Semaphorin receptors meet receptor tyrosine kinases on the way of tumor progression

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ABSTRACT

Semaphorins are extracellular signals known to guide migrating cells during developmental morphogenesis and in adult tissues. Semaphorin receptors, that is plexins and neuropilins, have been found in association with diverse receptor tyrosine kinases (RTKs), such as Met, ErbB2 and VEGFR2. These receptor complexes are formed in a cell-specific manner and can mediate distinctive signalling cascades, sometimes leading to divergent functional outcomes. This is particularly intriguing in cancer, since the same semaphorin has been found to mediate either tumor-promoting or tumor-suppressing functions, depending on the cancer type and cellular context. We will therefore review the current understanding about the role of RTKs in neuropilin and plexin signalling, putatively accounting for the multifaceted role of semaphorins in cancer.

Keywords: plexin; neuropilin; met; ErbB2; EGFR; VEGF

INTRODUCTION

Semaphorins

Around 20 membrane-bound or secreted proteins constitute the semaphorin family in vertebrates. In addition to their initially identified role in the wiring of the neuronal network,[1] semaphorins have been implicated in a variety of biological functions and developmental processes, often involving the guidance of cell migration and cell-to-cell interactions.[2] Notably, due to their regulatory role in angiogenesis and cancer cell behavior, they are emerging modifiers in tumor progression and potential therapeutic targets.[3]

All family members share a common domain, called *sema* domain, characterized by a typical seven-blade beta-propeller fold structure, also found in the alpha-integrins and in tyrosine kinase receptors of the Met family.[4] Semaphorins are then divided into eight subclasses, according to structural features beyond the*sema* domain, which are also characterized by distinctive properties.[5]

Semaphorin signals are mediated by the main receptor family of the Plexins, including nine members in vertebrates, which also carry the distinctive *sema*domain.[6] A subset of secreted semaphorins requires the presence of co-receptors called neuropilins (Nrp1/Nrp2). The analysis of crystal structures revealed that*sema* domain dimerisation is required for receptor binding and functional response; on the other hand, the mechanism responsible for plexin activation upon ligand engagement is less clear and partly controversial (reviewed by Hoto and Buck[7]).

Semaphorins and cancer

Semaphorin expression was found to be altered compared to normal tissues in various human tumors. Notably, semaphorin-receptors, plexins and neuropilins, are widely expressed in cancer cells, as well as in cells of the tumor microenvironment.[8] A growing number of reports has linked semaphorins and their receptors to multiple cancer hallmarks, such as invasion and acquisition of metastatic properties, tumor angiogenesis and activation of pro-tumorigenic inflammation, or, more rarely, regulation of proliferation and apoptosis.[9] Certain semaphorins can either promote or inhibit tumor progression depending on the implicated receptor complexes and the specific target cell.

Semaphorins seem to be important players in the tumor microenvironment (reviewed by Gu and Giraudo[10]). For instance, Sema4D or Sema6A have a pro-angiogenic activity by regulating endothelial cells,[11, 12] while others like class-3 Semaphorins can antagonize vascular endothelial growth factor (VEGF) signalling, and deploy endothelial cells repulsion.[13] Semaphorins also regulate the recruitment and activity of tumor-associated immune cells and macrophages,

which have been shown to regulate nearly all steps of tumor progression.[14] Thus, semaphorins are involved in many aspects of tumor biology, and they can be considered promising therapeutic targets in cancer.

Semaphorin signalling

Semaphorins have been shown to signal through many different pathways and effectors, which include their intrinsic GTPase-activating protein (GAP) activity for R-Ras, M-Ras and Rap1,[15, 16, 16] the indirect regulation of integrins and of other Rho GTPases, and the interaction with tyrosine kinases (for a general review, see reference[2]).

GAPs negatively regulate the activity of monomeric G proteins by promoting the hydrolysis of GTP to GDP. The intracellular domain of plexins contains two highly conserved GAP-like subdomains (C1 and C2) separated by a linker region. The conserved domains include three arginine residues necessary for catalytic GAP activity.[17, 18] The binding of Rnd1 to the linker region of PlexinA1 and PlexinB1 seems to be required for disrupting an inhibitory interaction between N-terminal and C-terminal halves of the segmented GAP domain.[19] The same function is played by Rnd2 for PlexinD1, while the GAP activity of PlexinC1 does not seem to depend on Rnd regulation.[16] A further level of control was recently revealed by Yang and colleagues,[20] who found that the GAP domain of insect d-PlexinA is phosphorylated by the cAMP-dependent protein kinase leading to the recruitment of 14-3-3ε adaptor protein, which in turn prevents plexin interaction with R-Ras and the ensuing semaphorin-induced cell-repulsion.

The primary functions of R-Ras are linked to integrin regulation, by increasing integrin-based cell adhesion to the extracellular matrix.[21] Hence, inhibition of integrin-mediated adhesion is a common and early event in semaphorin signalling,[18, 22] which impinges on cell migration and cytoskeletal changes, sometimes leading to the so-called 'collapsing' response. This is a typical behavior observed in semaphorin-treated cells *in vitro*, characterized by retraction of cellular processes (or axonal extensions) and cell rounding.[18]

Plexins of the B-subfamily can also interact with guanine nucleotide-exchange factors (GEFs) for RhoA, which promote the exchange of GDP for GTP and lead to RhoA activation.[23, 24, 25] This has been linked to the promotion of cell migration and to the activation of additional intracellular pathways, also involving tyrosine kinase signalling.

Upon ligand stimulation, different plexins were found to interact with cytoplasmic or receptor tyrosine kinases (RTKs) such as MET, ERBB2, VEGFR2, FYN, FES, PYK2 and SRC (reviewed by Franco and Tamagnone[26]). This often leads to the functional activation of the kinase, and thereby to the initiation of distinctive intracellular signalling cascades. Moreover, the cytoplasmic domain of plexins can get trans-phosphorylated on tyrosine residues, and this may have an impact on further signalling events, for example, by modulating domain conformation or creating phosphorylated docking sites for the recruitment of specific signal transducers. Notably, a specific plexin can alternatively associate with different tyrosine kinases, eliciting divergent signalling pathways and functional outcomes. These aspects will be extensively discussed in this review.

Plexin B1 and Met

Accumulating literature indicates that PlexinB1 may have an ambivalent function in cancer, acting either as a tumor promoter or as a tumor suppressor in different studies. This has sometimes been correlated with differential RhoA regulation in a cell-context dependent manner (see below). Notably, two major oncogenic RTKs were found to interact with PlexinB1: Met and ErbB2, as discussed in detail in this paragraph and the following.

Met, the plasma membrane receptor for Scatter Factor 1 (also known as Hepatocyte Growth Factor, HGF) was initially found to associate with PlexinB1 in liver progenitor cells.[27] Moreover, in response to PlexinB1-ligand Sema4D, tyrosine phosphorylation of both Met and PlexinB1 is induced, together with increased cell migration and invasion (see Figure 1a). Consistently, co-expression of PlexinB1 and Met, as well as Met phosphorylation, in breast and ovarian tumors correlates with metastatic spreading.[28] Met is furthermore activated and associated with overexpressed PlexinB1 in colon, liver, pancreas and gastric cancer cells.[29]Notably, Sema4D levels are upregulated in prostate, colon, breast and lung tumors compared to normal tissues.[30] Moreover, PlexinB1 was found to be overexpressed and mutated in invasive prostate cancer;[31] these mutations seem to affect the GTPase interacting domain of PlexinB1, but not its ability to synergize with RTKs and promote cell invasiveness.[32] Met tyrosine kinase receptor has also been implicated in PlexinB1-dependent pro-angiogenic activity mediated by Sema4D.[33]

