Semaphorin 3E Suppresses Tumor Cell Death Triggered by the Plexin D1 Dependence Receptor in Metastatic Breast Cancers

Jonathan Luchino, 1 Mélanie Hocine, 1 Marie-Claude Amoureux, 1 Benjamin Gibert, 2 Agnès Bernet, 2,3 Amélie Royet, 2,3 Isabelle Treilleux, 4 Patrick Lécine, 5,6 Jean-Paul Borg, 6 Patrick Mehnel, 2 Sophie Chauvet, 7 and Fanny Mann 1,*

1Aix-Marseille Université, CNRS, IBDM UMR 7288, 13288 Marseille, France
2Apoptosis, Cancer and Development Laboratory, Equipe labellisée “La Ligue”, LabEX DEVweCAN, Centre de Cancérologie de Lyon, INSERM U1052-CNRS UMR5286, Université de Lyon, Centre Léon Bérard, 69008 Lyon, France
3Netris Pharma, Centre Léon Bérard, 69008 Lyon, France
4Pathology Department, Centre Léon Bérard, 69008 Lyon, France
5CRCM, Equipe labellisée “La Ligue”, Inserm U1068-CNRS UMR7258, Aix-Marseille Université, Institut Paoli-Calmettes, 13009 Marseille, France
6CIRI, Inserm U1111-CNRS UMR5308, Université Lyon 1, ENS de Lyon, 69007 Lyon, France
*Correspondence: fanny.mann@univ-amu.fr
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SUMMARY

The semaphorin guidance molecules and their receptors, the plexins, are often inappropriately expressed in cancers. However, the signaling processes mediated by plexins in tumor cells are still poorly understood. Here, we demonstrate that the Semaphorin 3E (Sema3E) regulates tumor cell survival by suppressing an apoptotic pathway triggered by the Plexin D1 dependence receptor. In mouse models of breast cancer, a ligand trap that sequesters Sema3E inhibited tumor growth and reduced metastasis through a selective tumor cytocidal effect. We further showed that Plexin D1 triggers apoptosis via interaction with the orphan nuclear receptor NR4A1. These results define a critical role of Sema3E/Plexin D1 interaction in tumor resistance to apoptosis and suggest a therapeutic approach based on activation of a dependence receptor pathway.

INTRODUCTION

Anticancer therapies targeting signaling pathways that are disrupted or modulated and causally implicated in tumor progression may lead to enhanced, tumor-type-specific treatment for cancer patients. Specifically, dysregulation of genes initially identified as embryonic regulators of neuronal axon guidance has been observed in many forms of cancer. The increasing understanding of the role of neural guidance factors in the regulation of various phases of cancer development offers promise for the design of therapeutic strategies (Ballard and Hinck, 2012; Chen, 2012; Rehman and Tamagnone, 2013).

The semaphorins constitute one of the largest families of guidance molecules, which use plexin proteins as their main signal-transducing receptors. Abnormal expression levels of semaphorins and their receptors are commonly observed in cancers. For example, expression of the Plexin D1 receptor has been reported in tumor vasculature and in malignant cells in a wide range of human cancer types (Casazza et al., 2010; Roodink et al., 2005, 2009; Shalaby et al., 2012; Tseng et al., 2011). Expression levels of its Sema3E ligand appear to be positively correlated with increased metastatic disease in ovarian, melanoma, and colon cancers and with poor patient survival in colorectal and pancreatic cancers (Blainkin et al., 2012; Casazza et al., 2010; Tseng et al., 2011).

Most of our current knowledge of the function of Sema3E/ Plexin D1 signaling in cancer was obtained from experiments in which tumor cells were engineered to overexpress Sema3E...
and transplanted in animals. Two main, albeit antagonistic, effects have been reported. First, overexpression of Sema3E, which acts as a repulsive factor for Plexin-D1-expressing endothelial cells (Gu et al., 2005), leads to decreased neangiogenesis and reduced tumor growth (Casazza et al., 2010; Kigel et al., 2008; Sabag et al., 2012; Tseng et al., 2011). On the other hand, overexpression of a Sema3E fragment (p61-Sema3E), corresponding to the 61-kDa furin cleavage product of full-length Sema3E, contributes in an autocrine manner to cancer cell invasion and formation of distant metastases (Casazza et al., 2010, 2012; Christensen et al., 2005; Tseng et al., 2011). The molecular mechanisms by which Sema3E/Plexin D1 signaling influences metastatic spread are probably multiple and may depend on the type and location of the cancer. So far, two models have been proposed: ligand-induced activation of Plexin D1 on tumor cells facilitates cancer cell invasive migration through complex formation and transactivation of the epidermal growth factor receptor 2 (ErbB2) (Casazza et al., 2010) or through regulation of the subcellular location and activity of the Snail transcriptional repressor during epithelial-to-mesenchymal transition (EMT) (Tseng et al., 2011).

