRNA), growth of untreated H2052 cells was set up at 100%. Clonogenic survival was then plotted as a fraction relative to these untreated cells. \*,  $P < 0.05$ .

Plxn-A1 in additional MPM cells (H28, H2452, and Ist-mes1) showed a similar induction of apoptosis (Supplementary Fig. S1A). We also confirmed the expression of NP-1 and Plxn-A1 in additional malignant and normal mesothelial cells (Supplementary Fig. S1B). Thus, MPM cell survival required expression of either NP-1 or Plxn-A1.

To explore how Plxn-A1 regulates tumor cell survival, we selected pooled populations of H2052 cells that stably expressed high levels of VSV-tagged, tailless Plxn-A1 (*plexa1Δcyto*; Fig. 1C). H2052 cells expressing *plexa1Δcyto* required NP-1 for their survival (Fig. 1D, left) and failed to form colonies in soft agar (Fig. 1D, right). This indicates that Plxn-A1 cytoplasmic function is required for the anchorage-independent survival phenotype of these cancer cells.

**Increased Plxn-A1 does not promote anchorage-independent survival of nonmalignant mesothelial cells.** We asked whether overexpression of Plxn-A1 could confer apoptotic resistance and anchorage independence to nonmalignant mesothelial cells.

met-5A cells were infected with Plxn-A1 WT and selected pooled populations of cells expressing elevated protein levels of Plxn-A1 (Supplementary Fig. S2A) were used for experiments. As mentioned above, control met-5A died when NP-1 expression was inhibited by siRNA (Supplementary Fig. S2B). In contrast, met-5A overexpressing Plxn-A1 remained viable despite the absence of NP-1 (Supplementary Fig. S2B). However, met-5A cells infected with Plxn-A1 WT failed to grow in soft agar (Supplementary Fig. S2C). Notably, Plxn-A1 overexpression did not increase the basal levels of NP-1 (data not shown). Therefore, a signal functionally linked to Plxn-A1 supports the survival of nonmalignant mesothelial cells in a NP-1-independent manner.

**Sema6D acts as a ligand of Plxn-A1 involved in the survival of MPM cells.** Various members of semaphorins, such as Sema3A and Sema6D, directly or indirectly bind Plxn-A1 and activate Plxn-A1-mediated signal transduction (14). As we found that Plxn-A1 influences survival and anchorage independence of MPM cells,

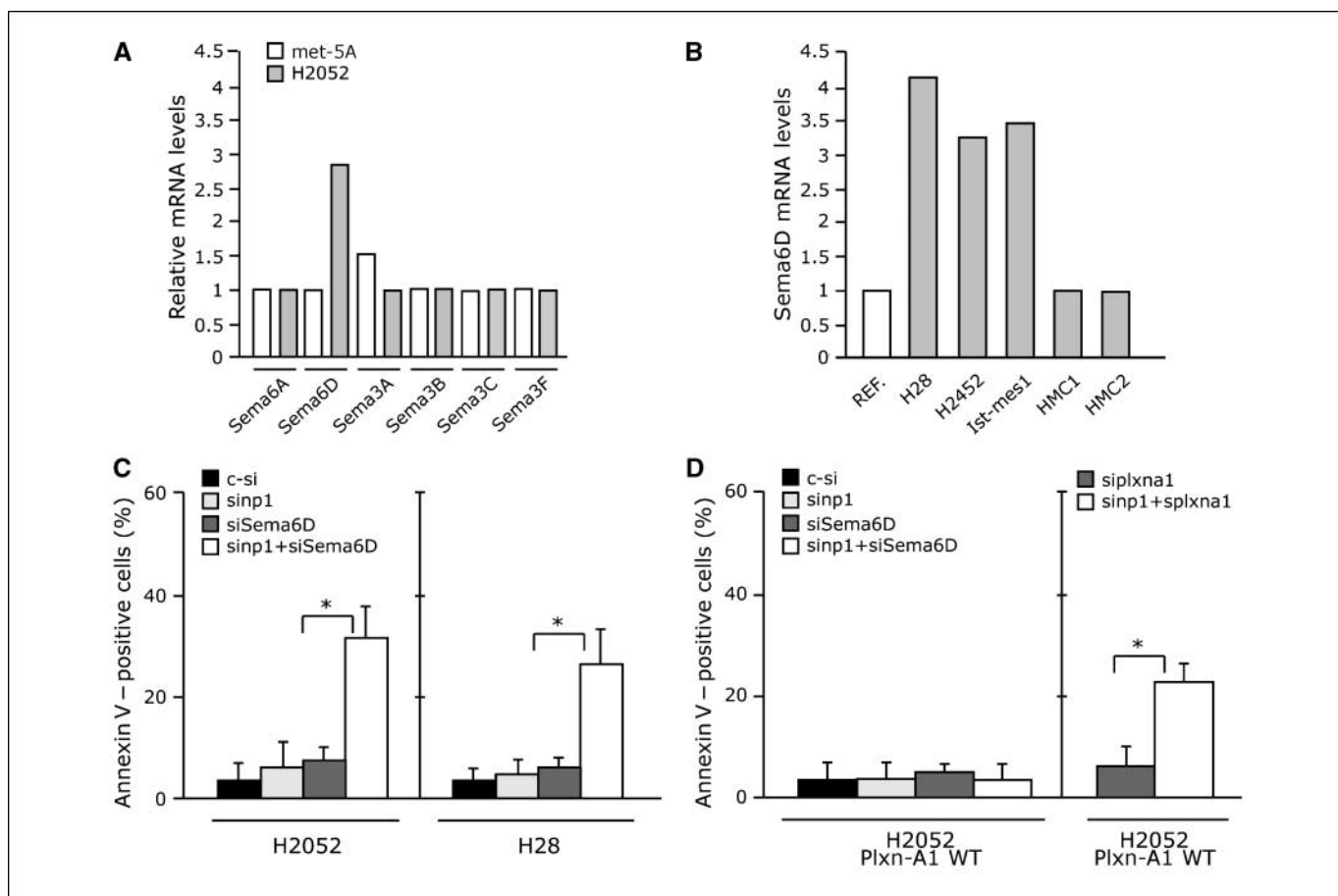
we postulated that malignant transformation is associated with constitutive activation of Plxn-A1 or linked to increased synthesis of Plxn-A1-binding semaphorins. In accord with the latter prediction, H2052 cells expressed more *Sema6D* mRNA than met-5A cells (Fig. 2A). Moreover, both H2052 and met-5A cells expressed multiple semaphorins potentially linked to Plxn-A1, but their mRNA levels remained constant (Fig. 2A). We also observed enhanced expression of *Sema6D* mRNA in H28, H2452, and Ist-mes1 cells (Fig. 2B).