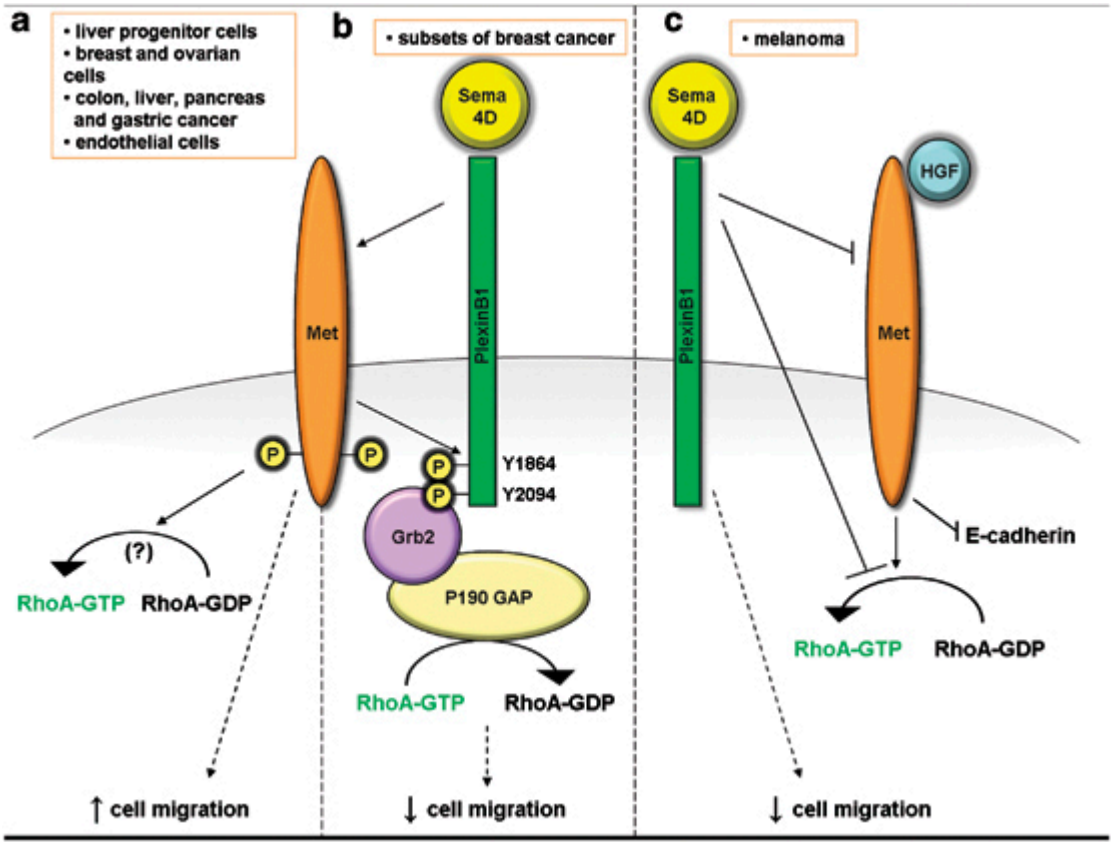

Figure 1.

Multifaceted role of Sema4D-PlexinB1 signalling in Met pathway. (a) PlexinB1 and Met are co-expressed and associated at the cell surface in different cells. In response to Sema4D stimulation, Met is trans-activated and becomes tyrosine phosphorylated in liver progenitor cells, some breast and ovarian cancers, colon, liver, pancreas and gastric cancer, and endothelial cells. This correlates with increased cell migration, invasiveness and angiogenesis. The role of RhoA regulation is not fully clear in this pathway. (b) In a subset of ER+ human breast cancer, Sema4D stimulation leads to PlexinB1 trans-phosphorylation at residues Y1864 and 2094, which serve as docking site for Grb2. In turn, Grb2 recruits p190GAP, resulting in RhoA inactivation and consequent inhibition of cell migration and invasion. (c) In melanoma cells, the PlexinB1-Met complex is not leading to RTK activation in response to Sema4D. Instead, Sema4D-PlexinB1 signalling inhibits the classical HGF-Met pathway, including reduced E-cadherin expression and RhoA activation. This eventually leads to decreased melanoma cell migration and invasion.

However, these findings are not concordant with the conclusions of other studies that suggest PlexinB1 as a tumor-suppressor protein. For instance, in a subset of oestrogen receptor-positive human breast cancers, low PlexinB1 expression is actually associated with tumors of higher grade, characterized by increased cell proliferation and poor outcome[34, 35](see Figure 1b). PlexinB1 expression was also found to be downregulated by the ERK pathway constitutively active in BRAF-mutated human melanomas[36] and, consistently, its expression is lost in metastatic and invasive melanomas *in vivo*.[37] Moreover, the forced expression of PlexinB1 in human melanoma cell lines results in the inhibition of colony formation in soft agar and reduced tumor growth in mouse xenografts.[36]

Melanomas may indeed represent a tumor type in which PlexinB1 may have an opposite role from what was described in other models (see Figure 1c). Notably, Met activation plays an important role in melanoma progression,[38] and it has been shown that PlexinB1 can inhibit Met signalling in both non-transformed and transformed melanocytes, consistent with the concomitant finding of activated Met and PlexinB1 loss in melanoma samples.[37, 39, 40] Indeed, PlexinB1 and Met are found in complex in melanocytes and their association is induced by Sema4D.[40]However, PlexinB1 overexpression in melanoma cells leads to decreased phosphorylation of Met and reduced migration in response to HGF,[37] while PlexinB1 knockdown leads to the opposite effects.[40] It could be envisaged as a balancing between the formation of PlexinB1-Met and Met-Met dimers in this tumor model, whereby Sema4D/PlexinB1 activation could lead to the inhibition of 'classical' oncogenic Met signalling. It has been demonstrated that Sema4D inhibits HGF-induced downregulation of

E-cadherin, an important mechanism by which melanoma cells can disrupt adhesion to keratinocytes[41] and commence migration.[39] The mechanisms underlying this crossregulation are currently unclear. Intriguingly, unpublished data mentioned by Soong and co-workers[40] indicate that PlexinB1 does not get phosphorylated in response to Sema4D in melanoma cells, despite the formation of PlexinB1-Met complex, further suggesting a context-dependent function of PlexinB1 in different tumor cells.

Still debated is also the role of RhoA regulation by Sema4D/PlexinB1 signalling. Rho family GTPases are implicated in numerous cellular processes, from cytoskeletal remodelling to cell migration, proliferation and apoptosis, and they also play a role in human cancers, including melanoma.[42, 43, 44] RhoA inactivation could have a role in the tumor suppressor function of PlexinB1 in melanomas. In fact, PlexinB1 can suppress Rho activity and abrogate HGF-induced Rho activation in melanoma cells.[45] RhoA inactivation was also found to ensue Sema4D stimulation in breast cancer cells co-expressing PlexinB1 and Met,[46] and Met-dependent phosphorylation of specific tyrosine residues of PlexinB1 (Y1864 and Y2094) is required for this effect[47] (see Figure 1b). By a screening approach, Sun and colleagues[47] identified Grb2 as the specific PlexinB1 interactor recruited in response to Met-mediated phosphorylation. In turn, Grb2 can mediate the recruitment of p190RhoGAP, a regulatory protein previously found in association with plexins, and mediating RhoA inactivation and inhibition of cancer cell migration.[48] The same mechanism cannot be applied to the melanoma model, because there is neither Met kinase activation in the complex, nor phosphorylation of PlexinB1 by Met. Further studies are necessary to clarify this point, for example in melanoma cells PlexinB1 could recruit p190GAP through other mechanisms, also independently from PlexinB1 phosphorylation. Notably, it would be interesting to study RhoA regulation by Sema4D in cells undergoing Sema4D-dependent Met activation associated with increased cell migration and invasion, which includes endothelial cells.[27, 33, 49]

Importantly, Swiercz and co-workers[46] reported another signalling mechanism involving alternative association of tyrosine kinases Met or ErbB2 with PlexinB1 in breast cancer cells. In this study, the authors report that PlexinB1 signaling leads to RhoA inactivation when coupled with Met, whereas it mediates RhoA activation when in complex with ErbB2 (this latter mechanism will be described in detail in the next paragraph). Distinct RTKs may achieve this divergent control on Rho activity in response to Sema4D via phosphorylation of specific tyrosine residues in the cytosolic tail of PlexinB1, as shown for Met.[47]

Plexins/neuropilins and EGFR family RTKs

PlexinB1 and ErbB2

PlexinB1 was also consistently shown to interact with ErbB2 transmembrane tyrosine kinase.[46, 50] Similar to what is described for Met, PlexinB1 associates with ErbB2 and, in the presence of Sema4D, it elicits RTK activation and phosphorylation of both receptors. Swiercz and colleagues[46, 50] also demonstrated that ErbB2-mediated phosphorylation of PlexinB1 leads to RhoA activation, due to the activity of PDZ-RhoGEFs associated with the plexin. By means of peptide- and mass-spectrometry-based approaches, the same authors clarified the specific tyrosine residues of PlexinB1 involved. In fact, tyrosines 1708 and 1732 phosphorylated by ErbB2 create a docking site for the SH2 domains of PLCγ signal transducer.[46] In turn, the SH3 domain of PLCγ interacts with the proline-rich C-terminal region of PDZ-RhoGEF leading to its activation (Figure 2).

