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Review

Emerging Role of Semaphorins as Major Regulatory Signals and Potential Therapeutic Targets in Cancer

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Semaphorins are mainly known as guidance signals in development, acting through receptors called Plexins. However, their role in cancer is rapidly emerging in the regulation of tumor angiogenesis, tumor growth, cancer cell invasiveness, and metastatic spreading. Intriguingly, activated plexins can transactivate receptor tyrosine kinases, such as MET, VEGFR2, FGFR2, and ERBB2, and lead to distinctive effects in a cell-context-dependent manner. Moreover, certain semaphorins concomitantly target endothelial and cancer cells, and can achieve remarkable inhibition of angiogenesis and tumor growth, associated with anti-metastatic activity. Altogether, these data validate the identification of semaphorin signals as promising therapeutic targets in cancer.

Introduction

Semaphorins

There are around 20 semaphorin genes in vertebrates that can be subdivided into multiple subclasses based on common structural features (Kolodkin et al., 1993; Semaphorin Nomenclature Committee, 1999). Members of subclass 3 are secreted, while the others are membrane-bound and, under certain circumstances, can be shed upon cleavage. All semaphorins have in common a *sema* domain, characterized by a seven-blade beta propeller structure, which contains sites for semaphorin dimerization and receptor binding (Liu et al., 2010; Nogi et al., 2010; Janssen et al., 2010).

Semaphorin Receptors

Main receptors for semaphorins are the plexins, a family comprising nine members in vertebrates (reviewed by Tamagnone and Comoglio, 2000). The extracellular domain of plexins also includes a *sema* domain that is putatively involved in ligand binding. A subset of the secreted semaphorins cannot interact with plexins alone, but require the presence of obligate coreceptor molecules called neuropilins (Nrp1/Nrp2) (Tamagnone et al., 1999; Takahashi et al., 1999), providing additional binding sites for ligands (see Figure 1) (for general reviews, see Kruger et al., 2005; Zhou et al., 2008; Neufeld and Kessler, 2008). Notably, neuropilins are also well-known coreceptors for vascular endothelial growth factors (VEGFs).

Semaphorin Signaling

Plexins are responsible for most known intracellular pathways triggered by semaphorins, while the short cytoplasmic tail of neuropilins is apparently devoid of signaling functions. The intracellular domain of plexins is largely conserved in the family and carries an unconventional split domain with GTPase-activating protein (GAP) activity for R-Ras, M-Ras, and Rap (Oinuma et al., 2004; Wang et al., 2012). Moreover, the cytoplasmic domain of different plexins was found to associate with other putative effector proteins, such as p190RhoGAP, PDZ-RhoGEFs, MICALs, FARP2 (reviewed by Zhou et al., 2008), and 14.3.3 proteins (Yang and Terman, 2012). Typical outcomes of plexin activation are inhibition of integrin-mediated cell-substrate adhesion and cytoskeletal remodeling. In experimental models, this leads to retraction of pseudopodia, and eventually to cell rounding, or to the typical "collapse" of axonal growth cone processes. In physiology, these mechanisms are thought to mediate semaphorin-dependent guidance of axonal extension and directional cell migration (Tran et al., 2007). Notably, semaphorin and plexin mouse knock-out models display defects in axon guidance, cardiovascular development (sometimes lethal), bone homeostasis, and immune response.

Additional Interactors and Signaling Pathways

Certain semaphorins were found to interact with additional molecules on the cell surface beyond their plexin receptors (see Figure 1), thereby mediating alternative signaling pathways in specific cellular settings. For instance, in lymphocytes, Semaphorin4D (Sema4D) can bind CD72 (Kumanogoh et al., 2000), and Sema4A the membrane receptor Tim-2 (Kumanogoh et al., 2002). Sema7A can engage integrin-beta1 through an RGD motif included in its amino acid sequence and trigger Focal Adhesion Kinase-dependent pathway (Pasterkamp et al., 2003). Moreover, other signaling receptors have been found in association with plexins and neuropilins on the cell surface; these include the adhesion molecule L1-CAM (Castellani et al., 2002) and the receptor tyrosine kinases (RTKs) OTK/PTK7, MET, RON, VEGFR2, and ERBB2 (reviewed by Franco and Tamagnone, 2008). In particular, it was shown that semaphorins can transactivate plexin-associated RTKs and promote cell growth and invasion, as opposed to the usual pathways leading to inhibition of migration. Thus, semaphorins can trigger multiple signaling cascades and often distinctive functions in a cell-context-dependent manner.