We then examined whether *Sema6D* was required for Plxn-A1-mediated tumor survival. As observed above with Plxn-A1 siRNA, knockdown of *Sema6D* by RNAi resulted in H2052 cell death when NP-1 expression was simultaneously inhibited (Fig. 2C). We confirmed these findings in an additional mesothelioma cell line, H28 (Fig. 2C). Plxn-A1 overexpression rescued cell death in H2052 cells exposed to *Sema6D* plus NP-1 siRNA but had no effect in those cells treated with Plxn-A1 plus NP-1 siRNA (Fig. 2D). Therefore, *Sema6D* acts upstream of plexin-A1 to promote tumor cell survival.

To support our findings and determine the relationship between Plxn-A1 and *Sema6D*, we examined whether the protein expression of Plxn-A1 might be positively correlated with *Sema6D* in human

MPM tissues. A significant correlation between the Plxn-A1-positive and *Sema6D*-positive tissues was found in a cohort of 29 MPM patients (Table 1). Tumor sections stained with *Sema6D* and Plxn-A1 taken from four of the analyzed patients are shown in Fig. 3.

**Plxn-A1 mediates tumor survival by increasing VEGF-R2 activity.** It is noteworthy that plexins use different coreceptors to exert a variety of biological effects. Indeed, Plxn-A1 forms a receptor complex with receptor-type tyrosine kinases such as VEGF-R2 or Off-track in a region-specific manner during chick cardiac morphogenesis (15). Moreover, Plxn-A1 may be associated with Trem-2 and DAP12 as observed in dendritic and bone marrow cells (26). However, Trem-2 and DAP12 expression was not detected in both malignant and nonmalignant mesothelial cells (data not shown). Therefore, to determine the molecular pathway that mediates Plxn-A1 intracellular signaling, we examined, by coimmunoprecipitation, the physical interactions between Plxn-A1 and either NP-1, VEGF-R2, or Off-track. H2052 cells expressing siRNA for *Sema6D* were cultured in the presence of the CM of *Sema6D*-expressing cells (*Sema6D*-CM) or the CM of cells transfected with vector alone (Control-CM; Fig. 4A). The association of Plxn-A1 with NP-1 was detected by coprecipitation either in the



**Figure 2.** *Sema6D* is involved in the Plxn-A1-mediated anchorage-independent survival of MPM cells. **A**, *Sema6A*, *Sema6D*, *Sema3A*, *Sema3B*, *Sema3C*, and *Sema3F* expression levels were measured by real-time PCR in both met-5A and H2052 cells. Taqman probe for human  $\beta$ -actin was used as internal control for each sample. **B**, *Sema6D* expression levels were measured by real-time PCR in the indicated malignant (H28, H2052, and Ist-mes1) and normal (HMC1 and HMC2) mesothelial cells. *Sema6D* expression levels were then normalized to the average ("REF" = 1) of values obtained in three different nontumor cells (mammary epithelial cells MCF-10A, HUVEC, and human T cells). **C**, cell death calculated using FITC-Annexin V in H2052 or H28 cells grown for 72 h in the presence of control siRNA (c-si), NP-1 siRNA (*sinp1*), *Sema6D* siRNA (*siSema6D*), or NP-1 siRNA plus *siSema6D* (*sinp1+siSema6D*). **D**, cell death assay results showing that overexpression of Plxn-A1 renders H2052 cells expressing *Sema6D* and NP-1 siRNA resistant to apoptosis. Statistical significance is shown.



**Table 1.** Positive correlation between Plxn-A1 and Sema6D expression in human malignant mesothelioma tissues

Plxn-A1	Sema6D		Total
	-	+	
-	5 (17.2)	3 (10.3)	8 (27.6)
+	5 (17.2)	16 (55.2)	21 (72.4)
Total (percentage)	10 (34.4)	19 (65.5)	29 (100)

NOTE: The 29 MPM specimens were analyzed by immunohistochemical staining antibodies specific to Plxn-A1 and Sema6D, respectively. Staining was interpreted as positive (+) if >5% of tumor cells showed specific staining. The expression pattern of Sema6D and Plxn-A1 was analyzed by using  $\chi^2$  test ( $P = 0.02$ ).