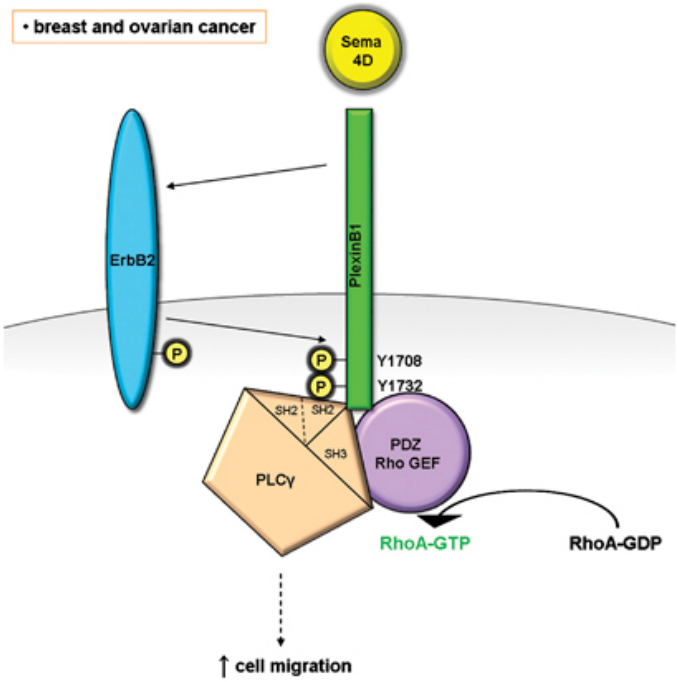

Figure 2.

PlexinB1-ErbB2 signalling in cancer cells. In subsets of breast and ovarian cancers, ErbB2 and PlexinB1 can interact, leading to phosphorylation of both the receptors. The phospho-tyrosines residues involved (Y1708 and Y1732) serve to recruit PLCγthrough its SH2 domains. The SH3 domain of PLCγ can then interact with PDZ-RhoGEF, which leads to RhoA activation and consequent increase in cell migration and invasiveness.

ErbB2 tyrosine kinase plays an important role in breast cancer, being overexpressed in 30% of the cases, especially those with high metastatic potential and worse prognosis.[51, 52] A recent work demonstrated that the endogenous or induced overexpression of ErbB2 is sufficient to elicit PlexinB1 phosphorylation in breast and ovarian cancer cells, leading to the activation of RhoA and RhoC GTPases, and the promotion of cell invasiveness, independent from Sema4D stimulation.[53] Moreover, when crossing in a PlexinB1-deficient background MMTVneu mice, a transgenic model developing slowly progressing breast cancers due to overexpression of wild-type ErbB2 in mammary glands, a strong decrease in metastasis formation was observed, with no effect on primary tumor growth. Consistently, patients with ErbB2-overexpressing breast cancer and low expression of PlexinB1 display a longer disease-free survival than patients expressing high levels of PlexinB1,[53] in agreement with previous findings.[35]Interestingly, in ErbB2-negative breast cancer patients, the authors observed an opposite statistical correlation between prognosis and PlexinB1 expression,[53]which could be consistent with the tumor-suppressive role of the plexin in melanoma, where ErbB2 is not aberrantly expressed.[36, 37, 54]

PlexinD1 and ErbB2

ErbB2 activation is also at the crossroad of the differential activity of Sema3E-receptor PlexinD1 in tumor and endothelial cells. In fact, Sema3E controls vascular development, acting as an inhibitory/repelling cue for endothelial cells.[55, 56] This effect is putatively dependent on the Rnd2-gated intrinsic GAP activity of PlexinD1 for R-Ras,[16] on RhoJ regulation,[57] or the induction of integrin endocytosis due to Arf6 activation[58, 59] (see Figure 3a). It is often seen that signalling pathways typical of embryogenesis and usually lost after development are reactivated in cancer. Indeed PlexinD1 expression, normally downregulated in adult tissues, is elevated in endothelial cells involved in tumor angiogenesis, but also in cancer cells,[60] suggesting that both cell types could be controlled by Sema3E in the tumor context. Notably, beyond the role of this pathway in angiogenesis, Casazza and coworkers[61] found that Sema3E and PlexinD1 levels positively correlate with the metastatic progression of human melanoma and colon cancer. This is actually consistent with the experimental evidence of a pro-invasive and metastatic activity of Sema3E-PlexinD1 signalling in cancer cells, also demonstrated by the loss of metastatic potential upon gene knockdown. Moreover, the authors demonstrated that this pro-invasive/metastatic activity is mediated by Sema3E proteolytic product p61 (generated by furin proprotein convertases[62]) via the trans-activation of ErbB2 tyrosine kinase associated with PlexinD1[61] (see Figure 3b). Inhibiting ErbB2 kinase impairs p61-Sema3E-induced migration and invasiveness of tumor cells,[61] which indicates that ErbB2 is a

major player of the pro-metastatic activity of Sema3E, even if the downstream effectors are currently unknown. Moreover, the mechanisms responsible for the selective activation of this pathway in cancer cells need to be clarified.

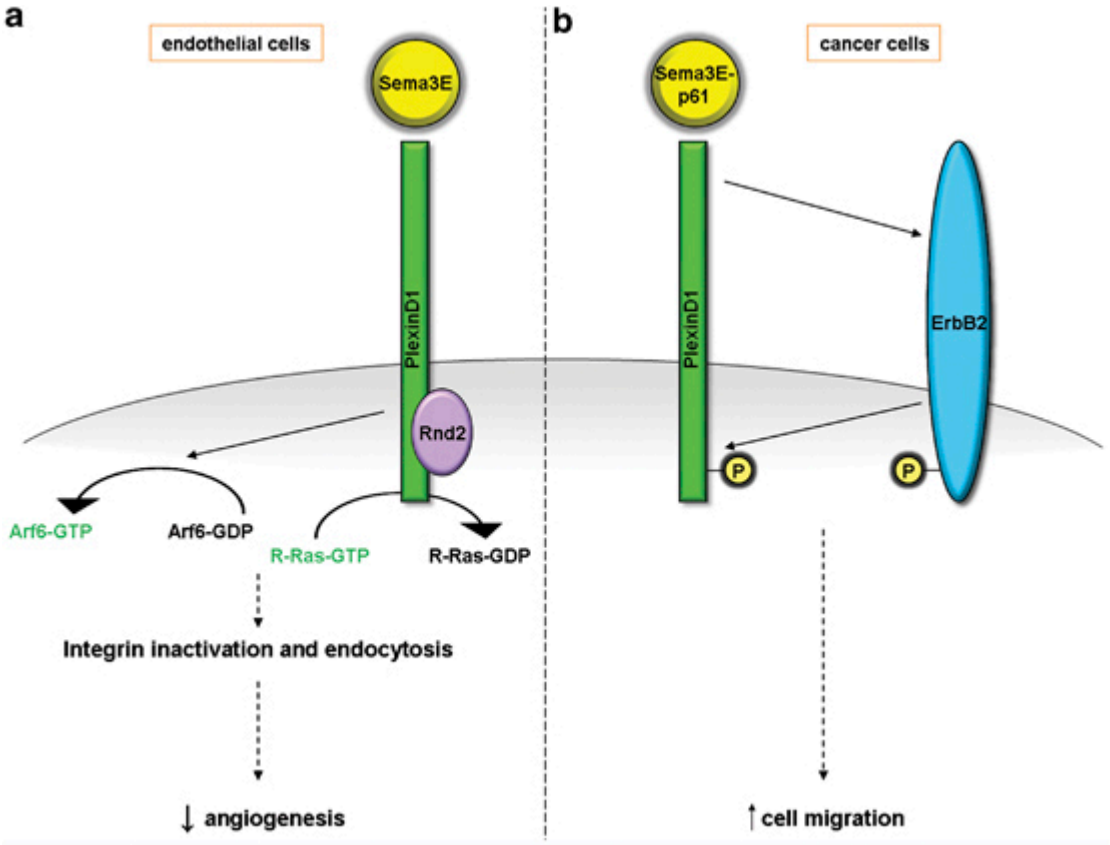

Figure 3.

