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Review

Divergent roles of Plexin D1 in cancer

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ABSTRACT

Plexin D1 belongs to a family of transmembrane proteins called plexins. It was characterized as a receptor for semaphorins and is known to be essential for axonal guidance and vascular patterning. Mutations in Plexin D1 have been implicated in pathologic conditions such as truncus arteriosus and Möbius syndrome. Emerging data show that expression of Plexin D1 is deregulated in several cancers; it can support tumor development by aiding in tumor metastasis and EMT; and conversely, it can act as a dependence receptor and stimulate cell death in the absence of its canonical ligand, semaphorin 3E. The role of Plexin D1 in tumor development and progression is thereby garnering research interest for its potential as a biomarker and as a therapeutic target. In this review, we describe its discovery, structure, mutations, role(s) in cancer, and therapeutic potential.

1. Discovery

Takagi and colleagues [1] discovered plexins in 1987 in the *Xenopus laevis* tadpole model. The authors were screening molecules that help to regulate neuronal interactions between the retinal axons and the optic tectum by using monoclonal antibodies (MAb). The optic tectum of an amphibian has a laminar structure with 9 defined layers. One MAb, MAb-B2, preferentially bound to the plexiform layers in the deeper part of the optic tectum. Molecular cloning indicated that the antigen for MAb-B2, a peptide with a molecular weight of about 200–220 kDa, was a new type-one membrane glycoprotein [2]. This new calcium-dependent, single-pass membrane protein was renamed *plexin* [3] following its preferential binding of retinal plexiform layers [4]. In 1996, Maestrini and colleagues [5] identified human Plexins. The authors were working on identifying unknown genes on the human X chromosome and found a cDNA in Xq28 that encoded a transmembrane membrane protein. The authors named this as SEX and reported that it shared structural similarities with the extracellular domain of the MET/HGF receptor. Additional screening of cDNA libraries revealed three more closely related sequences, SEP, OCT, and NOV that were located on human chromosomes 3p, 1, and 3q. They reported that this human gene family (SEX, SEP, OCT and NOV) encoded single-pass transmembrane proteins that had a large extracellular domain and a large cytoplasmic domain that was characterized by a conserved sequence, the SEX domain. The authors speculated that these receptors mediated their functions either through an enzymatic action or with the help of intracellular transducers that were yet to be identified, Furthermore, the authors reported that the cytoplasmic domains of these family members shared over 50% amino acid sequence similarity which is a unique structural characteristic [5]. In their subsequent work, the authors began referring to this family as Plexins [6] as their amphibian family counterparts had already been named plexins [3]. In 1999, Tamagnone et al. [6] reported 4 classes of plexins in vertebrates: A to D. They published that the human *Plexin* gene family had a minimum of nine members that were categorized into these four subfamilies. They showed that human Plexins belonging to the subfamilies B and C associated with membrane-bound classes 4 and 7 of semaphorins and postulated that the Plexins belonging to the A and D subfamilies would function as receptors for other subclasses of semaphorins [6].

2. Structure of Plexin D1

Plexins, type-1 transmembrane proteins, are categorized into 4 subfamilies based on their sequence similarity [7]: classes A (A1, A2, A3, and A4), B (B1, B2, and B3), C (C1), and D (D1) [8]. Over the years, the works by several groups have contributed towards unraveling the

Abbreviations: Dll4, Delta-like 4; EMT, epithelial-mesenchymal transition; EndMT, endothelial-mesenchymal transition; GAP, GTPase-activating protein; IPT, immunoglobulin-plexin-transcription; MAb, monoclonal antibody, monoclonal antibodies; MBS, Möbius syndrome; PSI, plexin, semaphorin, and integrin; Sema3E, semaphorin 3E; VEGF, vascular endothelial growth factor

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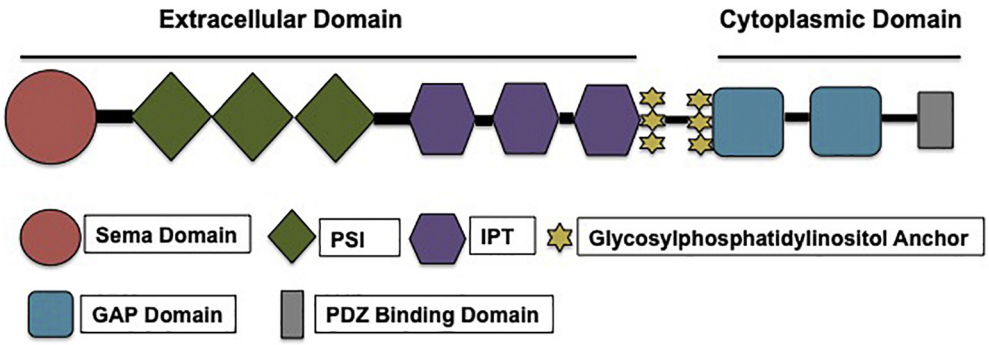

Fig. 1. Structure of Plexin D1.
The mature protein consists of around 1879 amino acids and has a molecular mass of about 208 kDa.

structure of Plexins. Kong and colleagues [7] presented the crystal structures of Plexin class A, Bell and group published crystal structures of Plexin B1 [9]; Wang and group [10] solved the Rho GTPase Binding Domains (RBD) of Plexins C1 and D1; Shang and group presented the structure of the intracellular domain of Plexin D1 [11]. Hota and Buck have extensively reviewed the structures of plexins in their review article [12].