Breast cancer in females is the most frequently diagnosed cancer. Despite significant advances in treatment metastatic breast cancer remains essentially incurable, indicating a critical need for identifying the mechanisms and genes responsible for tumor progression. Sema3E was identified as a gene expressed in murine mammary adenocarcinoma cell lines capable of metastasizing to the lung and bones, but only rarely expressed in nonmetastatic cells (Christensen et al., 1998). Sema3E transcript was also detected in human breast cancers, yet it is unclear whether its expression correlates with metastatic disease (Christensen et al., 2005). In this study, we investigated the involvement and mechanism of action of Sema3E/Plexin D1 signaling in metastatic breast cancer, and then further applied this information to the preclinical development of molecularly targeted anticancer agents.

RESULTS

Increased Sema3E Level Correlates with Metastatic Progression of Human Breast Cancers

To begin, we measured PLXND1, SEMA3E, and SEMA4A mRNA abundance by quantitative real-time PCR in a panel of 68 primary breast tumor biopsies obtained from patients with tumors localized to the breast (N0, 15 patients), with only axillary node involvement (N+M0, 40 patients), or with distant metastases (M+, 13 patients). Plexin D1 was detected in all tumor samples and average levels were relatively constant among the different classes of tumors. In contrast, expression of Sema3E gradually increased with progression and was significantly higher in M+ patients compared to N0 patients, with 4/13 (30.8%) of M+ patients showing at least a 5-fold increase in Sema3E expression levels (Figures 1 A and 1B). Immunohistochemical analysis confirmed elevated Sema3E expression in invasive breast cancers, despite unchanged Plexin D1 expression levels (Figure 1C; Figures S1A and S1B available online). Finally, expression of another ligand of the Plexin D1 receptor, Sema4A, in the breast tumors was low and did not vary with metastatic progression (Figures S1C and S1D). These results suggest that Sema3E/Plexin D1 signaling in human breast cancers correlates with distant metastatic disease.
Plexin D1 Triggers Apoptotic Cell Death when Dissociated from Its Semaphorin Ligands
To investigate Plexin D1 function, we transfected HEK293T cells with plasmids encoding human or mouse Plexin D1 receptor. A significant increase in cell death was observed, as measured by a trypan blue exclusion assay (Figure 2A), anti-active caspase-3 immunofluorescence (Figures 2B and 2C), and MTT assay (data not shown). Inhibitors of caspase-3/caspase-7 and caspase-9, but not of caspase-8, abolished Plexin-D1-induced cell death (Figure 2D). Similar results were observed when using small interfering RNA (siRNA) sequences against each individual protein. Since cell death was unlikely to account for this rescue because Plexin D1 death activity of Plexin D1 was dependent on the presence of its ligands, we speculated that a soluble recombinant protein capable of triggering cell death when dissociated from its semaphorin ligands (hPlxD1-RA) lacking intrinsic GTPase-activating protein (GAP) activity, which fails to mediate Sema3E-induced repulsion (Goldschneider and Mehlen, 2010). To determine whether Plexin D1 is a dependence receptor, we first expressed in HEK293T cells a Plexin D1 mutant (hPlxD1-RA) lacking intrinsic GTPase-activating protein (GAP) activity, which fails to mediate Sema3E-induced repulsion (Casazza et al., 2010; Uesugi et al., 2009). hPlxD1-RA displayed similar cell death induction in HEK293T cells as the wild-type receptor (Figures 2A–2C), indicating that Plexin D1 triggers apoptosis independently of its classical signaling properties as a GAP. We next tested whether the death activity of Plexin D1 was dependent on the presence of its ligands. Cell death induced by wild-type and mutant receptors was inhibited by adding 100 ng/ml recombinant Sema3E to the culture medium (Figure 2F). Changes in Plexin D1 expression were unlikely to account for this rescue because Plexin D1 protein levels were unaffected by Sema3E treatment (Figure S2A). We also tested the effect of Sema4A, which has been reported to bind to Plexin D1 with an affinity 10-fold lower than that of Sema3E (Toyofuku et al., 2007). When used at the same concentration as Sema3E, a soluble form of Sema4A was ineffective to save Plexin D1 protein levels from death; however, it did so when used at a concentration 10 times higher (1,000 ng/ml) (Figure 2G). Finally, a lack of rescue was observed with semaphorins that do not bind to Plexin D1 (including Sema3A, Sema3C, and Sema3F), added at 100 ng/ml (Figure 2G) or at higher concentrations (data not shown). Thus, Plexin D1 is a member of the dependence receptor family capable of triggering cell death when dissociated from its semaphorin ligands.