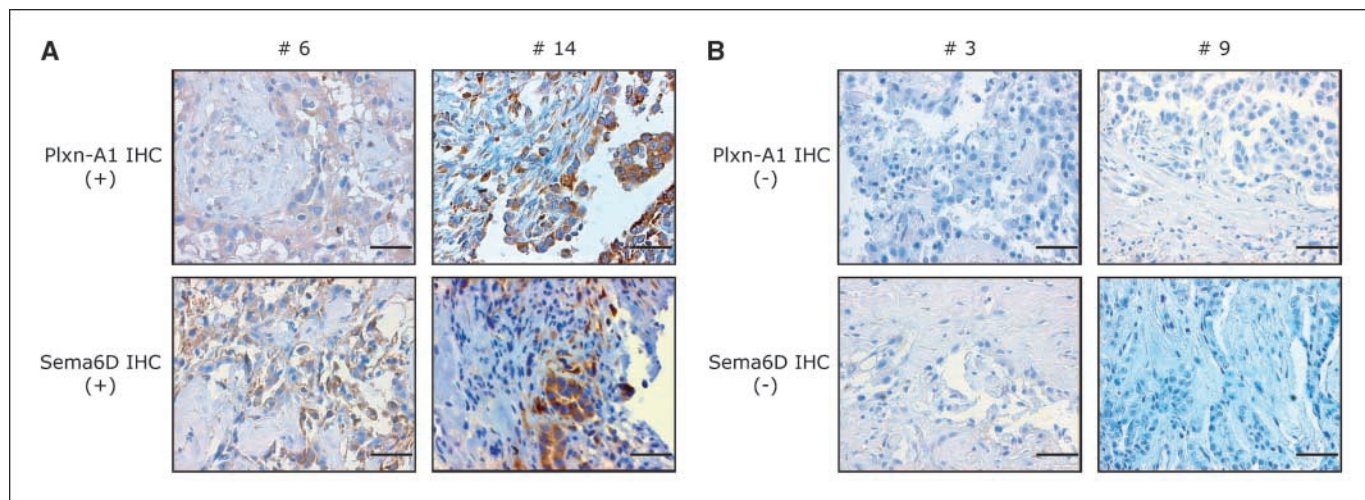
presence or absence of Sema6D-CM. Although both VEGF-R2 and Off-track interacted with Plxn-A1 in the absence of Sema6D-CM, Off-track was coimmunoprecipitated with Plxn-A1 at higher levels than VEGF-R2 (Fig. 4A). The presence of Sema6D-CM enhanced VEGF-R2 binding and suppressed Off-track association. This alteration in binding was accompanied to VEGF-R2 phosphorylation (Fig. 4B). These events are not detected in tumor cells transfected with Plxn-A1 siRNA (Fig. 4B). Interestingly, Sema6D-CM strongly enhanced VEGF-induced phosphorylation of VEGF-R2 (Fig. 4B), indicating a collaboration of Sema6D and VEGF signals by Plxn-A1/VEGF-R2 complex in tumor cells.

We next determined whether VEGF circuit is functionally involved in the survival of these cells. Although control antibodies had no effect, neutralizing antibodies against VEGF provoked the death of H2052 and H28 cells only when Sema6D expression was inhibited with siRNA (Fig. 4C). Similar results were observed when we used the Plxn-A1 siRNA instead of Sema6D siRNA (data not shown). These results suggest that both VEGF and Sema6D can support the survival of MPM cells. To determine whether Sema6D-