Semaphorins Are Versatile and Multifaceted Regulatory Signals in the Tumor Context

Accumulating evidence indicates that semaphorin signals can play a major role in the tumor context, beyond their established role in development (reviewed by Capparuccia and Tamagnone,

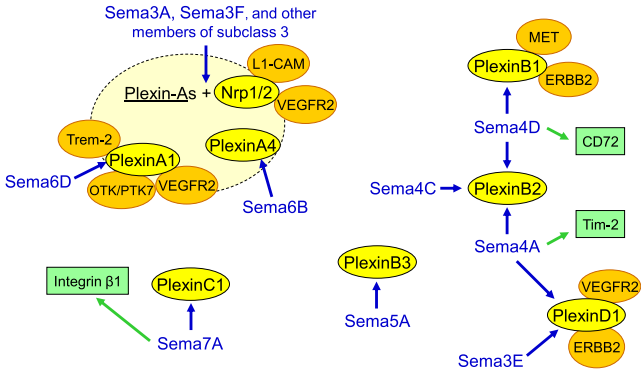

Figure 1. Schematic Representation of Main Semaphorin-Plexin Interactions

Plexins and plexin-associated molecules are indicated in ovals, while other putative semaphorin receptors are shown in green boxes.

2009). Cancer cells typically express both semaphorins and their receptors, and experimental data show that these signals can either promote, or inhibit, tumor cell migration, growth, and survival. Thereby, semaphorin functions in the tumor context may be partly due to a direct ("autocrine" or paracrine) regulation of cancer cells; a few specific examples will be discussed in this review. Notably, the expression of semaphorins and their receptors in tumors has been found up- or downregulated compared to normal tissues. The molecular mechanisms responsible for this regulation are largely unknown, but certain transcription factors and miRNAs have been implicated (e.g., Clarhaut et al., 2009; Coma et al., 2011; Urbich et al., 2012). On the other hand, mutations or other alterations of semaphorin or semaphorin receptor genes are rarely seen in human tumors (Balakrishnan et al., 2009), with the exception of PlexinB1 mutations reported in prostate cancer (Wong et al., 2007).

In addition to cancer cells, semaphorins and their receptors are expressed in normal cells of the tumor microenvironment. In particular, several semaphorins have been found to regulate endothelial cell function, either as inhibitors or promoters of angiogenesis (reviewed by Neufeld et al., 2012), and a number of them were recently shown to control tumor vasculature (diffusely discussed below). Certain semaphorins are furthermore importantly involved in the regulation of immune response (for a review see Takamatsu and Kumanogoh, 2012). For instance, Sema4D signaling was found to promote B lymphocyte activation, while Sema4A and Sema6D were implicated in T lymphocytes function. In one study, Sema3A promoted the cytokine storm induced by inflammatory signals (Wen et al., 2010), whereas others reported that Sema3A signaling inhibits T-cell function (Catalano et al., 2006; Lepelletier et al., 2006; Yamamoto et al., 2008). A recent report indicated that Sema4A is a chemoattractant for monocyte-macrophages (Meda et al., 2012); in addition, both Sema4A and Sema7A were found to induce monocytes to release pro-inflammatory and pro-angiogenic molecules (Meda et al., 2012; Holmes et al., 2002; Suzuki et al., 2007). Thus, specific semaphorins could have a role in controlling the recruitment of leucocytes into the tumor microenvironment, and their immuno-modulatory activity could have an impact on tumor progression; however, these potential functions have not been carefully investigated as yet.

A further semaphorin function potentially relevant to cancer is linked to their emerging role in bone homeostasis. In fact, signaling cascades triggered by Sema6D/PlexinA1, Sema4D/PlexinB1, and Sema3A/Nrp1 have been shown to play a crucial role in the regulation of osteoclast and osteoblast differentiation and the balance between osteolysis and osteodeposition (Takegahara et al., 2006; Negishi-Koga et al., 2011; Hayashi et al., 2012). As tumor cells can release all of the above signals within the tumor microenvironment, this could be relevant with respect to the development of bone metastatic sites.

