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Review

Emerging roles of semaphorin-3E in cancer

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ABSTRACT

Semaphorin-3E (Sema3E) is a secreted glycoprotein that has risen to prominence because of its widespread and often critical role as a signaling molecule in processes as diverse as axon guidance, angiogenesis, immune regulation, and cancer. Sema3E signals through the cell surface receptor plexin-D1, and in a capacity unique among class 3 semaphorins, Sema3E activates its receptor complex without requiring the presence of a neuropilin co-receptor. Sema3E was initially discovered in mice where its two major roles were quickly identified: as a key protein in directing axon growth during development, and as a negative regulator in cancer, where it was found to intensify cancer metastases. During development, Sema3E/ plexin-D1 functions to guide a regulated and directed pattern of vascular, somite, and neuronal growth in the developing embryo. Beyond development, Sema3E has been implicated in several disease states including inflammatory and vascular diseases, but most notably cancer. In cancer, Sema3E has been found to act directly on cancer cells by promoting cancer cell proliferation and metastasis; however, it has also been shown to have anti-angiogenic and tumor suppressor effects on some cancers. The discovery that furin-mediated cleavage of Sema3E could convert the protein into a pro-metastatic factor has now become a focal point of research on the molecule and likely explains the disparate observations of Sema3E's effects in cancer. The evidence is clear that Sema3E plays an important role in cancer, but a deeper understanding of the precise functions of the protein in different contexts and conditions is still required before Sema3E can be used as a therapeutic target or prognostic marker for the disease.

KEYWORDS: Sema3E, plexin-D1, development, cancer, disease, angiogenesis, metastasis, cell migration.

INTRODUCTION

Semaphorin 3E (Sema3E) is a member of the semaphorin family of secreted and membrane-associated proteins which were originally identified in humans as molecules that direct and regulate axonal and neuronal growth [1]. Since then, semaphorins have been found to participate in a diverse variety of developmental and post-developmental processes, including neuronal and vascular growth and patterning, tissue morphogenesis, immune cell regulation, and cancer development, as reviewed by Roth, 2009, and Yazdani & Terman, 2006 [2, 3].

Eight classes of semaphorins have been identified, with classes 3 through 7 found in vertebrates. While the other vertebrate semaphorins are membrane-bound, class 3 semaphorins are secreted proteins, of which seven members have been identified - Sema3A to 3G [4-6]. Sema3E is a class 3 secreted glycoprotein encoded by the

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SEMA3E gene that is found in chromosomal region 7q21.11 of humans, and in chromosomal region 5A1 of mice. The full-length Sema3E protein is 775 amino acids (a-a) long (containing a 25 a-a signal sequence and a 750 a-a mature protein) and has a predicted molecular weight of 89 kDa.

Class 3 semaphorins require binding to a neuropilin co-receptor to activate cell signaling. Neuropilin acts as a bridge that stabilizes the binding of semaphorin dimers to plexin molecules on each side of the dimer, forming a ternary complex that activates intracellular signaling [7]. The exception to this rule is Sema3E, which can bind directly to its receptor, plexin-D1, to activate cell signaling without the addition of a neuropilin co-receptor [8]. Sema3E binds to plexin-D1 in a ligand homodimer-receptor homodimer arrangement forming a complex that activates plexin-D1's intrinsic GTPase-activating protein (GAP) domain located on its cytoplasmic component [9].

1. Characteristics of Sema3E

1.1. Protein structure and function

Sema3E can be divided into four domains: an N-terminal sema domain, a PSI (plexins, semaphorins, and integrins) domain, an Ig-like C2-type domain, and a basic c-terminal domain (Figure 1A). The sema domain of the protein is a seven-blade beta propeller structure that is highly conserved [10]. It has a large size of approximately 500 a-a [11], and is involved in specific binding to neuropilins and plexins [12]. Particularly, a 70 a-a region within the sema domain is responsible for its binding and biological activity [13]. The positively-charged c-terminal domain is found to potentiate the biological activity of the protein [13], and is also necessary for binding to neuropilins [14]. The PSI domain, also known as the Met (hepatocyte growth factor receptor)-related sequence (MRS), is a cysteine-rich conserved 54 a-a motif that bears homology to the N-terminal region of β-integrins [15].