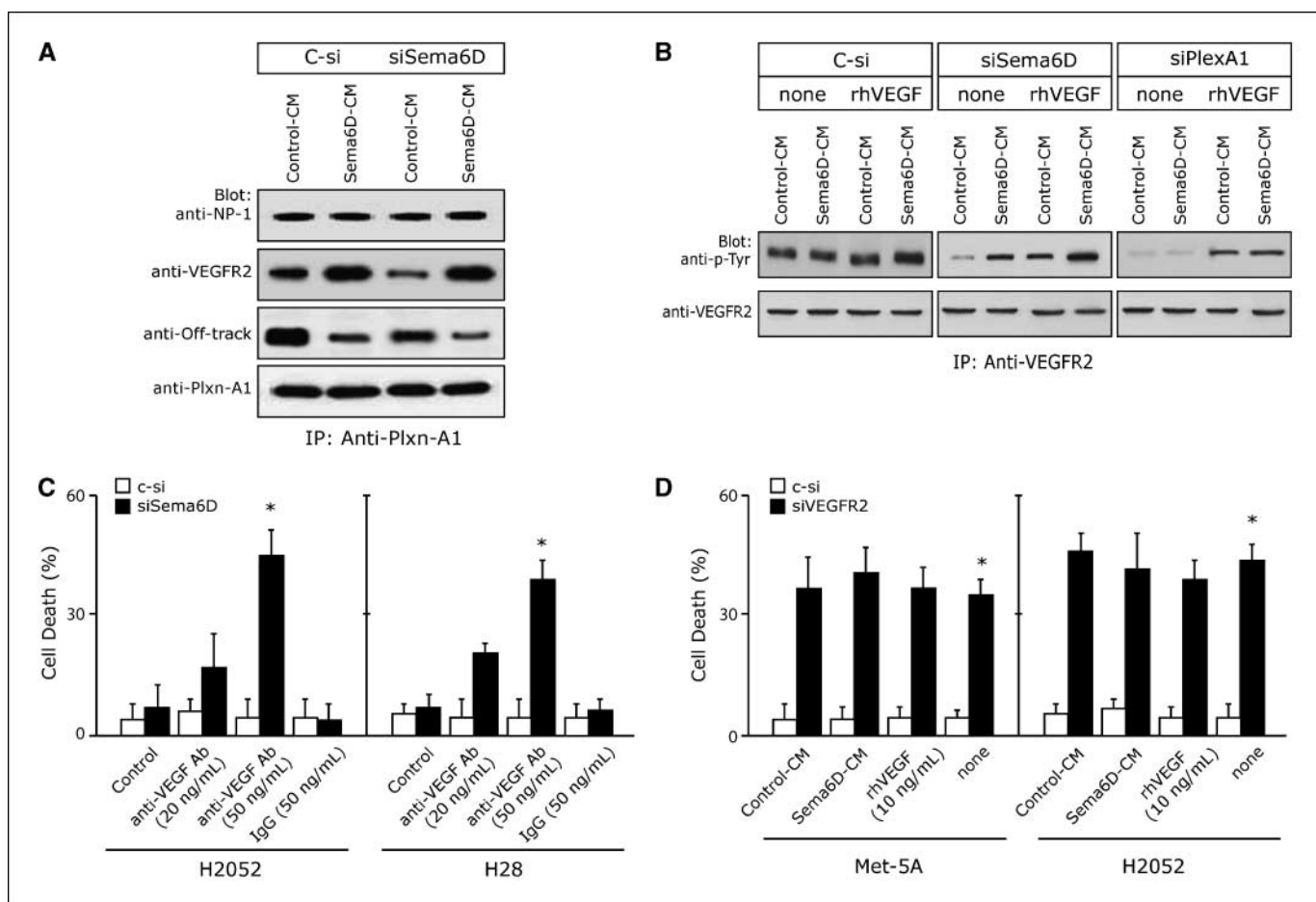
induced survival of MPM cells directly involves a VEGF-R2 activation, met-5A and H2052 cells were transiently transfected with either control or VEGF-R2 siRNA. Cells were then grown in the presence of Sema6D-CM or exogenous VEGF. Although control siRNA had no effect, the specific siRNA against VEGF-R2 induced cell death in both cell types (Fig. 4D). In the absence of VEGF-R2 expression, neither Sema6D-CM nor VEGF has shown any pro-survival effects (Fig. 4D). All these results indicate that even if it is possible that several signal transduction pathways are activated by Sema6D and VEGF, some of them will certainly lead to VEGF-R2 activation.

Accordingly, increasing concentration of VEGF reversed the inhibitory effect of NP-1 siRNA on H2052 cells expressing plexa1 $\Delta$ cyto (Supplementary Fig. S3A), whereas equimolar concentrations of fibroblast growth factor 2 (FGF2) and epidermal growth factor (EGF) were ineffective (data not shown), although MPM cells have got functional tyrosine kinase receptors for FGF and EGF (27, 28). Moreover, the addition of SU5416, a specific inhibitor of VEGF-R2 kinase activity, to met-5A Plxn-A1 WT cultures reduced cell survival in the presence of NP-1 siRNA (Supplementary Fig. S3B). These results support our observation that Plxn-A1 signaling modulates MPM cell survival by promoting a VEGF-R2-dependent pathway.

**Plxn-A1 mediates tumor cell survival via VEGF-R2-dependent activation of nuclear factor- $\kappa$ B.** We then studied whether Plxn-A1 signaling may activate the nuclear factor- $\kappa$ B (NF- $\kappa$ B) pathway, a known VEGF-induced signaling that is activated in multiple tumors (29). NF- $\kappa$ B reporter activity was increased in met-5A cells expressing Plxn-A1 WT, whereas H2052 cells, which exhibit constitutively active NF- $\kappa$ B, inhibited NF- $\kappa$ B activity significantly in the presence of either Sema6D or Plxn-A1 RNAi (Fig. 5A). Similar results were observed when cells were exposed to SU5416 (Fig. 5A). Western blotting of cell lysates showed that phosphorylation of the p65 subunit was greater in met-5A cells overexpressing Plxn-A1 but not in those treated with the specific inhibitor of VEGF-R2 (Fig. 5B). Accordingly, phosphorylation of the p65 subunit in H2052 cells was significantly down-regulated by either Sema6D or Plxn-A1 RNAi (Fig. 5B). Thus, activation of the Plxn-A1 pathway is important for the induction of NF- $\kappa$ B in the presence of VEGF-R activity.



**Figure 3.** Positive correlation of Plxn-A1 and Sema6D expression in human MPM patients. A and B, immunohistochemical (IHC) levels of Plxn-A1 and Sema6D from tumor sections (#6 and #14 represented Plxn-A1-positive and Sema6D-positive; #3 and #9 represented Plxn-A1-negative and Sema6D-negative) of four patients. The photographs were taken at 400-fold. Scale bar, 50  $\mu$ m.