Silencing Sema3E in Breast Cancer Cells Induces Apoptosis through the Dependence Receptor Plexin D1
To investigate whether Sema3E/Plexin D1 signaling regulates tumor cell viability, we used the mouse mammary 4T1 tumor cell line as a model of highly metastatic breast cancer cells (Aslakson and Miller, 1992). In vitro, 4T1 cells express Plexin D1 receptor and Sema3E but not Sema4A (Christensen et al., 1998) (Figure S2B). We found that transfecting 4T1 cells with a small hairpin RNA (shRNA) targeting Sema3E induced significant cell death, which was reversed by cotransfection of a Sema3E expression plasmid or by applying exogenous Sema3E protein (Figures 2H, 2I, and S2C). Similar results were obtained using a second shRNA construct (Figures S2C–S2E). One possible explanation for increased cell death is that Sema3E activates survival signaling mediated by Plexin D1. If that were indeed the case, removing Plexin D1 from tumor cells should result in a similar increase in cell death. However, treating 4T1 cells with siRNAs targeting Plexin D1 failed to affect cell viability (Figures 2J and S2F). Another explanation is that downregulation of Sema3E triggers cell death through unbound Plexin D1. Indeed, we observed that cell death induced by silencing of Sema3E expression was prevented in 4T1 cells that concomitantly received siRNAs against Plexin D1 (Figures 2K and S2G).

To examine whether the preceding results can be extended to other cancer cells, we knocked down Sema3E in MDA-MD-231 and MCF-7 cells, two human breast cancer cell lines that coexpress Sema3E and Plexin D1 (Kigel et al., 2008) (Figure S2H). Cell death induction was also observed in these conditions and was rescued by recombinant Sema3E (Figures 2L, 2M, and S2M). Finally, silencing Sema3E in MDA-MB-468 cells, which lack the death-triggering Plexin D1 receptor, did not promote cell death (Figures 2N and S2J).

Together, these observations argue that Sema3E production by cancer cells promotes their survival by inhibiting cell death mediated by the dependence receptor Plexin D1.

The SD1 Ligand Trap Blocks Sema3E/Plexin D1 Interaction and Induces Death of Breast Cancer Cells In Vitro
The results described above suggest that upregulation of Sema3E in metastatic breast cancers may protect tumor cells against the proapoptotic activity of Plexin D1, further favoring tumor development at primary and/or secondary sites. To provide support for this model, we attempted to develop a molecular compound that inhibits Plexin D1 interaction with its ligands. We reasoned that a soluble recombinant protein consisting of the Sema domain of human Plexin D1 (hereinafter called SD1) could act as a ligand-binding trap. The interaction between SD1 and Sema3E ligand was confirmed by in vitro pull-down assay (Figure 3A). A weak potential interaction between SD1 and Sema4A was not detected by coimmunoprecipitation (data not shown). SD1 was found to exert an inhibitory effect on Sema3E/Plexin D1 binding with a half-maximal inhibitory concentration (IC50) of 2.5 μg/ml (Figure 3B). Further validated that SD1 triggers death of 4T1 tumor cells in a dose-dependent manner, an effect that was reversed by adding excess of Sema3E (Figures 3C–3E). As expected, Sema4A was less effective than Sema3E in rescuing cells from death and required a concentration 10-fold higher than Sema3E (Figure 3F). These data demonstrate that a soluble compound that corresponds to the Sema domain of Plexin D1 acts as a ligand trap and triggers death of Plexin-D1-expressing cancer cells in vitro.

SD1 Inhibits Primary Tumor Growth and Metastasis
To determine whether the induction of cell death by SD1 interfered with tumor development and progression in vivo, we stably expressed in 4T1 cells a vector encoding SD1 (4T1-SD1) or a control vector (4T1-cont), together with a myristoylated Cherry (mCherry) encoding plasmid that allows transplanted cells to be visualized in vivo. Western blot analysis of cell culture
Figure 2. Unliganded Plexin D1 Receptor Induces Apoptosis in a Manner Analogous to Dependence Receptors

(A–C) HEK293T cells were transfected with an empty vector or vectors encoding human Plexin D1 (hPlxD1), mouse Plexin D1 (mPlxD1), or human Plexin D1 mutated in GAP activity domains (hPlxD1-RA). Cell death was measured by trypan blue exclusion assay (A) or by immunostaining of cleaved caspase-3 (B and C).

(D and E) HEK293T cells transfected with empty vector or hPlxD1-expressing vector were cultured in the presence of Z-DEVD-FMK (inhibitor of caspase-3/caspase-7), Z-LEHD-FMK (inhibitor of caspase-9), and Z-IETD-FMK (inhibitor of caspase-8) (D) or cotransfected with siRNAs targeting caspase-3, caspase-9, and caspase-8 (E). Cell death was measured by trypan blue exclusion.

(F and G) HEK293T cells were transfected with empty vector or vectors encoding hPlxD1 or hPlxD1-RA, and cultured in the presence of soluble Sema3E (F) or other semaphorin ligands, Sema3A, Sema3C, Sema3F, and Sema4A (G). Cell death was measured by trypan blue exclusion assay.