1.2. Cleavage by furin convertases

Many class 3 semaphorins undergo post-translational proteolytic cleavage that is necessary to yield a functional form of the protein. For instance, Semaphorins 3A, 3B and 3C (then identified as mouse SemD, SemA and SemE, respectively) are synthesized as inactive precursors, and only acquire their axon growth-inhibiting or chemorepulsive functions upon proteolytic cleavage by furin-like endoproteases [16].

Like other class 3 semaphorins, Sema3E also goes through proteolytic cleavage. Cleavage of full-length 89 kDa Sema3E by furin and furin-like convertases in the golgi apparatus occurs at the 560 a-a position, yielding a biologically active 61 kDa fragment, and a 25 kDa c-terminal fragment (Figure 1A) [17]. As such, Sema3E can exist in several post-translational forms: a full-length monomeric form of 87 kDa, a homodimeric form of 174 kDa, a 61 kDa furin-cleaved fragment, a 25 kDa c-terminal fragment, a 50 kDa homodimer of the c-terminal fragment, and a 110 kDa heterodimer of the 25 kDa c-terminal fragment complexed with the 87 kDa full-length protein (Figure 1B). The dimers are generated by the presence of disulfide bonds near the C-terminus. Importantly, there is evidence that the p61-Sema3E monomer, which lacks the disulfide linkages, differentially activates the plexin-D1 receptor compared to the full-length Sema3E homodimer; specifically, p61-Sema3E monomer acts as a growth attractant, while the full-length dimer acts as a growth repellent [17].

1.3. Receptors of Sema3E

1.3.1. Plexin-D1

Plexin-D1 is part of the plexin family of single-pass membrane-bound proteins with cysteine-rich extracellular domains, and comprises four subfamilies - A, B, C and D, which consists of ten members in all [18]. Plexin-D1 protein contains ten domains which are common to all plexin member proteins: a sema domain, three Met-related PSI domains, three glycine/proline-rich motifs, a single-pass transmembrane domain, and two highly conserved intracellular domains known as the sex-plexin (SP) domain, or the GTPase-activating protein (GAP) domains [18, 19] (Figure 2). Plexin-D1 is a large protein of 1925 a-a, and has a predicted molecular weight of 212 kDa [19]. Sema3E is the only class 3 semaphorin that can bind directly to, and activate plexin-D1 [8]. Other semaphorins that form complexes with, and signal through plexin-D1

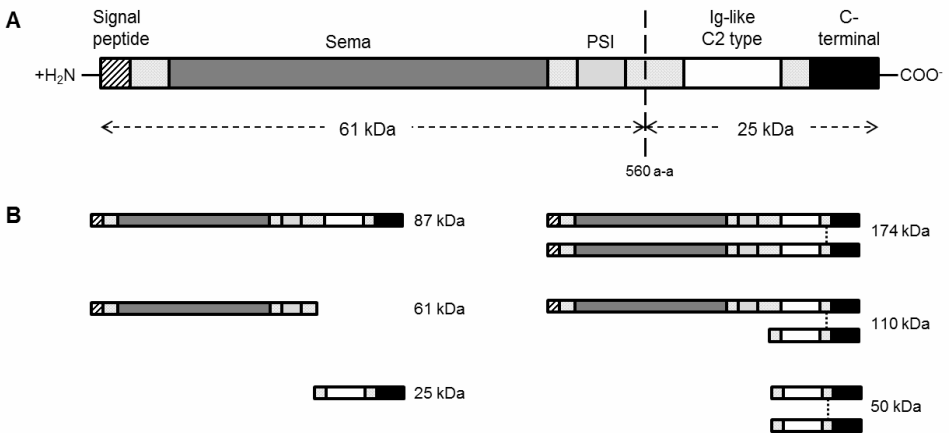

Figure 1. Structure of the Sema3E protein and its post-translational modifications. (A) Schematic diagram showing the domains of Sema3E. The full-length protein (89 kDa) is cleaved at the 560 a-a position by furin and furin-like proteases to yield a biologically active 61 kDa (p61-Sema3E) fragment and a 25 kDa c-terminal fragment. **(B)** The different post-translational modifications generated by furin-mediated cleavage of the full-length protein as well as homo- and heterodimerization, *via* disulfide linkage (dotted line), of the full-length protein or the full-length protein with the 25 kDa c-terminal fragment respectively.