**Figure 4.** Sema6D and Plxn-A1 regulate VEGF-R2 activity in MPM cells. **A**, H2052 cells expressing the indicated siRNA were incubated in the presence or absence of CM enriched with soluble Sema6D (*Sema6D-CM*) for 10 min. Proteins were immunoprecipitated (*IP*) from cell lysates and immunoblotted (*Blot*) using the indicated antibodies. **B**, lysates of H2052 cells transfected with the indicated siRNA and grown with or without Sema6D-CM and/or rhVEGF (5 ng/mL) were immunoprecipitated with anti-VEGF-R2 antibody. Immunoprecipitates were immunoblotted with the indicated antibodies. **C**, cell viability of both H2052 and H28 cells transfected with the indicated siRNA and treated with neutralizing antibodies against VEGF or control mouse nonimmune antibodies (IgG), as indicated. *Control*, no antibody added. **D**, effect of VEGF-R2 siRNA on cell viability of indicated cells. After transient transfection, cells were grown in the presence of Sema6D-CM or rhVEGF for additional 48 h. \*,  $P < 0.05$ .

To extend these findings, the expression of ~115 genes related to NF- $\kappa$ B-mediated signal transduction was analyzed using a NF- $\kappa$ B signaling pathway microarray in met-5A control cells and met-5A cells expressing plixin-A1 WT. Plxn-A1 expression significantly induced the expression of several NF- $\kappa$ B downstream genes, such as *intercellular adhesion molecule-3 (ICAM-3)*, *ICAM-2*, *Toll-like receptor-4 (TLR-4)*, *TLR-5*, *FOS*, *JUN*, and *interleukin-8 (IL-8)*; Fig. 5C). These findings indicate that activation of NF- $\kappa$ B pathway by Plxn-A1 translates into actual changes in NF- $\kappa$ B-responsive gene expression.

To directly determine if Plxn-A1-dependent activation of NF- $\kappa$ B is essential for the survival of mesothelial tumors, we inhibited NF- $\kappa$ B and tested the effects of Plxn-A1-dependent survival. Incubation with BAY 11-7082, a specific inhibitor of NF- $\kappa$ B, specifically induced cell death in the Plxn-A1 WT transfectants but had no effect on viability when NP-1 was expressed (Fig. 5D). Thus, activation of the NF- $\kappa$ B pathway is important for the Plxn-A1-mediated survival effects in the absence of NP-1.

**Plxn-A1 and Sema6D are regulated by crocidolite fibers and protect human mesothelial cells from apoptosis.** We tried to determine how Plxn-A1 and Sema6D expression is regulated. We assessed Plxn-A1 and Sema6D expression in three human primary

mesothelial cells and in met-5A cells after exposure with crocidolite fibers, the most pathogenic type of asbestos in the causation of MPM (1, 3). After 48 hours, cells were analyzed by Western blot using anti-Plxn-A1 and anti-Sema6D antibodies. Crocidolite strongly increased Plxn-A1 and Sema6D in all analyzed cells (Fig. 6A). Continuous exposure to crocidolite fibers ( $5 \mu\text{g}/\text{cm}^2$ ) induced a time-dependent expression of both Plxn-A1 and Sema6D proteins, with a maximum reached at 36 hours in two selected cell lines (HMC1 and met-5A; Fig. 6B). This action was at least partially mediated by increasing levels of Plxn-A1 and Sema6D mRNA in all tested cells (data not shown). These data suggest that induction of Plxn-A1 and Sema6D is promoted by asbestos fibers, providing a molecular explanation for Plxn-A1 and Sema6D expression in MPM tumors.

Because asbestos fibers are known to induce apoptosis in human and rabbit mesothelial cells (30), we measured the cytotoxicity in response to crocidolite fibers in met-5A and met-5A expressing Plxn-A1 WT in the presence or absence of Sema6D-CM stimulation. met-5A cells, when stimulated with Sema6D-CM, were significantly ( $P < 0.05$ ) less sensitive to asbestos-induced cytotoxicity than unstimulated cells (Fig. 6C). In contrast, met-5A cells expressing Plxn-A1 WT were intrinsically more resistant to asbestos-induced

cytotoxicity, irrespective of the presence of exogenous *Sema6D*-CM (Fig. 6D). Moreover, BAY 11-7082 almost completely abrogated the protective effect in *met-5A* exposed to *Sema6D*-CM and in *met-5A* expressing *Plxn-A1* WT (Fig. 6C and D). Similar results were observed when we used the VEGF-R2 inhibitor SU5416 instead of BAY 11-7082 (data not shown). This result strongly suggests that the identified *Sema6D*/*Plxn-A1*/VEGF-R2/*NF-κB* signaling may be responsible for cell survival in nonmalignant mesothelial cells after crocidolite exposure.