(H and I) 4T1 cells were transiently transfected with a control (nontargeting) shRNA or an shRNA specific to mouse Sema3E (shSema3E) and cell death was evaluated by trypan blue exclusion assay (H) or by active caspase-3 immunoreactivity (I). Knockdown effects were rescued by performing cotransfection with a plasmid encoding human Sema3E (hSema3E) or by addition of exogenous Sema3E ligand.

(J and K) 4T1 cells were transfected with control siRNA or siRNA targeted against mouse Plexin D1 (PlexD1 siRNA) alone (J) or together with an shRNA targeting Sema3E (K). Cell death was measured by trypan blue exclusion. The siRNA effect was rescued by reintroduction of siRNA-resistant hPlexD1 cDNA. (L–N) MDA-MB-231 (L), MCF-7 (M), and MDA-MB-468 (N) cells were transiently transfected with a control shRNA or an shRNA specific to human Sema3E (shSema3E), and cell death was evaluated by trypan blue exclusion. Cell death was rescued by cotransfection with a plasmid-encoding mouse Sema3E (mSema3E) or by adding soluble recombinant Sema3E protein.

Data are presented as mean ± SEM. *Significantly different with p < 0.05. **Significantly different with p < 0.01. ***Significantly different with p < 0.001. Scale bar is 200 μm. See also Figure S2.
supernatants confirmed SD1 secretion by 4T1-SD1 cells (Figure S3A). SD1 expression did not affect Plexin D1 or Sema3E protein levels in cells kept in vitro or transplanted to form solid tumors in mice (Figures S3B and S3C). We observed that, 72 hr after plating 4T1-SD1 cells, they began to die at a significantly higher rate than the 4T1-cont cells, presumably as a result of the accumulation of SD1 in the culture medium (Figure S3D). This effect was inhibited by siRNA-mediated knockdown of Plexin D1 expression (Figure S3E). Despite increased cell death, cell proliferation and migration of 4T1-SD1 cells was not different from that of control cells (Figures S3F–S3H). Similar results were obtained by analyzing different independent 4T1-cont (n = 2) or 4T1-SD1 (n = 2) clones (data not shown). Therefore, to produce a healthy 4T1-SD1 line destined for in vivo grafting procedures, the SD1-containing medium was replaced every other day by fresh culture growth medium.

In a first series of experiments, 4T1-SD1 and 4T1-cont cell lines were tested for their ability to form primary tumors following injection in syngeneic Balb/c mice, either subcutaneously or orthotopically in the mammary fat pad. Analysis of primary tumors collected 27 days after injection indicated that SD1 expression caused a significant reduction in tumor size in both subcutaneous (Figures 4A–4C) and orthotopic models (not shown). Thus, blocking ligand binding to Plexin D1 with SD1 inhibited tumor growth.

We next compared the metastatic potential of the two cell types. A decreased number of metastatic nodules at the surface of the lungs was found in animals that had received 4T1-SD1 cells compared to controls, both after subcutaneous (Figure 4D) and fat pad transplantation (data not shown). Reduced metastasis formation was confirmed after quantifying the total number of surface (Figure 4E) and internal (Figure 4F) nodules. This reduction may either reflect an antime-tastatic effect of SD1, or it may be secondary to limited primary tumor growth in the same animals.

To distinguish between these possibilities, a second set of mice was injected with 4T1-SD1 or 4T1-cont cells and sacrificed when primary tumors reached an average volume of 300 mm³. Because of their slow growth, 4T1-SD1-derived tumors were collected, in average, 14–19 days later than the control tumors (4T1-cont/subcutaneous: 46 ± 4 days postinjection [dpi]; 4T1-SD1/subcutaneous: 60 ± 3 dpi; 4T1-cont/fat pad: 31 ± 3 dpi; 4T1-SD1/fat pad: 50 ± 6 dpi). Comparison of lung metastasis in animals with primary tumors of similar size still revealed a reduced number of metastatic nodules in animals transplanted with 4T1-SD1 cells subcutaneously (Figures 4G–4K) and in fat pad (not shown). This result indicates that the Plexin D1 ligand trap SD1 exerts a direct inhibitory effect on metastasis formation, independent of primary tumor growth.
SD1 Inhibits Tumor Growth and Metastasis In Vivo by Inducing Tumor Cell Death