Among the 4 classes of plexins, Plexin D1 is considered the most structurally diverse. Plexin D1 and Plexin C1 have some features in their extracellular domains that are different from the other plexin family members [6]. The structure of Plexin D1 is shown in Fig. 1. Mature Plexin D1 protein comprises of about 1879 amino acids, and has a molecular mass of about 208 kDa [13].

Plexins are composed of an extracellular segment that typically has 10 domains; 1 membrane-spanning region, which is thought to be helical and conserved; and a cytoplasmic domain that has a GTPase-activating protein (GAP) domain and a Rho GTPase-binding domain insert [7]. Plexins have an N-terminal Sema domain that is trailed by a cysteine-rich plexin, semaphorin, and integrin (PSI) domain and an immunoglobulin-plexin-transcription (IPT) domain [8]. The sema domain's β-propeller structure has 7 blades and 2 extensive inserts; the latter is also known as an “extrusion region” [12]. The difference between the sema domain of plexins and semaphorins is that the sema domains of plexins do not dimerize [14]. A study in the mouse model reported that deletion of the sema domain of plexin A1 rendered it constitutively active suggesting that it is likely involved in regulating its activity [15]. We think that extending similar studies to Plexin D1 would be worthwhile, as it would potentially give us insight into if and how various domains contribute towards the regulation of its activity.

The PSI domains are composed of about 50 amino acid residues and generally have 8 cysteine residues [12]. The structure of Plexin D1 differs from other plexins in having only 6 of the conserved cysteine residues instead of 8 [16]. PSI domains can be positioned between the Sema and IPT domains or between IPT domains. These positioning aid in creating a segment between the comparatively rigid structures that helps appropriately orient the ligand and receptor-binding sites [12]. The IPT domains consist of immunoglobulin folds and share homology with several transcription factors. The immunoglobulin folds are relatively firm, and the loops within them and the linkers in between them provide a favorable binding site. The role of the IPT domain is yet to be elucidated [17]. A study in the zebrafish model demonstrated that a missense mutation in one of the IPT domains caused the receptor to become inactive [18]. The PSI and IPT domains have been postulated to be essential for receptor activation [12]. Several hypotheses have been advanced regarding an understanding of how plexins are activated: dimerization and GTPase binding, allosteric changes, and association with other proteins. However, no definitive conclusions have been reached [12]. Another hypothesis is that binding of semaphorins causes plexin monomers to form active dimers [19].

The cytoplasmic domains of Plexins are highly conserved and can interact with signaling molecules such as p21-activated kinase, Rho family GTPases, and others [8]. The intracellular domains share homology with Ras-GAP, and their GTPase-activating ability has been confirmed by several groups [12]. An unusual feature is the presence of an approximately 200-residue segment in the GAP homologous region. This segment was named the Rho GTPase binding domain after it was found to bind many Rho GTPases in Plexin families A and B. Binding of small Rho GTPase to this domain is thought to cause dissociation of the 2 GAP homologous regions and thereby activate the receptor [12]. The Rho GTPase-binding domain structure is similar to that of a ubiquitin fold [12]. Ubiquitin folds can help bind small GTPases belonging to the Ras family in a particular region of the structure [20]. With the help of x-ray crystallography and solution NMR spectroscopy techniques, Tong and colleagues [21] proposed a model where the Rho GTPase-binding domain participated in the receptor activation. The authors suggested that in its inactive, resting form the intracellular domain of plexins is dimerized via the Rho GTPase-binding domain. The binding of a GTPase disrupts the dimer and induces conformational changes. Furthermore, they observed that the ubiquitin-like tertiary fold played a role in bringing together two sequence motifs involved in Rho GTPases binding [21].

Wang and colleagues [22] demonstrated that plexins have GAP activity for Rap. All Plexins exhibit GTPase activating protein (GAP) activity towards R-Ras GTPases [23,24]. Many GAPs can stimulate guanosine triphosphate hydrolysis in both Ras and Rap GTPases, and their activities against these 2 substrates could be regulated in different ways. Plexins are thought to be a part of the family of dual-specificity GTPases [12]. The GAP domain of plexins has an unusual structure: it is divided into two segments by the Rho GTPase-binding domain (RBD) and shares little similarity with other Ras-GAPS in sequence [25].

The exact mechanism of activation of GAP activity is not yet known. However, some groups have put forth some potential mechanisms based on their respective findings. Wang and group [22] reported that plexins utilize an unconventional method for the GTP hydrolysis of Rap: in a normal state, Rap-GAP activity of plexins is normally autoinhibited and dimerization of plexins can stimulate Rap-GAP activity. Some reports suggest the involvement of Rnd subfamily in the R-Ras GAP activity of plexins. Plexins A1 and B1 need the protein Rnd1 to bind to their cytoplasmic domains for their R-Ras GAP activity [26,27]; Plexin D1 needs Rnd2 protein for its R-Ras GAP activity, and Plexin C1 does not need any of the Rnd proteins [23].