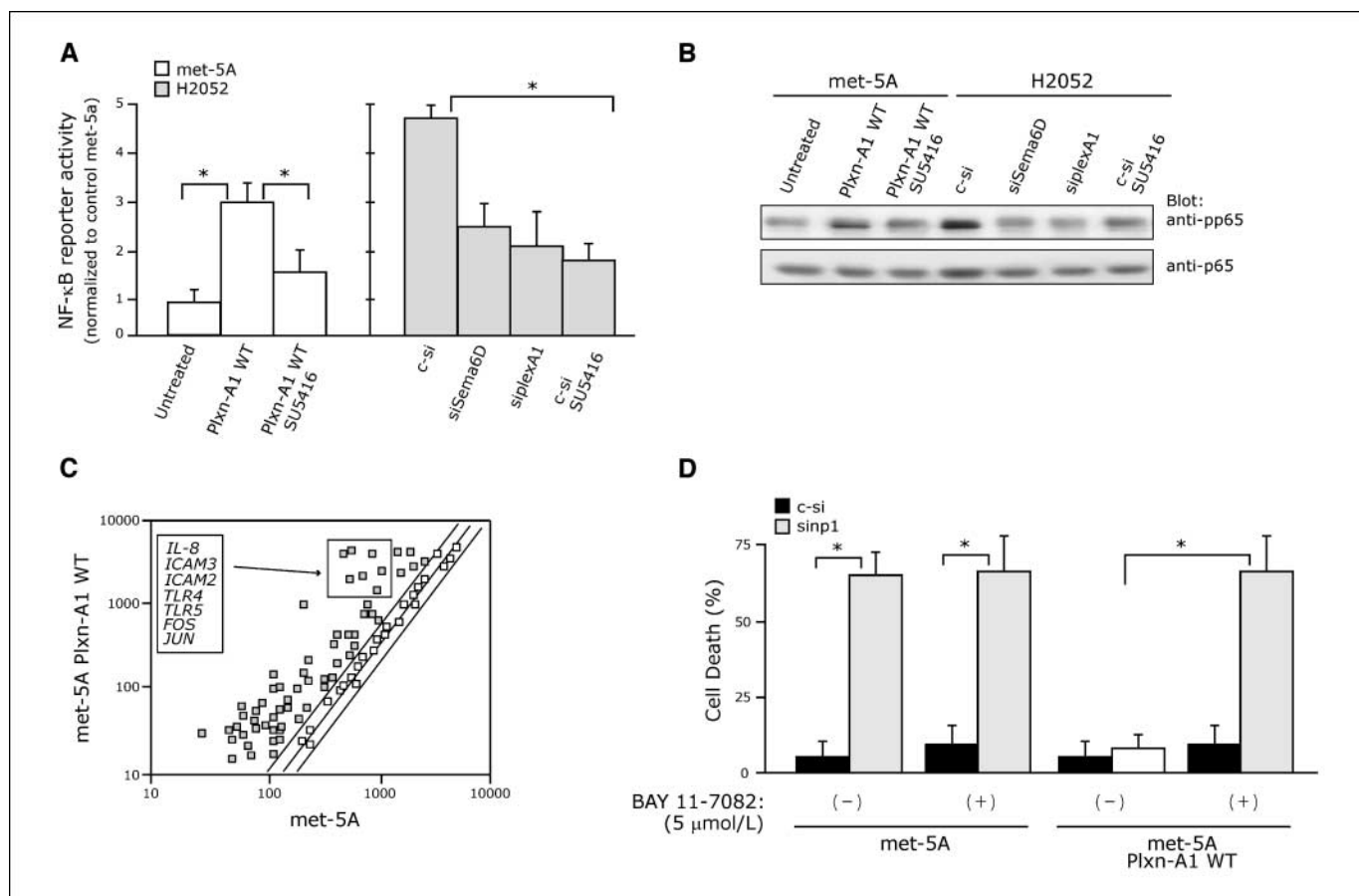
### Discussion

MPM poses considerable challenges for clinical oncologists because of its insidious nature and refractoriness to conventional multidisciplinary treatment regimens. Thus, research efforts are aimed at determining the mechanisms contributing to the pathogenesis of MPM.