To assess the mechanism by which SD1 affects tumor development, we analyzed a set of parameters responsible for regulating cancer growth. We excluded that SD1 affected density and size of blood vessels and lymphatic vessels or proliferation of tumor cells (Figures S4 A–S4F). In contrast, we observed a 3-fold increase in tumor cell death in primary tumors formed by 4T1-SD1 cells compared to control tumors (Figures 5 A–5F). To further test whether SD1 stimulated a death response exclusively in SD1-expressing cells (cell autonomously), as opposed to exerting a paracrine effect, Balb/c mice were coinjected subcutaneously with mCherry-labeled 4T1-SD1 cells and enhanced green fluorescent protein (eGFP)-labeled 4T1-cont cells (1:1 ratio). Analysis of caspase activities in the developed tumors showed that 4T1-cont cells died as frequently as 4T1-SD1 cells, indicating a paracrine effect of the SD1 compound (Figures 5 G and 5H). Interestingly, we noted that, unlike tumor cells, stromal cells did not show increased death in primary tumors formed by 4T1-SD1 cells (Figures 5 E–5H). However, analysis of stromal vascular cells, which express Plexin D1, revealed a small, but significant, death response in the presence of SD1 (Figures S4 G and S4H). These observations indicate that, consistent with the in vitro findings, SD1 induces death of Plexin-D1-expressing cells in vivo, and this activity may explain the decrease observed in primary tumor growth.

To metastasize to the lung, 4T1 tumor cells must disseminate from the primary tumor, invade the stroma to reach blood or lymph vessels, and then extravasate and home to other tissues. Sema3E/Plexin D1 signaling has been reported to induce EMT and cell migration in a way that can contribute to the metastatic potential of a tumor (Casazza et al., 2010; Tseng et al., 2011). However, 4T1-SD1 cells did not differ from control cells in their expression of canonical EMT markers, cellular morphology, or migratory properties (Figure S4 I; see also Figures S3 G and S3H). Increased apoptosis, on the other hand, may explain the SD1 antimetastatic effect. One possibility is that SD1 triggers apoptosis of circulating tumor cells as they travel to distant sites as single cells or small clusters. The existence of an autocrine loop for tumor cell survival was demonstrated in low-density cultures (350 cells/cm²), where isolated 4T1 cells showed increased cell death after addition of recombinant SD1 to the culture medium (Figure S4 J). However, isolated 4T1-SD1 cells cultured at the same density did not show increased cell death as compared to 4T1-cont cells, although they did so in high-density cultures (50,000 cells/cm²; Figure S4 K). The most likely explanation is that, in low-density cultures, cell-produced SD1 was too dilute to exert its effect on responding cells. It is therefore unlikely that the in vivo...
effect on metastasis reflects apoptosis of circulating 4T1-SD1 cells. Alternatively, SD1 expression may induce tumor cell death during the growth of disseminated tumor cells into macroscopic tumor nodules. In support of this idea, increase in activated caspase-3 immunostaining was evident in the pulmonary metastases of animals injected with 4T1-SD1 cells compared to controls (Figure 5I). Consistent with increased apoptosis, the metastatic nodules that formed in the 4T1-SD1-administered groups were smaller in size than those of control animals (Figure 5J). Thus, the antimetastatic effect of SD1 in vivo may be mediated by induction of tumor cell apoptosis in metastatic colonies.
We next asked whether the in vivo anticancer and prodeath effects of SD1 involved Plexin D1 dependence receptor signaling. To this end, the two cell lines, 4T1-SD1 and 4T1-cont, were stably transfected with an shRNA plasmid targeting Plexin D1 or with a control nontarget shRNA plasmid (Figures S5 A–S5D). The obtained clones exhibited unchanged SD1 and Sema3E expression levels (Figures S5 B, S5C, and S5E).

In vitro, Plexin D1 knockdown did not affect the proliferation or migration of tumor cells (Figures S5F–S5H). However, it rescued by up to 70% the death of SD1-expressing 4T1 cells that otherwise occurred after 72 hr in culture (Figure S5I). In vivo, we investigated the growth and metastatic behavior of the four different cell lines (called 4T1-cont/shCont, 4T1-cont/shPlxD1, 4T1-SD1/shCont, and 4T1-SD1/shPlxD1) in syngeneic mice injected subcutaneously. Plexin-D1-deficient 4T1 cells (4T1-cont/shPlxD1) formed primary tumors that reached an average size comparable to that of tumors that developed from control cells (4T1-cont/shCont) and formed a similar number of pulmonary metastases (Figures 6 A–6D). This lack of effect of Plexin D1 knockdown was surprising, given the previously reported role of autocrine Sema3E signaling in inducing metastatic behavior in cancer cells through activation of a Plexin D1–ErbB2 receptor complex (Casazza et al., 2010). However, analysis of ErbB2 gene expression by RT-PCR failed to detect significant levels of ErbB2 mRNA in the parental or clonally derived 4T1 cell lines used in the present study (Figure S5J) (Kaur et al., 2012). Thus, in the present model, ligand-induced classical Plexin D1 signaling appears dispensable for tumor development and dissemination.