Additionally, there is a possibility that Plexin D1 sequesters R-Ras through its Ras-GAP domain. This suggested mechanism would cause a localized and targeted inhibition of R-Ras activity instead of an overall reduction in R-Ras [28]. Two studies reported that concurrent binding of a semaphorin on the extracellular domain and a RhoGTPase on the intracellular domain is required for the GAP activity of plexins [29,30]. Bell and group [9] proposed a potential model for this. They suggest that binding of the semaphorin results in clustering of receptors on the extracellular domain and the resulting rearrangement would be

transferred to the intracellular N-terminal helix. This, in turn, would free up another binding site facilitating the bridging of 2-plexin molecules by RhoGTPase. These rearrangements would bring the catalytic machinery for the Ras-GAP activity in the appropriate position [9]. Liu and colleagues [31] also suggested a structural basis for the activation of GAP activity. They proposed that ligands that are in a constitutively dimeric form like A39R and Sema 7A bring inactive Plexin C1 into a dimerized state in which the orientation allows the activation of the GAP activity. Another study proposed that plexin activation could be regulated at multiple levels. They characterized a new protein segment in Plexin B1 called the coupling loop. Their data suggests this new segment could influence the interactions between RBD and GAP domains; hinder RBD dimerization and potentially impede the dimerization of the whole intracellular region of the plexins in synergy with Rho GTPase binding [32]. A study by He and colleagues [25] demonstrated using crystal structure for the intracellular portion of Plexin A3 that the C and N terminal regions of the GAP homologous regions form a GAP region that is similar to other Ras GAPs. Nevertheless, the GAP domain assumes a closed conformation and does not bind R-Ras and M-Ras in its substrate-binding site. This suggests that structurally plexins are in an autoinhibited state. Furthermore, the authors report that comparison with Plexin B1 RBD/Rnd1 complex indicates that binding of Rnd1 is not sufficient to induce a conformational change in the plexin. The authors support an allosteric activation mechanism that requires both semaphorin and a Rho GTPase and appropriate binding between the N-terminal portion, RBD and GAP domains in the intracellular region for the activation of GAP domain [25].

The C terminal of Plexin D1 has a type I PDZ-domain-binding motif, serine-glutamate-alanine [17], that facilitates its binding to proteins such as GIPC [33]. Burk and group [33] published that the interaction between Plexin D1 and GIPC enhances the colocalization of Plexin D1 with vesicular pools of active R-Ras, resulting in its inactivation. They further show that in the absence of the interaction between Plexin D1 and GIPC, there is missorting of Plexin D1 that causes loss of signaling. With the help of structural studies, Shang and colleagues [11] recently reported that, in the mouse model, plexin D1 binding released Gipc of its autoinhibitory state and promoted its interaction with myosin V. The authors postulate that this binding likely impacts plexin D1 endocytosis and the signaling it mediates. Additionally, their crystal structures form a relatively strong background for future studies aimed at investigating how these interactions are regulated and how disruption of these interactions might contribute to a disease state(s) [11]. While these studies provide some insight into the importance of Plexin D1 and GIPC interactions, further studies are required to understand the functional implications of these interactions fully.

Understanding the structure of Plexin D1 is essential for gaining insight into its different domains and their roles and its binding partners, as well as for understanding how binding occurs and how these interactions are coordinated and regulated. This knowledge would help reveal how Plexin D1 stimulates various signaling pathways and could ultimately lead to new therapeutic targets. For example, Janssen and colleagues [34] showed with the help of crystal structures the shared generic architecture between semaphorin-plexin complexes (human Plexin $B1_{1-2}$–Sema $4D_{ecto}$ and murine Plexin $A2_{1-4}$–Sema $6A_{ecto}$) and using biophysical and cellular assays proposed that a common mode of interaction stimulates all semaphorin-plexin complexes and the unique insertions between or within the sema domains regulate the binding specificity [34]. It would be exciting and valuable to extend such studies to Plexin D1 to understand its signaling better.

3. Mutations of Plexin D1

Mutations in human Plexin D1 have been associated with various pathological conditions. For example, Shma and colleagues [35] reported a homozygous mutation in the *Plexin d1* gene that affected a highly conserved residue. The resulting protein had an Arg 1299Cys change and is thought to have an unstable intracellular region that in turn impairs its anchoring and catalytic activity. This mutation is implicated in truncus arteriosus [35]. Roca and colleagues [36] reported that de novo mutations in the gene for *Plexin d1* were a cause for Möbius syndrome (MBS). Many MBS patients show various developmental abnormalities that are observed in *Plexin d1* mutant models; for example, in this study, two patients showed craniofacial bone abnormalities. Craniofacial bone abnormalities have been reported in other MBS patients as well. The authors described two de novo mutations in Plexin D1: one affected an arginine residue in the GTPase activating domain of the protein while another one affected a leucine residue in the IPT domain. Both of these mutations likely result in the loss of function [36].