Role of ErbB2 in Sema3E-PlexinD1 signalling. (a) In endothelial cells, Sema3E stimulation leads to Rnd2-gated GAP activity of PlexinD1 on R-Ras leading to inhibition of integrin-mediated adhesion. Moreover PlexinD1-induced Arf6 activation induces integrin endocytosis, finally leading to inhibition of angiogenesis. (b) The proteolytic product Sema3E-p61 has an opposite effect on cancer cells, via the cross-activation of ErbB2 tyrosine kinase and the phosphorylation of the PlexinD1-ErbB2 complex, associated with increased tumor cell migration and metastatic ability. The implicated pathway is not well characterized.

Notably, a point-mutated Sema3E isoform resistant to proteolytic processing, Uncl-Sema3E, was found to bind PlexinD1 and inhibit endothelial cells and angiogenesis *in vivo*, thereby suppressing tumor growth; in contrast, this isoform was unable to induce PlexinD1-ErbB2 association and kinase activation and consequently lacked any pro-metastatic activity.[63] Moreover, the authors found that Uncl-Sema3E can compete with endogenous p61-Sema3E for receptor binding, thus interfering with cancer cell metastatic behavior. This study highlighted Uncl-Sema3E as a potentially interesting new therapeutic tool, capable of concomitantly suppressing tumor growth and metastatic progression in multiple preclinical models.

Neuropilin-1 and EGFR

As mentioned above, a small family of co-receptor molecules, the neuropilins (Nrp1 and Nrp2), is involved in the receptor complexes for secreted semaphorins, providing an additional high-affinity binding site for the ligands. Neuropilins are also part of receptor complexes for VEGFs, with a predominant role in developmental angiogenesis.[64] Importantly, neuropilins have also been implicated in tumor growth and vascularisation, and mediate the effects of VEGFs and semaphorins on cell proliferation, cell survival and migration.[65, 66] Nrp1 appears to be mainly expressed in carcinomas, whereas Nrp2 is typically expressed in tumors derived from neural crest cells.[67, 68] Nrp1 levels often correlate with cancer progression and poor prognosis in different tumor types.[66] Notably, it has been reported that epidermal growth factor (EGF) can induce Nrp1 expression in tumor cells via EGF-Receptor tyrosine kinase signalling.[69, 70]

A recent study reported that Nrp1 overexpression can confer a selective advantage to cancer cells by promoting epidermal growth factor receptor (EGFR) signalling.[71] This is a major pathway activated in tumors, correlated with adverse prognosis. The authors demonstrated that the extracellular portion of Nrp1 promotes cancer cell survival and proliferation, independently from its VEGF-binding function, by associating with EGFR and eliciting its activation.[71] Notably, Nrp1-overexpressing tumors grown in mice showed increased EGFR tyrosine phosphorylation compared with controls. Current EGFR activation model implicates ligand-induced receptor oligomerization on the cell surface, followed by endocytosis.[72] Once in endocytic vescicles, EGFR can sustain prolonged intracellular signalling.[73, 74] Nrp1 has been found to play a role in endocytosis pathways involving VEGFR2 and secreted semaphorins.[75] Rizzolio and colleagues[71]reported that in response to epidermal growth factor or TGFα stimulation, a large fraction of Nrp1 co-localized with EGFR in early endosomes. Importantly, both EGFR clustering at the cell surface and the ensuing internalization were strongly impaired in Nrp1-silenced cells. As a consequence, by knocking-down Nrp1 in cancer cells, ligand-induced EGFR phosphorylation and activation of intracellular AKT effector pathway were inhibited.

Plexin/neuropilins and VEGFR

The cross-talk between plexins and VEGF-receptors was first reported in cardiovascular development, implicating Sema6D-induced PlexinA1 alternative association with OTK or VEGFR2-KDR in different cell populations, further leading to opposite functional outcomes.[76] Also Sema3E signalling was found to mediate opposite functions in different neuronal populations, depending on VEGFR2 association with PlexinD1.[77] In the cancer context, VEGFR2 signalling is pivotal in endothelial cells and angiogenesis,[78] but it was also implicated in cancer cell survival. As discussed below, semaphorin receptors were found to be relevant in the regulation of both these pathways.

Class-6 semaphorin receptors and VEGFRs

Emerging data seem to implicate class-6 Semaphorins and their plexin receptors as important regulators of VEGFR signalling both in tumor and in endothelial cells. It was reported that PlexinA1 and its ligand Sema6D are expressed and active in asbestos-related malignant pleural mesothelioma cells.[79] Malignant pleural mesothelioma is characterized by VEGF overexpression, which is involved not only in angiogenesis but also in directly sustaining tumor cell growth.[80] Catalano and colleagues[79] demonstrated that PlexinA1 and VEGFR2 are associated in a complex in malignant pleural mesothelioma cells. Moreover, in the presence of Sema6D, PlexinA1 promotes tyrosine phosphorylation of VEGFR2, leading to tumor cell survival and anchorage-independent growth, via VEGFR2-dependent activation of the transcriptional factor NF-kB. Importantly, expression of both Sema6D and PlexinA1 is induced by asbestos fibers, and Plexin-A1 overexpression in non-malignant mesothelial cells inhibits cell death induced by asbestos.[79]

PlexinA4 is a receptor for Sema6A and Sema6B.[81] It has been shown that knocking down PlexinA4 expression in primary endothelial cells inhibits VEGF signalling, as well as basic fibroblast growth factor-induced cell proliferation.[82]PlexinA4 was also found in association with VEGFR2 tyrosine kinase and enhancing VEGFR2 signalling. Notably, the knockdown of Sema6B expression in endothelial cells mimics the effects of PlexinA4 silencing, featuring a potential autocrine pro-proliferative autocrine circuit.[82]

Another recent study reported the pro-angiogenic function of the homologous ligand Sema6A. The authors found that Sema6A-depleted endothelial cells were more susceptible to apoptosis and cell death compared to controls, and responded poorly to VEGF stimulation.[11] Notably, Sema6A-silenced cells displayed reduced VEGFR2 expression, which could be rescued by treatment with recombinant soluble Sema6A-Fc, also recovering VEGF-dependent responses. Moreover, Segarra and colleagues[11] observed reduced tumor angiogenesis and tumor growth in Sema6A-null mice compared to wild-type littermates. The mechanism by which Sema6A regulates VEGFR2 expression remains to be clarified. In fact, the knockdown of either of the two known receptors, PlexinA2 and PlexinA4, individually or in combination, had no effect,[11] suggesting the existence of additional receptors for Sema6A. An alternative explanation could be a plexin-independent interaction in-cis between Sema6A and VEGFR2 in endothelial cells. These data are apparently in conflict with a previous study, reporting that the treatment of primary endothelial cells with a soluble extracellular domain of Sema6A inhibits VEGF signalling and angiogenesis;[83] notably, also in this case the involvement of plexins or alternative pathways was not clarified.

Neuropilins and VEGFRs

Neuropilins, in addition to being receptors for secreted semaphorins, were found to bind VEGFs and form receptor complexes with VEGF-R tyrosine kinases.[84, 85, 86,87] In particular, Nrp1 binds VEGF165 (but not VEGF121 isoform) and Nrp2 binds both VEGF165 and the smaller VEGF145. These VEGF-A isoforms differ by the presence or absence of specific domains consequent to alternative splicing.[88]Nrp1 and Nrp2 can also bind additional members of VEGF family: in particular, VEGF-B, VEGF-E and a splicing isoform of the Placental Growth Factor (known as PlGF2) can bind Nrp1,[89, 90] while VEGF-C and VEGF-D can bind Nrp2.[87, 91]

It has been shown that Nrp1 enhances VEGFR2 signalling and VEGF-induced chemoattraction of endothelial cells.[84, 86] One suggested mechanism implicates enhanced affinity of the ligand for VEGFR2 (and/or enhanced receptor clustering) in the presence of Nrp1.[86, 92] Notably, the formation of VEGFR2/Nrp1 complex seems to depend on the short intracellular sequence of Nrp1, including a consensus sequence for binding PDZ domains.[93] These are found, for example, in the adaptor protein GIPC/Synectin,[94] which plays a role in Nrp1-dependent angiogenesis, since synectin-deficient endothelial cells show reduced VEGFR2-Nrp1 complex formation.[93] Furthermore, GIPC/Synectin seems to be essential for Nrp1 endocytosis and trafficking, a mechanism regulating VEGFR signalling pathway.[95] Indeed, in response to VEGF165, Nrp1 and VEGFR2 undergo clathrin-dependent endocytosis,[75] a process that could facilitate their coupling to downstream effectors regulating endothelial cell migration.