Due to their versatile activity and wide receptor distribution in different cell types, semaphorin signals could mediate an intense cross-talk between cancer cells and the microenvironment, and play multiple concomitant functions potentially relevant to tumor progression. This complex scenario has been investigated for few family members by experiments in vivo, in mouse models. This review will mainly focus on recent studies that highlighted certain semaphorin signaling pathways as major modifiers of cancer progression and potential therapeutic targets relevant in translational perspective.

Semaphorins Are Potent Regulators of Tumor Angiogenesis In Vivo

Growing tumors can release strong and diverse pro-angiogenic stimuli and usually they become dependent on angiogenesis for growth and survival; this explains why anti-VEGF drugs, for example, can dramatically induce tumor shrinkage. However, refractoriness to drugs blocking individual pro-angiogenic factors may eventually arise due to the increased production of alternative signals sustaining tumor vessel development (Carmeliet and Jain, 2011). A further worrying aspect of VEGF-targeted drugs is the reported risk of inducing tumor invasion and metastatic spreading. Although the mechanisms underlying this process are still controversial, this seems to involve hypoxia-driven induction of tumor invasiveness (Semenza, 2012), and it is presently addressed by combined treatment with drugs directly targeting cancer cells.

Multiple semaphorins have been associated with the regulation of tumor vasculature, including Sema3A, Sema3E, Sema3F, and Sema3G (as inhibitory signals); Sema4D (as pro-angiogenic factor); and others (reviewed by Neufeld et al., 2012). These semaphorins may be released by cancer cells, as well as by stromal cells in the tumor microenvironment. Furthermore, endothelial cells themselves produce semaphorins controlling angiogenesis in autocrine manner. Importantly, the expression of certain semaphorins (and receptors) seems to be regulated in response to tissue hypoxia, including upon the "angiogenic switch" of growing tumors, as part of a balanced set of antagonistic signals controlling the development of new vessels (Coma et al., 2011; Moriya et al., 2010; Sun et al., 2009; Maione et al., 2009). However, this physiologic function of semaphorins seems to be distorted in cancer progression. For instance, the levels of anti-angiogenic factors Sema3A and Sema3F are often downregulated in advanced tumors (Maione et al., 2009; Bielenberg et al., 2004), while the pro-angiogenic Sema4D is typically increased (Basile et al., 2006). It was furthermore reported that Sema4D levels are induced by VEGF and inflammatory cytokines (Smith et al., 2011). Notably, in addition to being produced by cancer cells, Sema4D may be released into the tumor