4. Plexin D1 in cancer

The expression of Plexins is often dysregulated in cancers [37]. Plexin D1 expression is generally low in adult tissues [38] and is thought to be limited to a subset of activated fibroblasts and macrophages [39]. However, in several types of cancer, Plexin D1 is overexpressed in both tumor cells and their vasculature, including pancreatic [40], melanoma [39], ovarian [41], and colon cancers [42]. Thus, it is being considered as a marker for tumor vasculature [43] and is gaining prominence in cancer research [12]. Furthermore, to our knowledge, it is the only protein that is expressed aberrantly in both tumor vessels and tumor cells [43]. The canonical ligand of Plexin D1 is Sema 3E [17]. Overexpression of Sema 3E in pancreatic cancer has been associated with poor survival [44]. Plexin D1 can act as both a tumor promoter and a tumor suppressor as shown in Fig. 2 and discussed in the subsections below (Fig. 3).

4.1. Plexin D1 as a tumor promoter

Plexin D1 has been shown to promote tumor growth in a few studies [41,42,38]. Casazza and colleagues [42] reported that in colon cancer, p61-Sema 3E/Plexin D1 signaling promoted invasive and metastatic behavior of cancer cells. The authors also reported that in their mice xenograft models mice implanted with cancer cells with Plexin D1 knockdown there was reduced metastasis relative to their respective controls [42]. Tseng et al. [41] published that Sema 3E/Plexin D1 signaling in ovarian endometrioid cancer cells induced EMT through nuclear localization of Snail1 and thereby promoted tumor development by facilitating migration and EMT [41]. In addition to these, it was reported that in prostate cancer, Plexin D1 acted as a

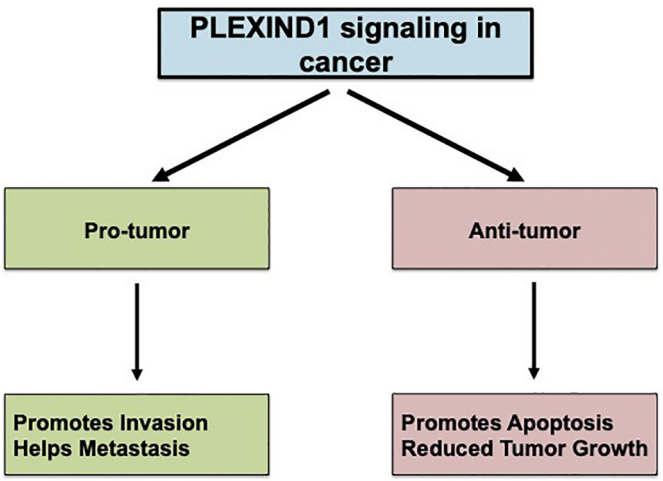

Fig. 2. Role of Plexin D1 mediated signaling in cancer.
Plexin D1 has been reported to mediate both pro- and anti-tumorigenic signaling in different cancer models.

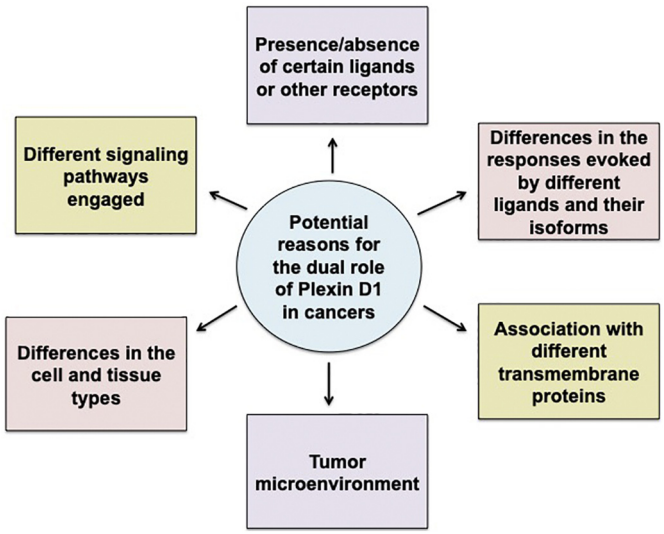

Fig. 3. Potential reasons for the dual role of Plexin D1 in cancers.
This chart lists the various factors that potentially influence the dual role of Plexin D1 in cancers. We think that it could be a possibility that one or maybe a combination of some of these factors determine whether Plexin D1 would function as a tumor suppressor or a tumor promoter.

transcriptional target, which was stimulated by Notch signaling, and helped in cancer cell migration and mediated Slug-dependent down-regulation of E-cadherin. The authors also reported that TCGA dataset mining showed a positive Spearman correlation coefficient between Plexin D1 and Notch1 levels in prostate cancer, colon and rectum adenocarcinoma, thyroid carcinoma and kidney renal clear cell carcinoma [38].

A recent study in mouse pancreatic microvascular endothelial cells reported that the microRNA-27b acts as a positive mediator of TGFβ mediated endothelial-mesenchymal transition (EndMT) and regulates the expression of plexin D1 [45] It would be interesting to extend this study to cancer cells and investigate if and how the regulation of Plexin D1 contributes to EndMT and thereby cancer.

Another hint of Plexin D1 being involved in tumor angiogenesis and metastasis is that the intracellular domain of Plexin D1 has consensus motifs for RAC/RhoA signaling [13], suggesting its involvement in cell motility and cytoskeletal rearrangements [39]. However, the biological relevance of these consensus motifs needs to be substantiated with further research.