Several studies indicated Nrp1 as a relevant therapeutic target in cancer, due to its involvement in multiple signalling pathways regulating tumor vasculature and cancer cells progression. In particular, two monoclonal antibodies targeting the extracellular domain of Nrp1 inhibited tumor angiogenesis and tumor growth in preclinical models.[96] Notably, only one of these antibodies blocked VEGF binding and VEGFR2 phosphorylation, while the other could interfere with Sema3A-dependent functions. These data suggested that Nrp1-targeting may be useful to hinder tumor growth by interference with multiple pathways. For instance, as mentioned above, Nrp1 also emerged as a relevant regulator of EGFR signalling.[71]

In addition to endothelial cells and tumor angiogenesis, VEGF is also known to regulate cancer cell proliferation, survival and migration.[64] Indeed, most tumor cells do not express VEGFR2, and VEGF signals are thought to act through VEGFR1, VEGFR3 and possibly neuropilins.[97, 98, 99] Thus, the functional role of neuropilins in cancer cells may be rather complex, considering all the signalling pathways in which they have been implicated, including the regulation of RTKs.

For instance, in medulloblastoma, the VEGF-family member placental growth factor (PlGF) involved in pathological angiogenesis and wound healing,[100] is co-expressed with its receptors Nrp1 and VEGFR1.[101] Indeed, clinical trials applying anti-PlGF antibodies in medulloblastoma patients achieved tumor regression[102, 103]and Snuderl and colleagues[104] demonstrated that stromal-derived PlGF represents a crucial growth factor for medulloblastoma cells survival. Moreover, while previous studies linked the inhibitory effect of anti-PlGF antibodies to the expression of VEGFR1 in tumor cells,[105, 106] Snuderl and colleagues[104]demonstrated that PlGF activity in medulloblastoma cells depends on Nrp1 and not VEGFR1.

Nrp1 may also play an important role in squamous skin carcinoma, where this receptor seems to regulate, in response to VEGF, the cancer stem cell (CSC) pool.[99] In this tumor model, CSCs are located in close proximity to endothelial cells in the perivascular niche; in fact, blocking VEGFR2 in endothelial cells caused a decrease in CSC pool size and self-renewal activity, associated with the reduction of microvascular density. It has been previously reported that *Vegfa*deletion in epidermal cells prevented squamous skin tumor development.[107, 108]Beck and colleagues[99] recently demonstrated an autocrine circuit of VEGF acting directly on epidermal cells and CSCs, and promoting cancer stemness and CSCs symmetric self-renewing divisions in Nrp1-dependent manner (see Figure 4). The implicated molecular mechanism is still partly unclear: Beck and colleagues suggest a cell-autonomous role of Nrp1 in epithelial cells, as anti-Nrp1 antibodies blocking VEGF binding suppressed the proliferation of purified $CD34^+$ cancer stem cells. Notably, a potential involvement of VEGFR1 in complex with Nrp1 could be envisaged, since it has been shown that deletion of VEGFR1 *(flt1)* in epidermal cells delays the onset of skin papilloma in k5-*Sos* transgenic mice.[107] Moreover, the authors demonstrated that VEGFR and EGFR pathways synergize in neoplastic cells. In fact, EGFR signalling upregulates VEGF, VEGFR1 and Nrp1 expression in a feed-forward loop, thus sustaining autocrine tumor cell proliferation.[107]

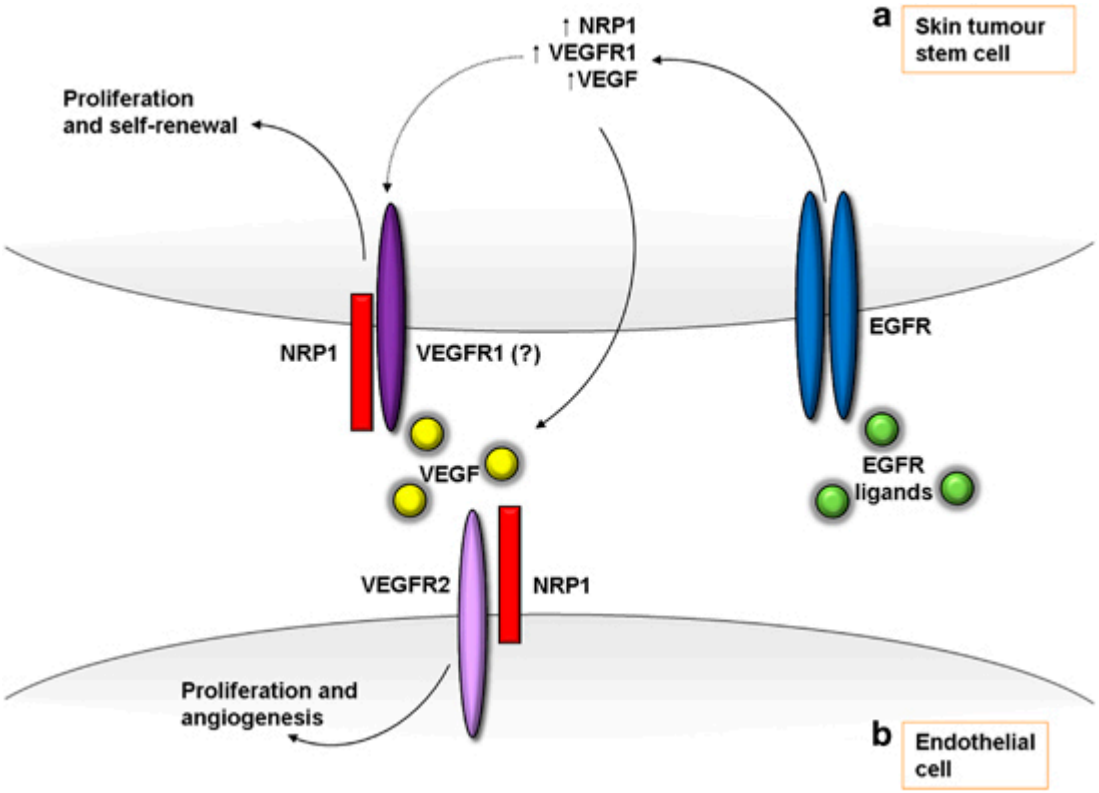

Figure 4.

Synergistic cross-talk between Nrp1- and EGFR-mediated signaling. (a) EGFR-mediated signalling leads to the upregulation of Nrp1, VEGFR1 and VEGF expression, which in turn promotes VEGF-Nrp1 signalling in epithelial cells, leading to cell proliferation and symmetric self-renewing. VEGFR1 might be implicated in the signalling cascade is association with Nrp1. (b) In endothelial cells, VEGF-VEGFR2 signalling promotes angiogenesis and the establishment of a perivascular niche which sustains the growth of cancer stem cells (CSC).

Concluding remarks

Thanks to a growing number of studies, the role of semaphorins, plexins and neuropilins in cancer starts to be understood. It appears clear that semaphorin signals can trigger multiple pathways depending on the receptor complex implicated, and in a cell-type specific manner. Tyrosine kinase receptors seem to be pivotal partners of semaphorin receptors in cancer cells, as well as in cells of the tumor microenvironment. This can account for the versatile and multifaceted activity mediated by certain semaphorins *in vitro*; moreover, it underlines the need to investigate their functions in different tumor types, also taking into account the functional state of oncogenic tyrosine kinases, such as Met, ErbB2 or EGFR.

Conflict of interest

The authors declare no conflict of interest.

REFERENCES

1. Kolodkin AL, Matthes DJ, Goodman CS. The semaphorin genes encode a family of transmembrane and secreted growth cone guidance molecules. *Cell*1993; 75: 1389–1399.
2. Kruger RP, Aurandt J, Guan KL. Semaphorins command cells to move. *Nat Rev Mol Cell Biol* 2005; 6: 789–800.