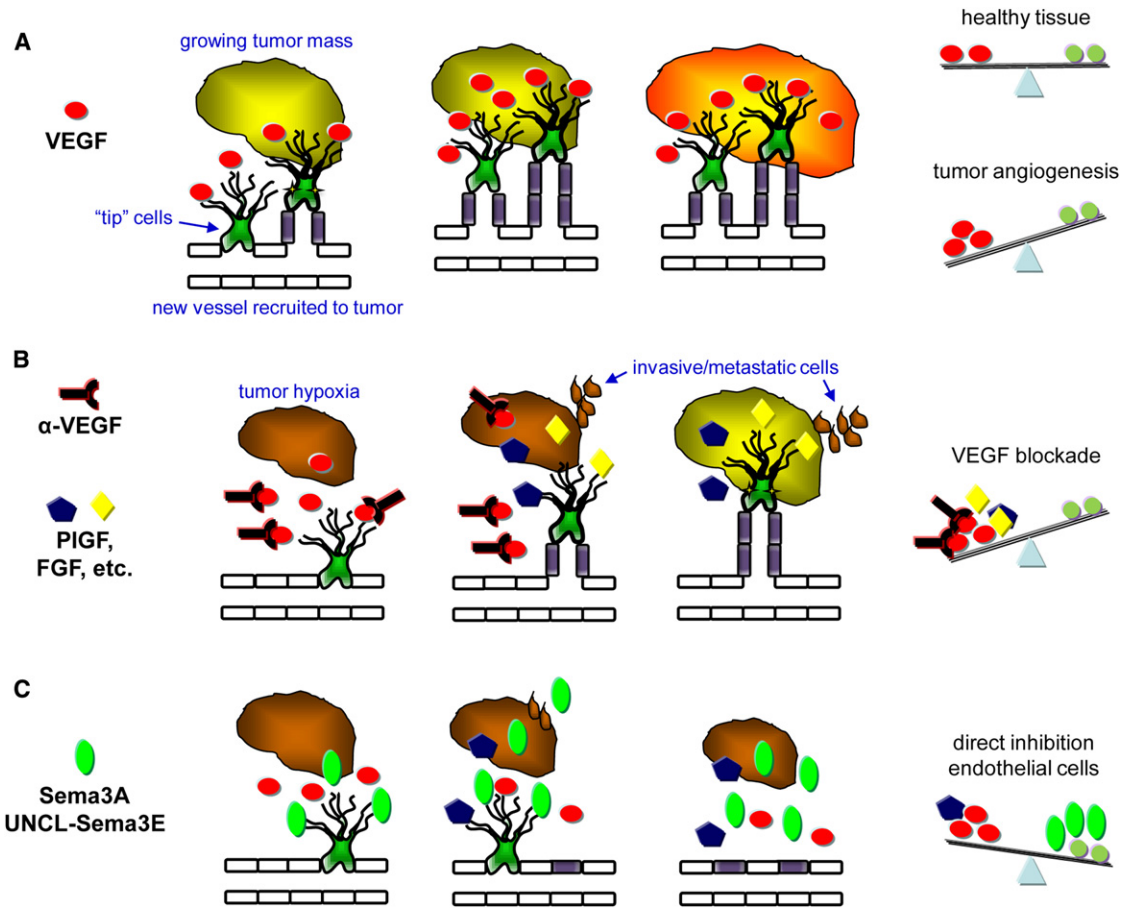

Figure 2. Semaphorins: A Novel Type of Anti-Angiogenic Factors Inhibiting Cancer Progression
(A) Unlike in normal tissues, the balanced activity of antagonistic signals controlling new vessel development is often distorted in tumors, which are characterized by predominance of pro-angiogenic signals (including VEGF, Sema4D, and others).
(B) Upon treatment with drugs blocking pro-angiogenic factors, tumors undergo hypoxia and shrinkage; but eventually they may become resistant by exploiting alternative pro-angiogenic signals. Moreover, tumor hypoxia may induce cancer cell invasiveness and metastatic spreading.
(C) Anti-angiogenic molecules directly targeting endothelial cells (such as Sema3A or Uncl-Sema3E) may be able to overcome these problems. Moreover, by directly inhibiting tumor cell invasiveness (with Sema3A, Uncl-Sema3E, but also anti-PlexinB1 antibodies), the metastatic spreading associated with tumor hypoxia could be prevented.

microenvironment by inflammatory cells, tumor-associated macrophages (Sierra et al., 2008) and, potentially, platelets (Zhu et al., 2007). Although genetic deletion in mice demonstrated that Sema4D is not required for developmental angiogenesis, experiments in vivo indicated that this semaphorin has a strong pro-angiogenic activity in tumor models (Sierra et al., 2008). While Sema4D loss or functional blockade in the microenviroment inhibited tumor growth and progression, this effect does not seem to rely exclusively on the main Sema4D-receptor PlexinB1, potentially implicating the alternative homolog PlexinB2 (Fazzari et al., 2007). Notably, Sema4D can control cancer cells in addition to tumor vasculature (discussed below), sustaining invasion and metastasis.

As an alternative to blocking the activity of pro-angiogenic factors, the direct targeting of endothelial cells by treatment with inhibitory semaphorins was assayed in preclinical tumor models in mice (see Figure 2). Sema3F was in fact the first semaphorin shown to have an anti-angiogenic activity in tumor models in mice (Bielenberg et al., 2004; Kessler et al., 2004). Sema3F binds to the coreceptor molecule Neuropilin-2 (Nrp2), which—in addition to interacting with plexins—is typically found in endothelial and lympho-endothelial cells in association with VEGFR2 and VEGFR3 (Kärpänen et al., 2006; Caunt et al., 2008). Intriguingly, derivatives of neural crest cells, including melanoma, also express Nrp2; and the forced overexpression of Sema3F in melanoma cells transplanted in mice blocked tumor angiogenesis, tumor growth, and metastatic spreading (Bielenberg et al., 2004). These data demonstrated that Sema3F is capable of inhibiting tumor angiogenesis and lympho-angiogenesis in vivo, and suggested that it may regulate a subset of cancer cells via Nrp2. Later studies have reported Nrp2 overexpression in human tumors (e.g., Dallas et al., 2008) and linked it with cancer progression; however, the implicated signaling pathways have not been clearly elucidated and they might reflect semaphorin-independent functions.