4.2. Plexin D1 as a tumor suppressor

Some studies have shown that Plexin D1 signaling can be antitumor as well. It was published that in a breast cancer mouse model, Plexin D1 promotes apoptosis through association with the orphan nuclear receptor NR4A1 in the absence of Sema 3E [37]. In this case, Plexin D1 acts as 'dependence receptor'. To date, it is the only Plexin family member that has been characterized as a dependence receptor [37]. Dependence receptors are known to mediate negative signaling. They trigger apoptosis when the ligand/stimuli are absent, but inhibit cell death in the presence of the respective stimulus [46]. In the breast cancer model, the authors show that in the absence of Sema 3E, Plexin D1 interacts with NR4A1 that causes the release of cytochrome *c* which in turn activates caspase 9, all of which subsequently results in mitochondria-mediated apoptosis. However, in the presence of the ligand, the association of Plexin D1 and NR4A1 is disturbed, perhaps due to the changes in the structure of the receptor, oligomeric state or due to the simultaneous binding of other molecules such as Rho GTPase Rnd2 or a combination of these causes [37]. This also suggests that the cancer cells need Sema 3E/Plexin D1 mediated signaling for their survival in this scenario.

4.3. Plexin D1 and angiogenesis

Several studies have reported that Plexin D1 is involved in developmental angiogenesis. Gu and colleagues [47] reported that in mouse model sema 3E-plexin D1 regulated the positioning of endothelial cells and patterning of the developing vasculature. Fukushima and group [48] demonstrated that sema 3E had antiangiogenic action on extra-retinal vessels that expressed plexin D1. The authors showed that binding of sema 3E to plexin D1 stimulated signaling that normalized angiogenic directionality in developing retinas and ischemic retinopathy. Their data suggests that in developing mouse retina model, astrocyte-derived VEGF generally enhances the expression of plexin D1 in growing blood vessels. Furthermore, increased plexin D1 expression in the extraretinal vessels inhibited disoriented projections of the endothelial filopodia induced by VEGF. Based on their findings, the authors of this study postulate that the effect of sema 3E therapy likely depends on the expression of plexin D1 on endothelial cells in various pathological settings. They suggest that Sema 3E therapy could help guide angiogenesis precisely to ischemic tissues provided that expression of Plexin D1 is limited to disoriented vessels. However, in disease settings where the expression of Plexin D1 in the abnormal vessels is broad, Sema 3E administration could potentially be an antiangiogenic therapy [48].

Kim and colleagues [49] showed that in the mouse retina model system, plexin D1 was specifically expressed by the endothelial cells present at the front of actively sprouting blood vessels and that VEGF secreted by the surrounding tissues directly controlled its expression. This, in turn, resulted in the regulation of Sema 3E-Plexin D1 signaling by VEGF through its influence over plexin D1 expression. The authors found that Sema 3E- Plexin D1 signaling negatively regulated the activity of the VEGF-induced Delta-like 4 (Dll4)-Notch signaling pathway. Thus, their data suggests that Sema 3E-Plexin D1 participate in angiogenesis through a feedback mechanism regulated by VEGF [49].

Zygmunt et al. [50] demonstrated in the zebrafish model that Sema-Plexin D1 signaling controls two distinct aspects of angiogenesis: sprout guidance and the spatial distribution of angiogenic ability within a primary vessel. The authors reported that Sema- Plexin D1 signaling confined angiogenic ability along the aorta and restricted angiogenic responses in segmental arteries. They found that this signaling antagonizes the pro-angiogenic VEGF signaling by ensuring an appropriate endothelial abundance of soluble flt1 (sflt1). sflt1 is an alternatively spliced form of the VEGF receptor Flt1 that functions as a high-affinity VEGF decoy [50].

Supporting anti-angiogenic signaling by Sema 3E- Plexin D1, Sakurai and colleagues [28] published that Sema 3E could act as an inhibitor of adult and tumor-induced angiogenesis. They reported that in mouse model Sema 3E mediated activation of Plexin D1 led to the disassembly of integrin mediated adhesive structures, which in turn inhibited the adhesion of endothelial cells to the extracellular matrix leading to the retraction of filopodia in endothelial tip cells. Furthermore, the study showed that this anti-angiogenic signaling involved inactivation of R-Ras and stimulation of Arf6. The authors utilized mouse model and administered recombinant Sema 3E via intraocular injections [28].

These findings suggest that there is a probability that Plexin D1 could be involved in tumor angiogenesis. It is known that during the early phases of development, Plexin D1 is present in the vasculature; however, adult vasculature lacks its expression [43]. Based on its role in developmental angiogenesis, and its aberrant expression in tumor vasculature, it is being investigated whether Plexin D1 promotes tumor angiogenesis [43]. We think that is a fascinating area of study and further studies, in different cancer models and with various ligands/isoforms, are required to fully explore the role(s) of Plexin D1 in tumor angiogenesis.

Table 1
Divergent roles of Plexin D1 mediated signaling in cancers.