3. Tamagnone L. Emerging role of semaphorins as major regulatory signals and potential therapeutic targets in cancer. *Cancer Cell* 2012; 22: 145–152.

4. Gherardi E, Love CA, Esnouf RM, Jones EY. The sema domain. *Curr Opin Struct Biol* 2004; 14: 669–678.

5. Zhou Y, Gunput RA, Pasterkamp RJ. Semaphorin signaling: progress made and promises ahead. *Trends Biochem Sci* 2008; 33: 161–170.

6. Tamagnone L, Comoglio PM. Signalling by semaphorin receptors: cell guidance and beyond. *Trends Cell Biol* 2000; 10: 377–383.

7. Hota PK, Buck M. Plexin structures are coming: opportunities for multilevel investigations of semaphorin guidance receptors, their cell signaling mechanisms, and functions. *Cell Mol Life Sci* 2012; 69: 3765–3805.

8. Capparuccia L, Tamagnone L. Semaphorin signaling in cancer cells and in cells of the tumor microenvironment--two sides of a coin. *J Cell Sci* 2009;122(Pt 11): 1723–1736.

9. Rehman M, Tamagnone L. Semaphorins in cancer: biological mechanisms and therapeutic approaches. *Semin Cell Dev Biol* 2013; 24: 179–189.

10. Gu C, Giraudo E. The role of semaphorins and their receptors in vascular development and cancer. *Exp Cell Res* 2013; 319: 1306–1316.

11. Segarra M, Ohnuki H, Maric D, Salvucci O, Hou X, Kumar A *et al*. Semaphorin 6A regulates angiogenesis by modulating VEGF signaling. *Blood* 2012; 120: 4104–4115.

12. Sierra JR, Corso S, Caione L, Cepero V, Conrotto P, Cignetti A *et al*. Tumor angiogenesis and progression are enhanced by Sema4D produced by tumor-associated macrophages. *J Exp Med* 2008; 205: 1673–1685.

13. Guttmann-Raviv N, Shraga-Heled N, Varshavsky A, Guimaraes-Sternberg C, Kessler O, Neufeld G. Semaphorin-3A and semaphorin-3F work together to repel endothelial cells and to inhibit their survival by induction of apoptosis.*J Biol Chem* 2007; 282: 26294–26305.

14. Muratori C, Tamagnone L. Semaphorin signals tweaking the tumor microenvironment. *Adv Cancer Res* 2012; 114: 59–85.

15. Oinuma I, Ishikawa Y, Katoh H, Negishi M. The Semaphorin 4D receptor Plexin-B1 is a GTPase activating protein for R-Ras. *Science* 2004; 305: 862–865.

16. Uesugi K, Oinuma I, Katoh H, Negishi M. Different requirement for Rnd GTPases of R-Ras GAP activity of Plexin-C1 and Plexin-D1. *J Biol Chem*2009; 284: 6743–6751.

17. Rohm B, Rahim B, Kleiber B, Hovatta I, Puschel AW. The semaphorin 3A receptor may directly regulate the activity of small GTPases. *FEBS Lett*2000; 486: 68–72.

18. Barberis D, Artigiani S, Casazza A, Corso S, Giordano S, Love CA *et al*. Plexin signaling hampers integrin-based adhesion, leading to Rho-kinase independent cell rounding, and inhibiting lamellipodia extension and cell motility. *FASEB J* 2004; 18: 592–594.

19. Wang H, Hota PK, Tong Y, Li B, Shen L, Nedyalkova L *et al*. Structural basis of Rnd1 binding to plexin Rho GTPase binding domains (RBDs). *J Biol Chem*2011; 286: 26093–26106

20. Yang T, Terman JR. 14-3-3epsilon Couples Protein Kinase A to Semaphorin Signaling and Silences Plexin RasGAP-Mediated Axonal Repulsion. *Neuron*2012; 74: 108–121.

21. Keely PJ, Rusyn EV, Cox AD, Parise LV. R-Ras signals through specific integrin alpha cytoplasmic domains to promote migration and invasion of breast epithelial cells. *J Cell Biol* 1999; 145: 1077–1088.

22. Serini G, Valdembri D, Zanivan S, Morterra G, Burkhardt C, Caccavari F *et al*. Class 3 semaphorins control vascular morphogenesis by inhibiting integrin function. *Nature* 2003; 424: 391–397.

23. Swiercz JM, Kuner R, Behrens J, Offermanns S. Plexin-B1 directly interacts with PDZ-RhoGEF/LARG to regulate RhoA and growth cone morphology.*Neuron* 2002; 35: 51–63.

24. Aurandt J, Vikis HG, Gutkind JS, Ahn N, Guan KL. The semaphorin receptor plexin-B1 signals through a direct interaction with the Rho-specific nucleotide exchange factor, LARG. *Proc Natl Acad Sci USA* 2002; 99: 12085–12090.

25. Perrot V, Vazquez-Prado J, Gutkind JS. Plexin B regulates Rho through the guanine nucleotide exchange factors leukemia-associated Rho GEF (LARG) and PDZ-RhoGEF. *J Biol Chem* 2002; 277: 43115–43120.

26. Franco M, Tamagnone L. Tyrosine phosphorylation in semaphorin signalling: shifting into overdrive. *EMBO Rep* 2008; 9: 865–871.

27. Giordano S, Corso S, Conrotto P, Artigiani S, Gilestro G, Barberis D *et al*. The semaphorin 4D receptor controls invasive growth by coupling with Met. *Nat Cell Biol* 2002; 4: 720–724.

28. Valente G, Nicotra G, Arrondini M, Castino R, Capparuccia L, Prat M *et al*. Co-expression of plexin-B1 and Met in human breast and ovary tumours enhances the risk of progression. *Cell Oncol* 2009; 31: 423–436.

29. Conrotto P, Corso S, Gamberini S, Comoglio PM, Giordano S. Interplay between scatter factor receptors and B plexins controls invasive growth.*Oncogene* 2004; 23: 5131–5137.

30. Basile JR, Castilho RM, Williams VP, Gutkind JS. Semaphorin 4D provides a link between axon guidance processes and tumor-induced angiogenesis.*Proc Natl Acad Sci USA* 2006; 103: 9017–9022.

31. Wong OG, Nitkunan T, Oinuma I, Zhou C, Blanc V, Brown RS *et al*. Plexin-B1 mutations in prostate cancer. *Proc Natl Acad Sci USA* 2007; 104: 19040–19045.

32. Zhou C, Wong OG, Masters JR, Williamson M. Effect of cancer-associated mutations in the PlexinB1 gene. *Mol Cancer* 2012; 11: 11.

33. Conrotto P, Valdembri D, Corso S, Serini G, Tamagnone L, Comoglio PM *et al*. Sema4D induces angiogenesis through Met recruitment by Plexin B1. *Blood*2005; 105: 4321–4329.

34. Rody A, Karn T, Ruckhaberle E, Hanker L, Metzler D, Muller V *et al*. Loss of Plexin B1 is highly prognostic in low proliferating ER positive breast cancers--results of a large scale microarray analysis. *Eur J Cancer* 2009; 45: 405–413.

35. Rody A, Holtrich U, Gaetje R, Gehrmann M, Engels K, von MG *et al*. Poor outcome in estrogen receptor-positive breast cancers predicted by loss of plexin B1. *Clin Cancer Res* 2007; 13: 1115–1122.

36. Argast GM, Croy CH, Couts KL, Zhang Z, Litman E, Chan DC *et al*. Plexin B1 is repressed by oncogenic B-Raf signaling and functions as a tumor suppressor in melanoma cells. *Oncogene* 2009; 28: 2697–2709.

37. Stevens L, McClelland L, Fricke A, Williamson M, Kuo I, Scott G. Plexin B1 suppresses c-Met in melanoma: a role for plexin B1 as a tumor-suppressor protein through regulation of c-Met. *J Invest Dermatol* 2010; 130: 1636–1645.

38. Otsuka T, Takayama H, Sharp R, Celli G, LaRochelle WJ, Bottaro DP *et al*. c-Met autocrine activation induces development of malignant melanoma and acquisition of the metastatic phenotype. *Cancer Res* 1998; 58: 5157–5167

39. Soong J, Chen Y, Shustef EM, Scott GA. Sema4D, the ligand for Plexin B1, suppresses c-Met activation and migration and promotes melanocyte survival and growth. *J Invest Dermatol* 2012; 132: 1230–1238.