In contrast, in conditions where tumor growth and metastasis were inhibited by SD1, Plexin D1 knockdown restored the ability of 4T1-SD1 cells to form large primary tumors and to disseminate to the lungs (Figures 6A–6D). The rescue effect was accompanied by a significant decrease of cleaved caspase-3 and...
Plexin D1 Functions as Sema3E Dependence Receptor

Cancer Cell

Plexin D1 Triggers Apoptosis via an NR4A1-Dependent Mechanism

We next attempted to determine the molecular mechanism by which Plexin D1 induces apoptosis. First, we expressed a Plexin D1 receptor deleted of its cytoplasmic domain (hPlxD1-ICD) in HEK293T cells. This mutant receptor lacked cell-killing activity (Figure 7A). Conversely, the soluble cytoplasmic domain of Plexin D1 (hPlxD1-ICD) was sufficient to achieve functional death signaling (Figure 7A). To identify proapoptotic components that may interact with Plexin D1 intracellular domain, we screened a human embryonic brain cDNA library using hPlxD1-ICD as bait in a yeast two-hybrid assay. This screen identified the orphan nuclear receptor NR4A1, also known as Nur77, as a potential interactor of Plexin D1 (data not shown).

Expression of NR4A1 has been reported in multiple cancer cell lines and in patients with different types of tumor, including breast cancer patients (Alexopoulou et al., 2010). NR4A1 exerts a dualistic function in cancer, where it can promote tumor by favoring cell growth and survival but can also exert tumor suppression activity via a proapoptotic effect (Lee et al., 2011; Mohan et al., 2012; To et al., 2012). The interaction between NR4A1 and Plexin D1 was confirmed by pull-down assay in lysates of HEK293T cells coexpressing both proteins (Figure 7B). Interestingly, when cells were grown in the presence of Sema3E, the cytoplasmic distribution of NR4A1 both in 4T1 cells in vitro and in 4T1 experimental tumors (Figures 7D and 7E), as has been reported in human breast tumors (Alexopoulou et al., 2010). Consistent with a cytoplasmic role of NR4A1 in Plexin-D1-induced cell death, blockade of nuclear export abolished the cell death response induced by SD1 on 4T1 cells (Figure 7F). Moreover, the death effect induced by SD1 still occurred in the presence of transcription inhibitor, indicating that the death pathway does not require transcriptional activity (Figure 7F). In addition, in 4T1 cells transfected with NR4A1 siRNAs, expression of a nuclear-export-deficient NR4A1 mutant failed to rescue the pro-apoptotic effect of SD1 as the wild-type NR4A1 did (Figures 7C and S6). Thus, Plexin-D1-mediated cell death requires the extranuclear pathway of NR4A1 action. We therefore tested whether the mitochondrial pathway of apoptosis is activated in 4T1 cells treated with SD1. Disruption of mitochondrial membrane integrity was observed in the SD1-treated cells (Figures 7G and 7H). Moreover, we found that SD1 treatment triggered the release of cytochrome c to the cytoplasm, and its blockade with the BAX channel blocker IMAC2 suppressed the death effect of SD1 on 4T1 cells (Figures 7I and 7J). Together, these results support a model in which the unliganded Plexin D1 receptor interacts with cytoplasmic NR4A1, which acts on the mitochondria to induce cytochrome c release and subsequent activation of the caspase cascade.

Early Preclinical Evaluation of Plexin D1 Ligand Trap in Models of Breast Cancer

Because the SD1 trap appears to exert a tumor-growth- and metastasis-inhibiting effect, we developed a biologic interfering with Plexin D1/ligands interaction(s) that may be compatible with preclinical and clinical development. Similar to the Trap-VEGF currently in phase II clinical trials (Gaya and Tse, 2012), we generated various SD1 variants fused to human IgG1 Fc. These variants were then assessed in in vitro assays and through systemic delivery in mouse models. A variant named SD1-v2, which comprises in addition to the Sema domain the two adja-cent cysteine-rich Plexin, Semaphorin, Integrin (PSI) domains of Plexin D1, was analyzed further and successfully produced in a large-scale format. SD1-v2 showed the same death effect as SD1 on murine and human breast tumor cell lines (Figure S7A). In a first series of experiments, 4T1 cells were injected subcutaneously into nude mice, and the tumors were allowed to develop until they reached a volume of 100 mm³. At this time, mice received intraperitoneal injections of SD1-v2 or PBS, five times a week for 2 weeks. The SD1-v2 injections slowed the growth of primary tumors (Figure 8A). In addition, SD1-v2-treated animals had significantly fewer metastases in the lungs as compared to vehicle controls (Figure 8B). We conducted similar experiments with human MDA-MB-231 breast cancer cells, which, like 4T1 cells, formed smaller tumors when transduced to express SD1 stably (Figures S7B–S7M). SD1-v2 treatment of nude mice bearing orthotopically implanted human MDA-MB-231 cells significantly delayed primary tumor growth (Figure 8C). The effect of SD1-v2 on secondary tumors was not evaluated because of the very low incidence of lung macrometastases observed in mice bearing MDA-MB-231 primary tumors (Rose et al., 1994). Together, these data show that targeting endogenous Plexin D1 ligands can achieve inhibition of established primary tumors and metastases in preclinical models of breast cancer.