Cancer type	Stimulant	Mechanism	Reference
Tumor promoter			
Prostate Cancer	Notch signaling	Facilitated cancer cell migration and mediated Slug-dependent down regulation of E-cadherin	38
Ovarian endometrioid cancer	Sema 3E	Induced EMT through nuclear localization of Snail1	41
Colon cancer	p61-Sema 3E	Increased metastasis and invasive characterestic	42
Tumor suppressor			
Breast Cancer	Absence of Sema 3E	Interacts with NR4A1; Mitochondria-mediated apoptosis	37

This table summarizes the currently known mechanisms engaged by Plexin D1 to mediate its functions in different cancers.

5. Potential causes underlying this dual role Plexin D1 in cancer

Several factors have been attributed to this differential function of Plexin D1 in cancers wherein it can both promote and inhibit cancer development and progression. One of them is the difference in the signaling mechanisms engaged (Table 1). For instance, Casazza et al. [42] reported that in endothelial cells, paracrine Sema 3E- Plexin D1 signaling caused cell repulsion leading to decreased blood vessel density and tumor growth whereas autocrine signaling in tumor cells expressing Sema 3E, transactivation of Plexin D1 – associated ErbB2/Neu oncogenic kinase promoted invasion and metastasis.

Another cause for this opposing role could be the differences in the signaling responses evoked by different ligands and their various isoforms. For instance, the proteolytic fragment p61 of secreted Sema 3E facilitates cell invasiveness and metastatic spreading while an uncleavable variant of Sema 3E does not promote metastatic spreading, but rather hinders it by competing with endogenous p61-Sema 3E. In this study, the authors utilized a Sema 3E isoform that had a point mutation and was resistant to cleavage mediated by furin [51].

Besides these, a reason for this differential role could be the differences in cell and tissue types. Plexin D1 can evoke different responses by activating divergent signaling pathways even when stimulated by the same ligand in cancer cells versus endothelial cells [42]. For example, in cancer cells, Plexin D1/Sema 3E signaling promoted cell invasiveness and metastasis via transactivation of ErbB2/Neu oncogenic kinase. However, in endothelial cells, it has a cell repelling activity [42]. This suggests the involvement of either the tumor microenvironment and/or some other factor(s) in deciding the outcome of the signaling cascade. Plexins and semaphorins are known to promote metastasis through autocrine signaling loops and through regulation of communication between the various cell types present within the tumor [14]. This is one way in which tumor microenvironment is potentially contributing to the signaling outcome.

Furthermore, the association of Sema 3E- Plexin D1 with different transmembrane proteins can evoke different responses. Plexins engage tissue and cell lineage-specific co-receptors and cytoplasmic protein kinases that might be contributing to the diverse functions and outcomes [8]. For instance, in mouse corticofugal and striatonigral neurons expressing plexin D1 but lacking mNRP1, sema 3E acts as a repellent; however, in subiculo-mammillary neurons, where both plexin D1 and Nrp1 are expressed, Sema 3E signals changes to attraction and/or stimulation of axonal growth [16,52].

NRP1 is a co-receptor for Plexin D1 [33]. Plexins and NRP1 could influence the function of each other [12]. For example, Chauvet and colleagues [52] demonstrated that association of Nrp1 with plexin D1 converted axonal repulsion by plexin D1 to attraction during brain development. There is a possibility that the role of Plexin D1 in cancer is also influenced by the presence or absence of its co-receptors. However, this idea needs in-depth studies to be validated. There are very few publications about the role of Plexin D1 in cancer and so far none of the reports, to our knowledge, have discussed the involvement of NRP1 in the Plexin D1 mediated tumor development or tumor suppression. We think that studies elucidating the role of NRP1 in Plexin D1 mediated tumor growth or inhibition would be critical for fully understanding how and why Plexin D1 functions as a tumor promoter in certain cancers and as a tumor inhibitor in others.

6. Therapeutic importance of Plexin D1

6.1. Plexin D1 as a biomarker

Roodink and colleagues [39], using immunohistochemistry analysis, demonstrated that the expression of Plexin D1 is upregulated in several clinical solid tumors, particularly in tumor vasculature and malignant cells in addition to fibroblasts and macrophages. However, in the corresponding normal tissues, Plexin D1 expression is limited to fibroblasts and macrophages that have likely been activated [39]. In another study by the same group, the authors reported the expression of Plexin D1 in the tumor vasculature and on tumor cells in human brain tumors [43]. Furthermore, with the help of in situ hybridization assays, the authors demonstrated that in a mouse model of brain metastasis the expression of *Plexin D1* is restored during tumor angiogenesis [43]. These findings suggest that Plexin D1 could potentially serve as a marker for tumor vessels [39,43] and tumor vasculature [43]. Shalaby et al. [53] have proposed that Plexin D1 could be a biomarker for cervical cancer. Taken together, these findings suggest that Plexin D1 might be a clinically relevant marker tumor marker.