40. Soong J, Scott G. Plexin B1 inhibits MET through direct association and regulates Shp2 expression in melanocytes. *J Cell Sci* 2013; 126(Pt 2): 688–695.

41. Li G, Schaider H, Satyamoorthy K, Hanakawa Y, Hashimoto K, Herlyn M. Downregulation of E-cadherin and Desmoglein 1 by autocrine hepatocyte growth factor during melanoma development. *Oncogene* 2001; 20: 8125–8135.

42. Ridley AJ. Rho family proteins: coordinating cell responses. *Trends Cell Biol*2001; 11: 471–477.

43. Narumiya S, Tanji M, Ishizaki T. Rho signaling, ROCK and mDia1, in transformation, metastasis and invasion. *Cancer Metastasis Rev* 2009; 28: 65–76.

44. Routhier A, Astuccio M, Lahey D, Monfredo N, Johnson A, Callahan W *et al*. Pharmacological inhibition of Rho-kinase signaling with Y-27632 blocks melanoma tumor growth. *Oncol Rep* 2010; 23: 861–867.

45. McClelland L, Chen Y, Soong J, Kuo I, Scott G. Plexin B1 inhibits integrin-dependent pp125FAK and Rho activity in melanoma. *Pigment Cell Melanoma Res* 2011; 24: 165–174.

46. Swiercz JM, Worzfeld T, Offermanns S. ErbB-2 and met reciprocally regulate cellular signaling via plexin-B1. *J Biol Chem* 2008; 283: 1893–1901.

47. Sun T, Krishnan R, Swiercz JM. Grb2 mediates semaphorin-4D-dependent RhoA inactivation. *J Cell Sci* 2012; 125(Pt 15): 3557–3567.

48. Barberis D, Casazza A, Sordella R, Corso S, Artigiani S, Settleman J *et al*. p190 Rho-GTPase activating protein associates with plexins and it is required for semaphorin signalling. *J Cell Sci* 2005; 118(Pt 20): 4689–4700.

49. Giacobini P, Messina A, Morello F, Ferraris N, Corso S, Penachioni J *et al*. Semaphorin 4D regulates gonadotropin hormone-releasing hormone-1 neuronal migration through PlexinB1-Met complex. *J Cell Biol* 2008; 183: 555–566

50. Swiercz JM, Kuner R, Offermanns S. Plexin-B1/RhoGEF-mediated RhoA activation involves the receptor tyrosine kinase ErbB-2. *J Cell Biol* 2004;165: 869–880.

51. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* 1987; 235: 177–182.

52. Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE *et al*. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science* 1989; 244: 707–712.

53. Worzfeld T, Swiercz JM, Looso M, Straub BK, Sivaraj KK, Offermanns S. ErbB-2 signals through Plexin-B1 to promote breast cancer metastasis. *J Clin Invest* 2012; 122: 1296–1305.

54. Kluger HM, DiVito K, Berger AJ, Halaban R, Ariyan S, Camp RL *et al*. Her2/neu is not a commonly expressed therapeutic target in melanoma -- a large cohort tissue microarray study. *Melanoma Res* 2004; 14: 207–210.

55. van der ZB, Hellemons AJ, Leenders WP, Burbach JP, Brunner HG, Padberg GW *et al*. PLEXIN-D1, a novel plexin family member, is expressed in vascular endothelium and the central nervous system during mouse embryogenesis.*Dev Dyn* 2002; 225: 336–343.

56. Gu C, Yoshida Y, Livet J, Reimert DV, Mann F, Merte J *et al*. Semaphorin 3E and plexin-D1 control vascular pattern independently of neuropilins. *Science*2005; 307: 265–268.

57. Fukushima Y, Okada M, Kataoka H, Hirashima M, Yoshida Y, Mann F *et al*. Sema3E-PlexinD1 signaling selectively suppresses disoriented angiogenesis in ischemic retinopathy in mice. *J Clin Invest* 2011; 121: 1974–1985.

58. Sakurai A, Gavard J, nnas-Linhares Y, Basile JR, Amornphimoltham P, Palmby TR *et al*. Semaphorin 3E initiates antiangiogenic signaling through plexin D1 by regulating Arf6 and R-Ras. *Mol Cell Biol* 2010; 30: 3086–3098.

59. Sakurai A, Jian X, Lee CJ, Manavski Y, Chavakis E, Donaldson J *et al*. Phosphatidylinositol-4-phosphate 5-kinase and GEP100/Brag2 protein mediate antiangiogenic signaling by semaphorin 3E-plexin-D1 through Arf6 protein. *J Biol Chem* 2011; 286: 34335–34345.

60. Roodink I, Raats J, van der ZB, Verrijp K, Kusters B, van BH *et al*. Plexin D1 expression is induced on tumor vasculature and tumor cells: a novel target for diagnosis and therapy? *Cancer Res* 2005; 65: 8317–8323.

61. Casazza A, Finisguerra V, Capparuccia L, Camperi A, Swiercz JM, Rizzolio S*et al*. Sema3E-Plexin D1 signaling drives human cancer cell invasiveness and metastatic spreading in mice. *J Clin Invest* 2010; 120: 2684–2698.

62. Christensen C, Ambartsumian N, Gilestro G, Thomsen B, Comoglio P, Tamagnone L *et al*. Proteolytic processing converts the repelling signal Sema3E into an inducer of invasive growth and lung metastasis. *Cancer Res*2005; 65: 6167–6177.

63. Casazza A, Kigel B, Maione F, Capparuccia L, Kessler O, Giraudo E *et al*. Tumour growth inhibition and anti-metastatic activity of a mutated furin-resistant Semaphorin 3E isoform. *EMBO Mol Med* 2012; 4: 234–250.

64. Guttmann-Raviv N, Kessler O, Shraga-Heled N, Lange T, Herzog Y, Neufeld G. The neuropilins and their role in tumorigenesis and tumor progression.*Cancer Lett* 2006; 231: 1–11

65. Pellet-Many C, Frankel P, Jia H, Zachary I. Neuropilins: structure, function and role in disease. *Biochem J* 2008; 411: 211–226.

66. Rizzolio S, Tamagnone L. Multifaceted role of neuropilins in cancer. *Curr Med Chem* 2011; 18: 3563–3575.

67. Bielenberg DR, Pettaway CA, Takashima S, Klagsbrun M. Neuropilins in neoplasms: expression, regulation, and function. *Exp Cell Res* 2006; 312: 584–593.

68. Ellis LM. The role of neuropilins in cancer. *Mol Cancer Ther* 2006; 5: 1099–1107

69. Parikh AA, Liu WB, Fan F, Stoeltzing O, Reinmuth N, Bruns CJ *et al*. Expression and regulation of the novel vascular endothelial growth factor receptor neuropilin-1 by epidermal growth factor in human pancreatic carcinoma. *Cancer* 2003; 98: 720–729.

70. Akagi M, Kawaguchi M, Liu W, McCarty MF, Takeda A, Fan F *et al*. Induction of neuropilin-1 and vascular endothelial growth factor by epidermal growth factor in human gastric cancer cells. *Br J Cancer* 2003; 88: 796–802.

71. Rizzolio S, Rabinowicz N, Rainero E, Lanzetti L, Serini G, Norman J *et al*. Neuropilin-1-dependent regulation of EGF-receptor signaling. *Cancer Res*2012; 72: 5801–5811.

72. Hofman EG, Bader AN, Voortman J, van den Heuvel DJ, Sigismund S, Verkleij AJ *et al*. Ligand-induced EGF receptor oligomerization is kinase-dependent and enhances internalization. *J Biol Chem* 2010; 285: 39481–39489.

73. Sorkin A. Internalization of the epidermal growth factor receptor: role in signalling. *Biochem Soc Trans* 2001; 29(Pt 4): 480–484.

74. Sigismund S, Argenzio E, Tosoni D, Cavallaro E, Polo S, Di Fiore PP. Clathrin-mediated internalization is essential for sustained EGFR signaling but dispensable for degradation. *Dev Cell* 2008; 15: 209–219.

75. Salikhova A, Wang L, Lanahan AA, Liu M, Simons M, Leenders WP *et al*. Vascular endothelial growth factor and semaphorin induce neuropilin-1 endocytosis via separate pathways. *Circ Res* 2008; 103: e71–e79.