Recent reports highlighted other semaphorin molecules that potently inhibit tumor angiogenesis in vivo, independent of VEGF blockade, and concomitantly control invasive/metastatic

progression. Sema3A was actually the first semaphorin found to inhibit endothelial cells (Miao et al., 1999). This was initially associated with the fact that Sema3A coreceptor Neuropilin-1 (Nrp1) also binds VEGF and mediates its pro-angiogenic activity. It was later shown that Sema3A- and VEGF-binding sites on Nrp1 are largely independent (Appleton et al., 2007); moreover, the antagonistic activity of these signals in endothelial cells seems to implicate distinct signaling pathways, rather than binding competition to Nrp1. Sema3A-receptor complexes are thought to include plexins of A-subfamily, beyond Nrp1, especially PlexinA4 and/or PlexinA1. Notably, a recent report (discussed below) suggests that these plexins may play a role in the regulation of both endothelial and cancer cells (Kigel et al., 2011). Sema3A was found to inhibit integrin-mediated adhesion to the extracellular matrix, which is crucially required for endothelial cell migration and survival (Serini et al., 2003; Casazza et al., 2011). Upon delivery in different preclinical tumor models in mice, Sema3A displayed a strong anti-angiogenic effect associated with inhibition of cancer cell motility and metastatic spreading in different tumor models (Maione et al., 2009, 2012; Casazza et al., 2011; Chakraborty et al., 2012). According to one study, the treatment with Sema3A can achieve "normalization" of the tumor vasculature in spontaneous tumor models in mice, possibly by pruning aberrant tumor vessels (Maione et al., 2009). In another study, the sustained treatment with Sema3A resulted in extensive vessel disruption and hypoxia in transplanted tumors (Casazza et al., 2011), suggesting that vessel normalization may be obtained in a specific therapeutic window. Moreover, Sema3A directly inhibited cancer cell invasiveness dependent on Nrp1 expression (Casazza et al., 2011). Importantly, Nrp1 levels are typically higher in tumors compared to respective normal tissues, and Nrp1 expression seems to confer a selective advantage in tumor progression (reviewed by (Rizzolio and Tamagnone, 2011), which may implicate fewer risks of developing resistance to Sema3A-mediated inhibition. Of note, combined treatment with Sema3A could prevent the metastatic spreading induced by VEGF-blocking drugs in a spontaneous tumor model in mice (Maione et al., 2012).

Another secreted semaphorin found to inhibit tumor angiogenesis is Sema3E. The role of Sema3E in developmental angiogenesis is well established, even if multiple signaling cascades have been implicated (reviewed by Tamagnone and Mazzone, 2011). Importantly, Sema3E signaling in endothelial cells does not require neuropilins, but rather depends on the specific receptor PlexinD1 (Gu et al., 2005). PlexinD1 is expressed at high levels in developing vessels, especially in the endothelial "tip" cells that lead vascular sprouts. In the adult, PlexinD1 expression is mainly found in sites of neo-angiogenesis, including tissues recovering from ischemic insults, and growing tumors (Roodink et al., 2005; Moriya et al., 2010; Fukushima et al., 2011). Intriguingly, Sema3E-PlexinD1 signaling was found to trigger VEGFR2 activation in certain neurons (Bellon et al., 2010). Instead, PlexinD1 activation in endothelial cells inhibits their migration and restricts sprouting angiogenesis, antagonizing pro-angiogenic signals; in fact, during development, Sema3E is strictly localized in order to define permissive and non-permissive areas for vascularization (Gu et al., 2005). Recent studies implicated ARF6 activation by PlexinD1 in the negative regulation of integrin function mediated by Sema3E in endothelial cells (Sakurai et al., 2010, 2011). Notably, forced Sema3E overexpression in experimental tumor models resulted in a dramatic anti-angiogenic effect, associated with strong suppression of tumor growth (Casazza et al., 2010).