Most tumors require angiogenesis mediated by neovasculature to grow over 2 to 3 mm^3 [43], which has generated substantial interest in antiangiogenic therapies. Most studies that have focused on inhibiting angiogenesis were directed at blocking the VEGF-A pathway [39,54,55]. Although several therapeutic studies in animal tumor models have had positive results, these therapies did not have curative effects in clinical studies [39,56,57]. Various reasons have been attributed for these results, including heterogeneity of the tumor vasculature. Typically, the patients who are candidates for these therapies have tumors in which all maturation stages are represented, thereby rendering only a small portion of the tumor susceptible to angiogenesis inhibition [39]. Besides, in organs such as the brain and liver, where the vascular density is intrinsically high, tumor growth and metastasis can be facilitated by preexisting vessels [58–60]. Thus, these tumors are relatively less susceptible to antiangiogenic therapies. Furthermore, antiangiogenic therapies could promote the tumor microenvironment to adapt or continue tumor growth, or both, by co-option of an existing capillary bed [61–63]. Therefore, we think that markers that specifically differentiate tumor vasculature from normal vasculature must be found for effective vascular targeting.

Plexin D1 is a promising marker for tumor vessels and cells for some cancers because it is highly expressed in tumor cells [39] but not in adult vasculature [43]. However, further in-depth investigations are needed to fully validate this possibility because in certain cancers, such as breast carcinomas and vulvar squamous cell carcinoma, its expression is very low or absent [39]. It would be essential to extend the analysis of Plexin D1 expression to other cancer types as well as to stages and grades to reach definitive conclusions.

A thought that needs to be substantiated by research is that Plexin

D1 could be a marker of the reactive oxygen-driven tumor and high levels of reactive oxygen species (ROS). A rationale for this idea: Hemangioma is a benign vascular tumor and arises from multipotent stem cells [64]. The hemangioma stem cells are the source for all cells including endothelial cells and the adipocytes involved in hemangioma involution [64]. Huang and colleagues [65] published that GLUT1-positive endothelial cells in infantile hemangioma behaved like a facultative stem cell population and the expression of Plexin D1 is elevated in them. NADPH oxidases (Nox) are a family of NADPH-dependent enzymes that generate reactive oxygen species and consist of 7 members in humans [66]. NADPH oxidase 4 (Nox4), an H_2O_2 producing oxidase [67], is elevated in hemangioma [66]. It has also been reported that topical eosin (a potent Nox inhibitor) application resulted in the regression of ulcerated hemangiomas of infancy [68].

A recent review article suggested viewing cancer as a duality to discover and/or determine the most optimal treatment as the enormous mutational burdens present in the tumors make it impossible to sequence and target all the mutations present in a patients' tumor sample [69]. We think that in future if Plexin D1 is validated as a biomarker for either tumor vasculature or reactive oxygen-driven tumor, then its expression in the tumors could be helpful to determine the best treatment option for the patient. For example, if Plexin D1 is established as a biomarker for reactive oxygen-driven tumor, then certain treatments such as radiation therapy and certain cytotoxic chemotherapies that are known to generate reactive oxygen could be ruled out for patients whose tumors have high Plexin D1 expression.

6.2. Plexin D1 as a therapeutic target

The findings of some studies suggest that potentially targeting Plexin D1 or the signaling it mediates may have substantial therapeutic implications. In this section, we are describing some of the possible therapeutic benefits and risks involved in targeting Plexin D1 or the signaling it engages in pathological conditions.

6.2.1. Potential benefits of therapeutic targeting of Plexin D1

Targeting Plexin D1 has been shown to allow for concurrent targeting of two different tumor compartments—tumor cells and tumor vessels [43]. After intravenous injections in mice, phages that had single domain antibodies against Plexin D1 on their surface were specifically observed in cerebral melanoma metastases and were associated with the tumor vessels. Roodink et al., the authors of this study, observed that the injected single-domain antibodies accumulated in the tumor vasculature and less so, possibly non-specifically, in the tumor interstitium. The authors suggested that administering antibodies against Plexin D1 might be a viable route for the delivery of cytotoxic molecules to tumors because the tumor vessels would probably allow extravasation of the antibodies [43]. However, these hypotheses need to be validated experimentally.

Plexin D1 is involved in developmental angiogenesis as discussed above in Section 4.3. For instance, it negatively regulates angiogenesis when it is expressed in the tips of endothelial cells. In endothelial cells, the GAP domain of Plexin D1 is activated and adhesion mediated by integrins is suppressed [14]. Therefore, a possibility exists that inhibiting Plexin D1 could eventually lead to antiangiogenic effects in cancer as well [43]. It would be beneficial to understand and test if this antiangiogenic signaling mediated by Plexin D1 could be used for therapeutic purposes.

A study in the zebrafish model demonstrated the involvement of Plexin D1 in the regulation of the type V collagen microenvironment in visceral adipose tissue (VAT) and, thereby, body fat distribution and insulin sensitivity. The study showed that Plexin D1 regulated VAT growth and thereby body fat distribution; it was involved the establishment of a hyperplastic growth conducive ECM microenvironment, and that lack of z. plexin D1 protected the zebrafish from high-fat dietary supplement induced insulin resistance and glucose intolerance. Thus, their findings suggest that Plexin D1, besides cancer, could be a target for treating metabolic disease(s) [70].