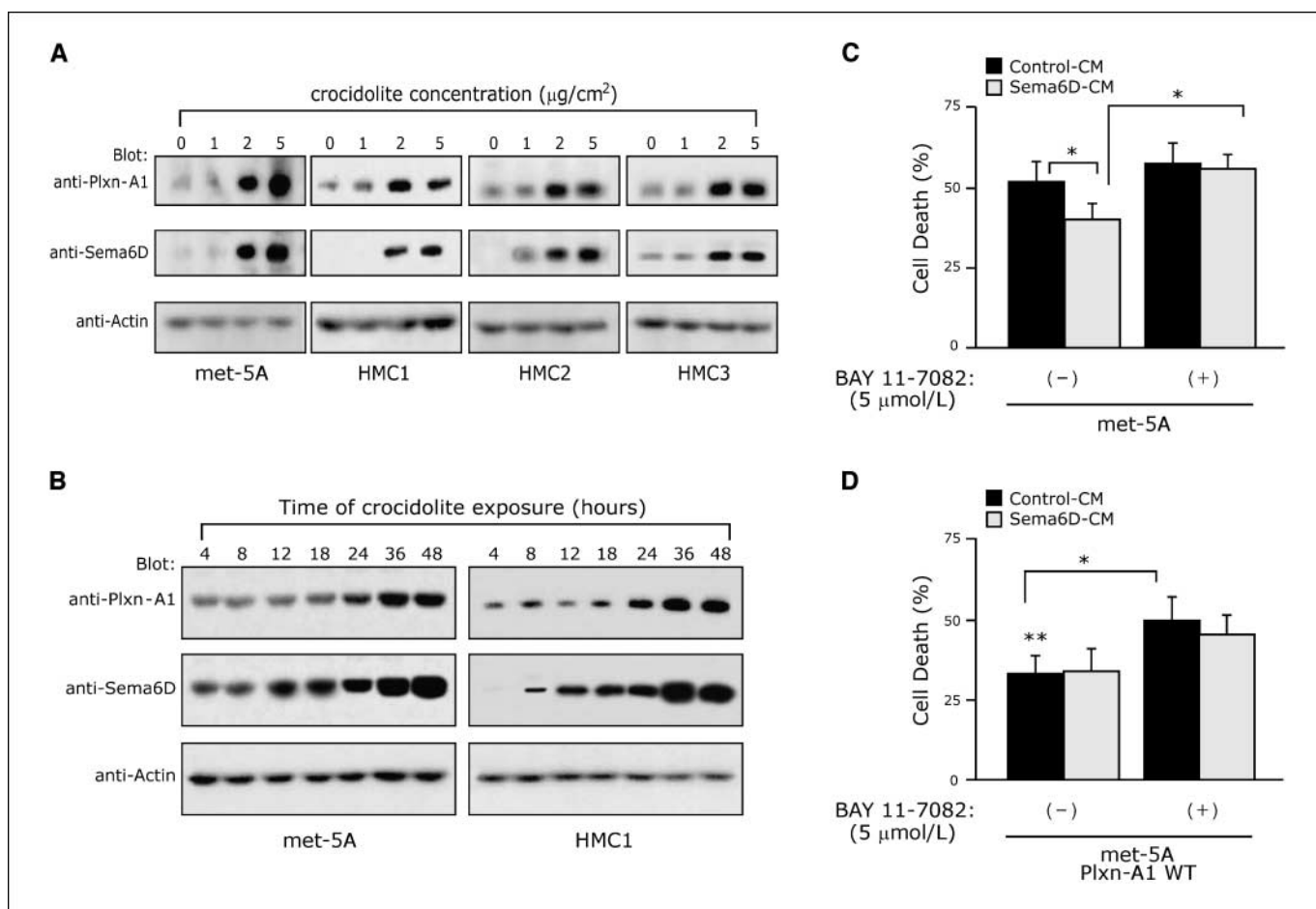
In the present study, we used MPM model to investigate whether anchorage-independent growth and survival of cancer cells, a key step toward malignant transformation and therapeutic resistance, depends on semaphorins and their receptors, the neuropilins and the plexins. These experiments were initiated because a growing body of evidence indicates that various semaphorins can either

promote or inhibit tumor progression through the promotion or inhibition of processes such as tumor angiogenesis, metastasis, and cell survival (14). For instance, several class 3 semaphorins, such as *Sema3B* and *Sema3F*, have been characterized as tumor suppressors. By contrast, other semaphorins, such as *Sema4D*, have been found to function as proangiogenic factors and to promote tumor progression. We found that malignant transformation is associated with a strong expression of *Plxn-A1* and *Sema6D* and that these molecules, in combination with NP-1, are necessary and sufficient to induce the survival of MPM cells. Our immunohistochemical analysis of human MPM specimens offers *in vivo* evidence that links *Plxn-A1* and *Sema6D* to MPM carcinogenesis.

To our knowledge, this is the first demonstration that *Sema6D* and *Plxn-A1* receptor, in addition to their role on cardiogenic precursors during cardiac development (15, 31), also exerts a protumorigenic activity in cancer cells. The exact mechanism of this activity still remains to be elucidated. It might simply depend on autocrine circuit where *Sema6D* is the ligand that binds *Plxn-A1* on cancer cells or it might reflect a dual signaling activity of *Sema6D* and *Plxn-A1* on cancer cells. Indeed, it was shown that *Sema6D* can function both as ligand and receptor for *Plxn-A1*. This phenomenon is known as “forward” and “reverse” signaling, respectively (15, 31). In our model, forward signaling through



**Figure 5.** *Plxn-A1* activates *NF-κB*, which accounts for increased cell survival. **A**, *NF-κB* reporter activity in *met-5A* and *met-5A* *Plxn-A1* WT cells as well as in H2052 cells transfected with the indicated siRNA and grown with or without SU5416 (5 μmol/L). **B**, immunoblotting for *NF-κB*, p65 subunit, and Ser<sup>536</sup>-phosphorylated p65 (*pp65*) in the indicated cell lysates. **C**, the expression of genes related to *NF-κB* signaling pathway was analyzed in *met-5A* cells and *met-5A* *Plxn-A1* WT cells by *NF-κB* signaling pathway microarray as described in Materials and Methods. Results are shown as a dot plot of individual genes, comparing the expression levels found in *met-5A* and *met-5A* *Plxn-A1* WT cells. *IL-8*, *ICAM-3*, *ICAM-2*, *TLR-4*, *TLR-5*, *FOS*, and *JUN* are genes in the panel significantly ( $P < 0.001$ ) regulated >2-fold. **D**, effect of *NF-κB* inhibition with BAY 11-7082 on cell viability of indicated cells transfected with control siRNA (*c-si*) or NP-1 siRNA (*siNP1*). \*,  $P < 0.05$ .



**Figure 6.** Asbestos induces Plxn-A1 and Sema6D proteins, which protect from asbestos-induced cytotoxicity. *A*, Western blot analysis of the indicated cells treated with different doses of crocidolite. Total protein extracts were analyzed for Plxn-A1 and Sema6D protein expression using anti-Plxn-A1 and anti-Sema6D antibodies. Anti-actin antibodies were used as control of protein loading. 0, untreated cells at time zero. *B*, time course analysis of the Plxn-A1 and Sema6D protein expression on indicated cells exposed to crocidolite fibers in a range of 4 to 48 h. *C* and *D*, Live/Dead Assay on met-5A cells (*C*) and infected met-5A Plxn-A1 WT cells (*D*) exposed to crocidolite fibers (3 µg/cm<sup>2</sup>) and grown with or without Sema6D-CM (dilution, 1:4) in the presence or absence of SU5416 (5 µmol/L) for 36 h. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ , significantly different compared with control met-5A reported in *C*.