Sema3E Is a Dual-Activity Factor Independently Controlling Endothelial and Cancer Cells

Importantly, in addition to inhibiting endothelial cells, Sema3E was found to control the behavior of cancer cells through an independent signaling cascade. In fact, this secreted semaphorin is proteolytically converted into a fragment, known as p61-Sema3E, which plays a distinctive autocrine function in cancer cells by driving invasion and metastasis without significantly impinging tumor growth (Casazza et al., 2010). This activity is strictly dependent on the transactivation of the oncogenic tyrosine kinase ERBB2, associated with PlexinD1, in response to p61-Sema3E stimulation. By expression profiling analysis, it was found that higher Sema3E expression in primary tumors (colorectal and melanoma) correlates with increased metastatic progression (Casazza et al., 2010). Notably, another group reported that Sema3E-PlexinD1 signaling induces epithelial-to-mesenchymal transition in ovarian endometrioid cancer cells (Tseng et al., 2011).

In a recent study, it was shown that a mutated non-processable version of Sema3E (Uncl-Sema3E) is unable to elicit ERBB2 transactivation and pro-metastatic signaling in cancer cells (Casazza et al., 2012). Yet, Uncl-Sema3E remains fully competent for binding PlexinD1 and triggering its inhibitory activity in endothelial cells, which leads to decreased angiogenesis and tumor growth in mice. Moreover, the same molecule "competed out" the autocrine signaling of endogenous p61-Sema3E in cancer cells and blocked their invasive and metastatic behavior (Casazza et al., 2012). Uncl-Sema3E was effective in multiple tumor models in mice, including spontaneous RipTag2 pancreatic tumors, and transplanted LLC tumors nonresponsive to VEGF blockade. This is consistent with the idea that these semaphorins exert a direct anti-angiogenic activity on endothelial cells, rather than interfering with pro-angiogenic signals (Figure 2). Moreover, a concomitant direct inhibitory effect of Uncl-Sema3E in cancer cells was implicated to prevent hypoxia-driven invasion and metastasis. Together with the examples discussed in the previous section, these data highlight the relevance of anti-angiogenic semaphorins that can directly target endothelial cells and potently suppress tumor growth in mouse models without promoting cancer invasiveness, and even concomitantly reducing metastatic spreading. An open question concerns the possibility of developing functional antibodies or small molecules, alternative to the large recombinant semaphorin proteins tested so far in mouse models, in order to trigger plexin-mediated inhibitory signals or block signaling pathways elicited by endogenous semaphorins.

PlexinB1 Plays Tumor-Type-Specific Functions in Cancer Progression

PlexinB1 is another family member coupled with RTKs on the cell surface, in particular with MET (Giordano et al., 2002) and ERBB2 (Swiercz et al., 2004). These interactions have a major role in Sema4D-PlexinB1 signaling, as they can mediate distinctive and sometimes divergent pathways in a cell-context-dependent manner, for instance leading to opposite regulation of RhoA