76. Toyofuku T, Zhang H, Kumanogoh A, Takegahara N, Suto F, Kamei J *et al*. Dual roles of Sema6D in cardiac morphogenesis through region-specific association of its receptor, Plexin-A1, with off-track and vascular endothelial growth factor receptor type 2. *Genes Dev* 2004; 18: 435–447.

77. Bellon A, Luchino J, Haigh K, Rougon G, Haigh J, Chauvet S *et al*. VEGFR2 (KDR/Flk1) signaling mediates axon growth in response to semaphorin 3E in the developing brain. *Neuron* 2010; 66: 205–219.

78. Kerbel RS. Tumor angiogenesis. *N Engl J Med* 2008; 358: 2039–2049.

79. Catalano A, Lazzarini R, Di NS, Orciari S, Procopio A. The plexin-A1 receptor activates vascular endothelial growth factor-receptor 2 and nuclear factor-kappaB to mediate survival and anchorage-independent growth of malignant mesothelioma cells. *Cancer Res* 2009; 69: 1485–1493.

80. Strizzi L, Catalano A, Vianale G, Orecchia S, Casalini A, Tassi G *et al*. Vascular endothelial growth factor is an autocrine growth factor in human malignant mesothelioma. *J Pathol* 2001; 193: 468–475.

81. Suto F, Ito K, Uemura M, Shimizu M, Shinkawa Y, Sanbo M *et al*. Plexin-a4 mediates axon-repulsive activities of both secreted and transmembrane semaphorins and plays roles in nerve fiber guidance. *J Neurosci* 2005; 25: 3628–3637.

82. Kigel B, Rabinowicz N, Varshavsky A, Kessler O, Neufeld G. Plexin-A4 promotes tumor progression and tumor angiogenesis by enhancement of VEGF and bFGF signaling. *Blood* 2011; 118: 4285–4296.

83. Dhanabal M, Wu F, Alvarez E, McQueeney KD, Jeffers M, MacDougall J *et al*. Recombinant semaphorin 6A-1 ectodomain inhibits *in vivo* growth factor and tumor cell line-induced angiogenesis. *Cancer Biol Ther* 2005; 4: 659–668.

84. Soker S, Takashima S, Miao HQ, Neufeld G, Klagsbrun M. Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. *Cell* 1998; 92: 735–745.

85. Fuh G, Garcia KC, de Vos AM. The interaction of neuropilin-1 with vascular endothelial growth factor and its receptor flt-1. *J Biol Chem* 2000; 275: 26690–26695.

86. Soker S, Miao HQ, Nomi M, Takashima S, Klagsbrun M. VEGF165 mediates formation of complexes containing VEGFR-2 and neuropilin-1 that enhance VEGF165-receptor binding. *J Cell Biochem* 2002; 85: 357–368.

87. Gluzman-Poltorak Z, Cohen T, Herzog Y, Neufeld G. Neuropilin-2 is a receptor for the vascular endothelial growth factor (VEGF) forms VEGF-145 and VEGF-165 [corrected]. *J Biol Chem* 2000; 275: 18040–18045.

88. Park JE, Keller GA, Ferrara N. The vascular endothelial growth factor (VEGF) isoforms: differential deposition into the subepithelial extracellular matrix and bioactivity of extracellular matrix-bound VEGF. *Mol Biol Cell* 1993; 4: 1317–1326.

89. Makinen T, Olofsson B, Karpanen T, Hellman U, Soker S, Klagsbrun M *et al*. Differential binding of vascular endothelial growth factor B splice and proteolytic isoforms to neuropilin-1. *J Biol Chem* 1999; 274: 21217–21222.

90. Migdal M, Huppertz B, Tessler S, Comforti A, Shibuya M, Reich R *et al*. Neuropilin-1 is a placenta growth factor-2 receptor. *J Biol Chem* 1998; 273: 22272–22278.

91. Karpanen T, Heckman CA, Keskitalo S, Jeltsch M, Ollila H, Neufeld G *et al*. Functional interaction of VEGF-C and VEGF-D with neuropilin receptors.*FASEB J* 2006; 20: 1462–1472

92. Whitaker GB, Limberg BJ, Rosenbaum JS. Vascular endothelial growth factor receptor-2 and neuropilin-1 form a receptor complex that is responsible for the differential signaling potency of VEGF(165) and VEGF(121). *J Biol Chem*2001; 276: 25520–25531.

93. Prahst C, Heroult M, Lanahan AA, Uziel N, Kessler O, Shraga-Heled N *et al*. Neuropilin-1-VEGFR-2 complexing requires the PDZ-binding domain of neuropilin-1. *J Biol Chem* 2008; 283: 25110–25114.

94. Cai H, Reed RR. Cloning and characterization of neuropilin-1-interacting protein: a PSD-95/Dlg/ZO-1 domain-containing protein that interacts with the cytoplasmic domain of neuropilin-1. *J Neurosci* 1999; 19: 6519–6527.

95. Horowitz A, Seerapu HR. Regulation of VEGF signaling by membrane traffic.*Cell Signal* 2012; 24: 1810–1820.

96. Pan Q, Chanthery Y, Liang WC, Stawicki S, Mak J, Rathore N *et al*. Blocking neuropilin-1 function has an additive effect with anti-VEGF to inhibit tumor growth. *Cancer Cell* 2007; 11: 53–67.

97. Bachelder RE, Crago A, Chung J, Wendt MA, Shaw LM, Robinson G *et al*. Vascular endothelial growth factor is an autocrine survival factor for neuropilin-expressing breast carcinoma cells. *Cancer Res* 2001; 61: 5736–5740

98. Bachelder RE, Lipscomb EA, Lin X, Wendt MA, Chadborn NH, Eickholt BJ *et al*. Competing autocrine pathways involving alternative neuropilin-1 ligands regulate chemotaxis of carcinoma cells. *Cancer Res* 2003; 63: 5230–5233.

99. Beck B, Driessens G, Goossens S, Youssef KK, Kuchnio A, Caauwe A *et al*. A vascular niche and a VEGF-Nrp1 loop regulate the initiation and stemness of skin tumours. *Nature* 2011; 478: 399–403.

100. Carmeliet P, Moons L, Luttun A, Vincenti V, Compernolle V, De MM et al. Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. Nat Med 2001; 7: 575–583.

101. Slongo ML, Molena B, Brunati AM, Frasson M, Gardiman M, Carli M et al. Functional VEGF and VEGF receptors are expressed in human medulloblastomas. Neuro Oncol 2007; 9: 384–392.

102. Lassen U, Nielsen DL, Sorensen M, Winstedt L, Niskanen T, Stenberg Y et al. A phase I, dose-escalation study of TB-403, a monoclonal antibody directed against PlGF, in patients with advanced solid tumours. Br J Cancer 2012;106: 678–684.

103. Martinsson-Niskanen T, Riisbro R, Larsson L, Winstedt L, Stenberg Y, Pakola S et al. Monoclonal antibody TB-403: a first-in-human, Phase I, double-blind, dose escalation study directed against placental growth factor in healthy male subjects. Clin Ther 2011; 33: 1142–1149.

104. Snuderl M, Batista A, Kirkpatrick ND, Ruiz de AC, Riedemann L, Walsh EC et al. Targeting placental growth factor/neuropilin 1 pathway inhibits growth and spread of medulloblastoma. Cell 2013; 152: 1065–1076.

105. Yao J, Wu X, Zhuang G, Kasman IM, Vogt T, Phan V et al. Expression of a functional VEGFR-1 in tumor cells is a major determinant of anti-PlGF antibodies efficacy. Proc Natl Acad Sci USA 2011; 108: 11590–11595.

106. Bais C, Wu X, Yao J, Yang S, Crawford Y, McCutcheon K et al. PlGF blockade does not inhibit angiogenesis during primary tumor growth. Cell 2010; 141: 166–177.

107. Lichtenberger BM, Tan PK, Niederleithner H, Ferrara N, Petzelbauer P, Sibilia M. Autocrine VEGF signaling synergizes with EGFR in tumor cells to promote epithelial cancer development. Cell 2010; 140: 268–279.

108. Rossiter H, Barresi C, Pammer J, Rendl M, Haigh J, Wagner EF et al. Loss of vascular endothelial growth factor a activity in murine epidermal keratinocytes delays wound healing and inhibits tumor formation. Cancer Res 2004; 64: 3508–3516.

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