6.2.2. Potential risks involved in therapeutic targeting of Plexin D1 and possible approaches to circumvent these risks

A few concerns surround the therapeutic targeting of Plexin D1. This membrane-bound receptor has crucial role(s) in axonal guidance, nervous system development, and vascular patterning. Its homozygous deletion in mice results in neonatal death, defects in axial skeletal morphogenesis and structure of the cardiac outflow tract, and deformities in the peripheral vasculature [71]. A few normal adult tissues like heart, testis, and liver express Plexin D1 transcripts at low levels. Because Plexin D1 expression is not restricted to tumors, it might be important to target only cancer tissue therapeutically [14]. Another reason why Plexin D1 targeting needs to be specific to tumor cells is that Plexin D1 signaling seems to be cell-type specific. For example, Wang and colleagues [72] published that depending on the type of neuron, Plexin D1 mediated different synaptogenic outcomes. Thus, in the context of cancer, while hypothetically it would be beneficial to downregulate Plexin D1 in tumor cells, aberrant down-regulation of Plexin D1 in normal cells might negatively impact its normal biological function(s).

Worzfeld and Offermanns [14] suggested two plausible approaches to target Plexin D1 directly in their review article. One method they proposed is administering antibodies against Plexin D1 and the second way is using a soluble form of Plexin D1 that could act as a decoy receptor and ligand trap [14]. However, as described in the paragraph above, targeting of Plexin D1 needs to directed specifically to the tumor cells as otherwise, it may impair the physiological function(s) of Plexin D1 in normal cells and tissues.

A possible approach to evade this problem would be to target the ligands engaged by Plexin D1, and some studies have been focused on the possibility of mitigating Plexin D1 signaling by targeting its canonical ligand, Sema 3E. The results are variable. A few studies have shown that Sema 3E mediates anti-angiogenic signaling. For example, Sakurai and colleagues [28] demonstrated that Sema 3E/Plexin D1 signaling could decrease angiogenesis through a pathway that involves inactivation of R-Ras and stimulation of Arf6. Another study showed that Sema 3E could inhibit tumor development. In this study, the authors used lentivirus system to direct the expression of semaphorins and found that in their glioblastoma model, Sema 3D and Sema 3E exhibited the most substantial anti-tumorigenic effects and mice implanted with U373MG or U87MG glioblastoma cells expressing Sema 3D, and Sema 3E had almost two folds longer survival compared with their controls. Furthermore, the tissues derived from these mice had considerably decreased concentrations of blood vessels indicating that inhibition of angiogenesis was likely the primary cause behind the tumor inhibition [73]. In contrast, Casazza et al. [42] reported that overexpression of Sema 3E resulted in fewer tumor vessels and less tumor growth, but it enhanced the invasiveness of the cancer cells, as well as transendothelial migration and metastasis. Similarly, Luchino et al. [37] showed that Sema 3E facilitated the survival of tumor cells by inhibiting the apoptotic pathway stimulated by Plexin D1. These various observations of the therapeutic potential of Sema 3E/Plexin D1 could be attributed to differences in cell and tumor types, models used, and the way Sema 3E is overexpressed. Furthermore, Sema 3E differs from other class 3 semaphorins because it binds to Plexin D1 and not to neuropilins [73]. This suggests that the co-receptor(s) engaged by Plexin D1 probably also impact the signaling outcome. In addition, Roodink et al. [74] showed that in human melanoma, Plexin D1 contributed to the invasive and metastatic nature of the cancer, but Sema 3E was not the stimulating ligand.

Another probable approach would be to target/inhibit other proteins that are a part of the signaling pathways engaged by Plexin D1 to mediate tumor growth and progression. However, more extensive studies need to be done on the pathways it employs to mediate its role(s) in

tumor growth and progression. This knowledge is vital for understanding how Plexin D1 influences tumor progression and for identifying other key players that could either serve as biomarkers or be therapeutically targeted.

Collectively, all these reports suggest that Plexin D1 seems to hold considerable therapeutic significance. However, further studies are required to determine whether it would serve better as a biomarker or a therapeutic target; and determine whether the type and stage of cancer would have any influence on this.

7. Conclusions

Plexin D1, a transmembrane protein, plays well-established roles in axonal guidance and vascular patterning. Some of the emerging literature suggests that it is involved in several cancers. The function of Plexin D1 in cancer is not yet fully elucidated. Some studies suggest that it mediates pro-tumorigenic signaling while some other studies indicate that it facilitates anti-tumorigenic signaling. Furthermore, it seems to hold therapeutic significance as a biomarker and maybe as a therapeutic target as well. We think that this is a fascinating area of study that warrants further in-depth investigations. However, the role of Plexin D1 in cancer is a relatively new area of research, and several fundamental questions remain unanswered at this point: what influences the function of Plexin D1 in cancer? ligands, co-receptors, tumor microenvironment or all of them; what is the biological significance of aberrant expression of Plexin D1 in tumor cells and tumor vasculature?; does the role of Plexin D1 in a particular cancer depend upon its type and stage?. We think that finding answers to all these questions will give the scientific community an insight into the role of Plexin D1 in cancer(s) and most likely yield new biomarker(s) and ultimately help us find new therapeutic target(s).

Declaration of Competing Interest

Both authors declare no conflicts of interest.

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