DISCUSSION

The Dependence Receptor Plexin D1 Activates the NR4A1-Mediated Mitochondrial Apoptotic Pathway

Among the nine vertebrate plexins, Plexin D1 is the most structurally divergent family member (Tamagnone et al., 1999) and so far the only one to be characterized as a dependence receptor. Examples of dependence receptors include other surface caspase-9 immunostaining in tumors of 4T1-SD1/shPlxD1 cells compared to 4T1-SD1/shCont tumors (Figures 6E–6H). To exclude the possibility of nonspecific effects, we confirmed these results using a second shRNA sequence (Figures S5K–S5M). Together, these data indicate that the inhibitory effect of SD1 on cancer progression involves engagement of prodeath signaling from the Plexin D1 dependence receptor expressed in tumor cells.
receptors initially described in the context of axon guidance (DCC, UNC5H, Neogenin, and EphA4), as well as receptors for trophic factors (RET, IR, IGF1r, MET, ALK, TrkA, and TrkC) and morphogens (Patched) (Goldschneider and Mehlen, 2010). The mechanisms by which dependence receptors exert their cell-killing effects are diverse and, in most cases, still incompletely understood. One of the best-studied cases of dependence receptor death signaling concerns Patched, which directly recruits an atypical caspase-activating complex, the “dependosome,” that includes the cytoplasmic adaptor protein DRAL, TUCAN, and caspase-9 (Fombonne et al., 2012; Mille et al., 2009). The present data show that Plexin D1 engages an alternative mechanism for activation of caspase-9 via the mitochondrial pathway. In the absence of ligand, Plexin D1 interacts with...
cytoplasmic NR4A1, which triggers mitochondrial apoptosis through release of cytochrome c, an activator of the caspase-9 apoptosome. In the presence of Sema3E ligand, Plexin D1–NR4A1 interaction is disrupted, possibly as a result of changes in receptor structure, oligomeric state, and/or concurrent binding of the Rho GTPase Rnd2 (Janssen et al., 2010; Siebold and Jones, 2013; Uesugi et al., 2009).

How NR4A1 induces the mitochondrial apoptosis pathway is incompletely defined. The best-defined mechanism involves translocation of NR4A1 to the outer mitochondrial membrane, where it binds to the apoptosis regulator B cell lymphoma 2 (Bcl-2) (Li et al., 2000). NR4A1 binding induces a conformational change in Bcl-2 that exposes its previously hidden BH3 domain, resulting in conversion of Bcl-2 from an antiapoptotic to a proapoptotic molecule (Lin et al., 2004). In this case, the initiation of apoptosis may require proteolytic processing of Plexin D1 intracellular domain, as shown for many other dependence receptors (Goldschneider and Mehlen, 2010), to allow translocation of an active Plexin D1–NR4A1 complex to the mitochondria. However, direct mitochondrial targeting of cytoplasmic NR4A1 is not always required for cell death because it has been suggested that NR4A1 can stimulate other cytosolic proapoptotic molecules, such as BAX, to associate with the mitochondria (Wilson et al., 2003). Future studies will continue to define the mechanism of action of cytoplasmic NR4A1 and how its proapoptotic function is regulated by Plexin D1.

Disruption of Semaphorin/Plexin D1 Signaling Prevents Breast Cancer Growth and Metastasis

The dependence receptor model predicts that such receptors act as conditional tumor suppressors by triggering apoptosis of cancer cells in settings of ligand limitation. Aggressive/metastatic cancers may have evolved several strategies to escape dependence-receptor-mediated cell death by downregulation, or mutation, of the receptor and/or downregulation of its downstream effector molecules (Castets et al., 2012; Coissieux et al., 2011; Genevois et al., 2013). Interestingly, recent analyses revealed a negative association between NR4A1 expression levels in breast cancer and histologic grade, with reduced expression of NR4A1 in higher grade and metastatic tumors (Alexopoulou et al., 2010; Muscat et al., 2013). This reduction may potentially contribute to the survival of aggressive tumors. Alternatively, cancer cells may inhibit the dependence-receptor-mediated cell death pathway by autocrine production of the trophic ligand (Bouzas-Rodriguez et al., 2010; Fitamant et al., 2008). Consistent with this second model, we showed here that expression of the high-affinity ligand for Plexin D1, Sema3E, increases with tumor progression in human breast cancers. Thus, production of the antiapoptotic ligand Sema3E may represent a cell death evasion mechanism that has implications in tumor growth and progression.