activity (Swiercz et al., 2008). This possibly explains the controversial literature related to the functional role of PlexinB1 in human tumors. In fact, in addition to its pro-angiogenic activity in endothelial cells, a number of studies reported pro-invasive functions of Sema4D-PlexinB1 signaling in a variety of cancer cell types (Giordano et al., 2002; Swiercz et al., 2008; Sierra et al., 2008; Binmadi et al., 2012; Ye et al., 2010; Qiang et al., 2011). Moreover PlexinB1 is overexpressed and frequently mutated in prostatic cancer, and some of these genetic changes were associated with a "switch" of receptor signaling accompanied by increased invasiveness and metastasis (Wong et al., 2007). On the other hand, in melanoma, oncogenic signaling by mutated constitutively active BRAF was found instead to suppress PlexinB1 expression (Argast et al., 2009); furthermore, experimental evidence indicated a tumor suppressor function of overexpressed PlexinB1 in melanoma cells (Argast et al., 2009; Stevens et al., 2010). One group recently addressed this issue in vivo by breeding an ERBB2-driven mouse breast carcinoma model in a PlexinB1 null background (Worzfeld et al., 2012). It emerged that PlexinB1 expression in vivo is not required for tumor growth or tumor angiogenesis, consistent with previous data, but it has a major role in promoting metastatic spreading. The specific role of Sema4D was not elucidated in this study; however, in vitro experiments suggested that PlexinB1 expression in cancer cells is required to mediate RhoA/RhoC activation elicited by overexpressed and constitutively active ERBB2 kinase (Worzfeld et al., 2012). Notably, low levels of PlexinB1 in ERBB2-overexpressing human breast tumors significantly correlated with better patient prognosis. Moreover, interfering with PlexinB1 was proposed as complementary therapeutic approach to block invasion and metastasis of ERBB2-positive tumors. Beyond the analysis of gene-deficient mouse models and RNAi approaches in vitro, this conclusion was based on the application of PlexinB1-blocking antibodies to cultured cells (Worzfeld et al., 2012). In fact, the use of interfering antibodies in vivo to manipulate Sema4D/PlexinB1 function in tumors has not been tested as yet. Interestingly, in a previous study, co-expression of PlexinB1 and MET tyrosine kinase in human breast and ovarian cancers was similarly found to correlate with metastatic progression (Valente et al., 2009). On the other hand, another group reported opposite prognostic significance of PlexinB1 expression in a subset of patients with estrogen receptor-positive breast cancer, indicating better survival in PlexinB1-expressing tumors and poor prognosis upon PlexinB1 loss (Rody et al., 2009). Thus, depending on the tumor type, PlexinB1 expression may be considered as a negative or a positive prognostic factor. Emerging data seem to suggest a similar scenario of tumor context-dependence for the homologous receptor PlexinB3, interacting with Sema5A (Artigiani et al., 2004). For instance, it was reported that an increased expression of Sema5A and PlexinB3 significantly correlates with invasion and metastasis in human gastric and pancreatic tumors (Pan et al., 2009; Sadanandam et al., 2010). On the other hand, another group found that Sema5A-PlexinB3 signaling inhibits the migration of human glioma cells and leads to inactivation of the pro-invasive protein fascin (Li et al., 2012). Further studies are required to elucidate whether this reflects the involvement of distinct receptor complexes and alternative signaling pathways, as seen for PlexinB1.

PlexinA4 Is Involved in Multiple Semaphorin Receptor Complexes, Responsible for Different Functions in Cancer and Endothelial Cells

In a recent study, PlexinA4 was reported to associate with RTKs VEGFR2 and FGFR2, and thereby promote signaling pathways leading to increased viability of endothelial and tumor cells (Kigel et al., 2011). Similar effects were observed upon overexpression of PlexinA4-ligand Sema6B, potentially suggesting that elevated Sema6B and/or PlexinA4 levels in tumors may correlate with progression. Intriguingly, it was proposed that PlexinA4-RTK receptor complexes may be functionally alternative to PlexinA4-PlexinA1 heterodimers responsible for Sema3A anti-angiogenic signals (Kigel et al., 2011). Thereby PlexinA4 might mediate opposite biological functions depending on the implicated semaphorin ligand and plexin-associated signal transducers. Based on these data, it could be envisaged that cancer treatment with Sema3A (or related molecules) could lead not only to direct inhibition of tumor cell migration and angiogenesis, but also prevent stimulatory signaling cascades by titrating out the bivalent receptor component PlexinA4.

Conclusions

In summary, inhibitory semaphorins that restrict cell migration and angiogenesis during development are often downregulated in advanced tumors, and proved to be effective tumor suppressor molecules in preclinical mouse models. Other semaphorins and plexins, such as Sema4D, PlexinB1, Sema3E and PlexinD1, are frequently upregulated in tumors as they support cancer progression and metastatic spreading. They have been proposed as prognostic predictors and recombinant molecular tools capable of blocking these pathways and effectively reducing tumor angiogenesis and metastatic spreading in preclinical trials in mice. Because the network of semaphorin signals is rather complex and cell-context-dependent, further studies may help to establish the relevance of individual family members as prognostic predictors and therapeutic targets for specific tumor types in humans. Moreover, biotechnological studies will aid in the development of improved molecular tools interfering with plexin activation and applicable for use in clinical trials.

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