Plxn-A1 is necessary for tumor cell survival. Whether Plxn-A1 mediates a reverse signaling by Sema6D or somehow involves other types of receptors still remains unexplored.

To determine the molecular pathway that mediates Plxn-A1 forward signaling, we showed that at least some of the Plxn-A1 signals ultimately promoted the NF- $\kappa$ B pathway and that this is achieved by inducing VEGF-R2 activation. We have shown a direct association between Plxn-A1 and VEGF-R2 in MPM cells. The binding of Plxn-A1 to VEGF-R2 and, in turn, VEGF-R2 phosphorylation are most likely affected by a CM enriched of soluble Sema6D (Sema6D-CM). Moreover, this Sema6D-CM synergistically enhanced VEGF-induced tyrosine phosphorylation of VEGF-R2 in a Plxn-A1-dependent manner (Fig. 4). These observations indicate that in MPM tumors, Plxn-A1 may function as a modifier of VEGF pathway(s) through the formation of Plxn-A1-VEGF-R2 complex. This is consistent with the report showing that Sema6D through Plxn-A1 activates VEGF-R2-mediated signal transduction and controls cardiac morphogenesis (15).

How the Sema6D/Plxn-A1/VEGF-R2 signaling can affect anchorage-independent growth and survival of tumor cells is presently unknown. Previous data have shown that VEGF-R2 can promote cell survival through the activation of NF- $\kappa$ B pathway (reviewed in

ref. 30). In addition, a growing body of evidence implicates NF- $\kappa$ B-dependent pathway in anchorage-independent growth and tumor progression (32). At present, there are no data that clearly link semaphorins to NF- $\kappa$ B pathway(s). We showed that NF- $\kappa$ B transcriptional activity is significantly induced by Plxn-A1 overexpression. Moreover, p65 phosphorylation was blocked by treatment with VEGF-R2 inhibitors as well as by Plxn-A1 or Sema6D siRNA (Fig. 5). This suggests a key role of NF- $\kappa$ B pathway(s) in Plxn-A1-mediated signaling. Analysis of the activation status of NF- $\kappa$ B showed that NF- $\kappa$ B was constitutively active in MPM cells even when the cells were serum starved and it was not stimulated any further by cell treatment with autocrine growth factors, such as VEGF, FGF2, and EGF. In contrast, in H2052 cells transfected with plxn-A1 siRNA, NF- $\kappa$ B activity was very low in serum-starved cells. This activity was then readily increased by stimulation with VEGF, EGF, and, to a lesser degree, FGF2 (data not shown). Hence, in plxn-A1-negative MPM cells, FGF and EGF are able to induce NF- $\kappa$ B activation, although they did not activate VEGF-R2. Nevertheless, even if FGF2 and EGF induce cell proliferation of plxn-A1-negative MPM cells, VEGF only replaced Sema6D or plxn-A1 in the induction of cell survival (Supplementary Fig. S3). Together, these data suggest that plxn-A1 initiates the assembly



of a signaling complex consisting of several transmembrane and intracellular proteins. Complex formation or clustering of several of these multiprotein complexes most likely induces VEGF-R2 to activate its various downstream signaling cascades. Among them, one is represented by NF- $\kappa$ B, but we cannot completely exclude the possibility that other downstream signaling cascades are involved in plexin-A1 pathway(s).

Tight control of VEGF-R2 signaling is crucial for MPM progression. We had previously shown that VEGF-R2 activation and subsequent MPM cell proliferation were mediated by an autocrine VEGF circuit (2). This, in turn, had causally been related to SV40 infection, which is associated to MPM (33). However, in normal mesothelial cells, VEGF induces expression of Sema3A that inhibits VEGF-mediated cell signaling (6). This suggests that a dysregulation of semaphorins and/or their receptors may be responsible for tumor-promoting effects of VEGF. We showed here that a long exposure of mesothelial cells to asbestos fibers leads to a progressive increase in Plxn-A1 and Sema6D (Fig. 6), linking the semaphorins pathway(s) to the early stages of asbestos-induced transformation of mesothelial cells. Moreover, the identified Sema6D/Plxn-A1 signaling represents an important survival pathway for human mesothelial cells that confers resistance to cell death after crocidolite exposure (Fig. 6). Therefore, our results might suggest that Sema6D antagonizes the inhibitory effects of Sema3A and then promote tumor cell survival. This point is currently under investigation.

In summary, we have provided evidence that a Plxn-A1-dependent pathway is critical in human MPM cells by regulating cell survival and anchorage-independent growth. This is achieved by controlling VEGF-R2-dependent NF- $\kappa$ B activity. Further studies will be necessary to elucidate the mechanism of tumor-promoting effect of Plxn-A1 in human tumors and to determine whether other members of plexin family can function as tumor suppressors or stimulators *in vivo*. Moreover, forward and reverse Plxn-A1 signaling must be considered. However, understanding the function of Plxn-A1 in the regulation of malignant transformation may help to unravel the molecular mechanisms involved in early MPM oncogenesis and may open novel therapeutic avenues to interfere with this process.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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