The results of the present study differ from those of previous research using gene knockdown of Sema3E, which failed to identify a role of this semaphorin in cancer cell survival and tumor growth (Casazza et al., 2010). We have repeated these experiments using 4T1 clones stably expressing Sema3E shRNAs and confirmed that the tumors grew normally (data not shown). Two different, but not exclusive, hypotheses may be advanced to reconcile the apparent inconsistency between results based on Sema3E knockdown and Plexin D1 ligand sequestration by SD1. First, knocking down Sema3E ligand expression in 4T1 cells had a deleterious effect on cell survival, thus severely reducing the colony number during the process of stable clone selection. Moreover, the few recovered clones did not manifest increased rate of cell death when expanded in vitro (unlike transiently transfected 4T1 cells), indicating that Sema3E-negative clones that have survived the selection process were resistant...
to apoptosis induced by unliganded Plexin D1 (data not shown). This selection bias was circumvented during the generation of stable clones expressing the SD1 ligand trap by selecting and expanding the cell lines under nonapoptotic conditions (i.e., by washing out the SD1-containing medium regularly). A second possibility is that in vivo Sema3E–deficient 4T1 cells were saved from Plexin-D1-induced cell death by another ligand expressed in the tumors. Interestingly, we observed that Sema4A, which is not expressed by 4T1 cells in vitro, is present in 4T1-derived tumors. Taken together, these results raise the possibility that autocrine Sema3E ligand might cooperate with Sema4A expressed in growing tumors to protect cancer cells against the proapoptotic activity of Plexin D1.

Targeting the Plexin D1 Dependence Receptor as a Potential Therapeutic Strategy for Breast Cancer

Although Sema3E is a potent inhibitor of tumor neoangiogenesis, the use of recombinant Sema3E for cancer therapy is compromised by its adverse prometastatic effect (Casazza et al., 2010). Interestingly, it has been reported that a furin-resistant form of Sema3E can inhibit metastasis while retaining its antiangiogenic and antitumor activity (Casazza et al., 2012). This anti-metastatic property can be explained by failure of the mutated ligand to enhance ErbB2 recruitment to Plexin D1 and its subsequent transactivation (Casazza et al., 2012). The present findings open additional possibilities for the rational design of selective antimetastatic drugs that induce tumor cell death both in primary and secondary tumors by targeting the Sema3E/Plexin D1 pathway. This approach may be valuable for the treatment of metastatic breast cancers, including ErbB2-negative cancers.

Another antiapoptotic ligand that inhibits dependence receptor function in breast tumors is Netrin-1 (Fitamant et al., 2008). Although both Netrin-1 and Sema3E are upregulated in human breast cancers with metastatic propensity, they are often found overexpressed in distinct sets of tumors (data not shown). The mechanisms leading to aberrant expression of Sema3E or Netrin-1 in cancer are presently unclear. This understanding might be important in the future to link patients' tumor types to the effectiveness of treatments targeting distinct dependence receptor pathways and to the development of personalized anti-cancer medicine.

EXPERIMENTAL PROCEDURES

A more detailed description of the experimental procedures and reagents used in this study can be found in the Supplemental Experimental Procedures.

Quantitative RT-PCR and Immunohistochemistry on Human Breast Tumor Samples

Human tissue samples were obtained from the Biological Resource Center (Centre Léon Bérard, Agreement number: DC-2008-99), after approval by the institutional review board and ethics committee of Centre Léon Bérard, with fully informed patient consent. To assay PLXND1, SEMA4E, and SEMA4A expression in breast tumors, total RNA was isolated and reverse-transcribed using the iScript cDNA Synthesis kit (BioRad). The hydroxymethylbilane synthase (HMBS) housekeeping gene was used as internal control. Real-time quantitative RT-PCR was performed on a LightCycler 480 apparatus (Roche) using the LightCycler TaqMan Master kit (Roche) and probes 42, 25, 5, and 26, respectively (Universal ProbeLibrary; Roche). Average gene expression is given as the ratio between expression in each sample and the average of expression in the N0 samples. Immunohistochemical analysis was performed as described (Bernet et al., 2007). The pathologist selected representative areas from breast carcinomas.

Animal Models and In Vivo Procedures

All animal procedures were carried out in accordance with accepted standards of animal care, under the agreement number E-10-055-21 for animal experimentation delivered by the French Ministry of Agriculture. In vivo studies were conducted in 6- to 8-week-old female BALB/c ByJ mice or CD-1 nude (nu/nu) athymic mice (Charles River Laboratories). Mice were injected with 4.5 x 10^5 4T1 or 2 x 10^6 MDA-MB-231 cells subcutaneously into the right posterior flank or into the fat pad of the mammary gland. When tumors reached a volume of 100 mm^3, PBS or 20 mg/kg of body weight SD1-v2 were administered intraperitoneally five times per week for 2 weeks. Tumor size was measured externally using a caliper, and the width (A) and length (B) of the developing tumor was converted to volume using the equation V = 0.52 x A^2 x B. The primary tumors and the lungs of the animals were dissected and processed as described (Casazza et al., 2010).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.ccr.2013.09.010.

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