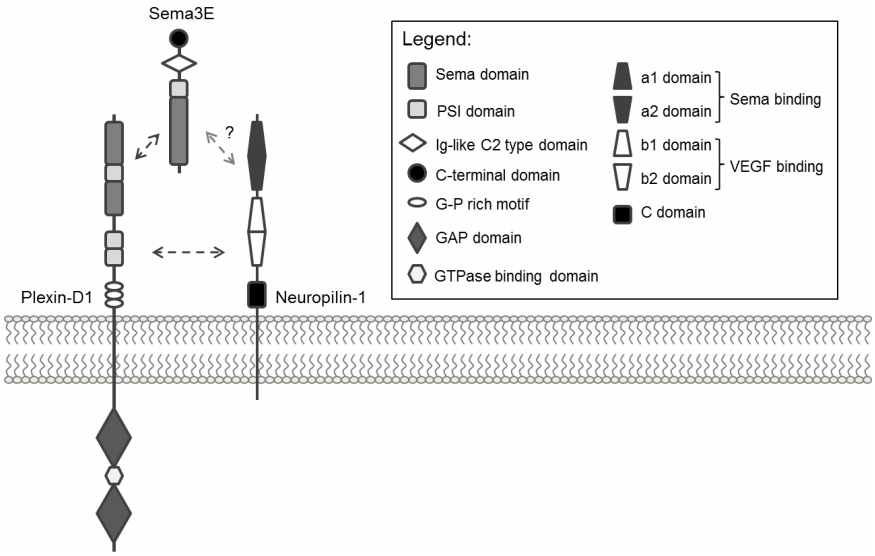

Figure 2. Structure of Sema3E, plexin-D1 and Neuropilin-1. Sema3E is unique amongst class 3 semaphorins in that it can bind directly to a plexin to activate intracellular signaling without the need to bind a co-receptor, such as a neuropilin. Sema3E has been shown to bind to plexin-D1, which can also interact with Neuropilin-1, to activate cell signaling. The direct binding or interaction of Sema3E to Neuropilin-1 is unclear and unknown at this point.

include Sema3A, Sema3C, Sema3D, and Sema4A [20-22]. However, unlike Sema3E, these semaphorins require neuropilin co-receptors, which stabilize their interactions with the plexin- D1 receptor and activate the plexin receptor's downstream signaling pathways.

1.3.2. Neuropilins

There are two members of the neuropilin family of type I transmembrane glycoproteins, neuropilin-1 and neuropilin-2. Each neuropilin interacts with specific class 3 semaphorins; neuropilin-1 specifically interacts with Sema3A, 3B, and 3E; neuropilin-2

interacts with Sema3F and 3G; both neuropilins bind Sema3C at similar affinities [3, 23-26]. Neuropilin-1 is a 130 kDa protein that is 923 a-a long. The neuropilin protein consists of two complement-binding (CUB) or a1/a2 domains, which interact with the sema domain of semaphorins [27], and two coagulation factor-like domains or b1/b2 domains, which interact with PSI and Ig-like domains of semaphorins [23]. The intracellular/cytoplasmic component of the protein is a short 40 a-a long sequence that is not known to have any enzymatic or signaling function [28] (Figure 2).

1.4. Sema3E/plexin-D1 signaling pathways

As previously mentioned, Sema3E is unique amongst class 3 semaphorins in that it can bind directly to its primary receptor plexin-D1 to activate cell signaling without the need to bind a co-receptor [8]. Prototypically, a Sema3E homodimer binds to a plexin-D1 homodimer directly forming a heterotetramer complex which undergoes conformational changes that initiate intracellular signaling [9]. However, there is evidence for monomeric Sema3E activation of the plexin-D1 receptor under certain conditions [17]. The co-receptor of Sema3E is neuropilin-1, which can complex with plexin-D1 to initiate intracellular signaling, most notably in a gating mechanism by which the presence of neuropilin-1 in addition to plexin-D1 changes Sema3E's activity towards developing axons from repulsion to attraction [29] (Figure 3A). However, the mechanisms of Sema3E signaling *via* neuropilin-1 have not been well-elucidated in the literature. Henceforth this review will focus mostly on Sema3E/plexin-D1 signaling pathways, which are illustrated in Figure 3.

Intracellular signaling by plexin-D1 is mediated through domains found on its cytosolic tail, two Ras-associated GAP domains and a Rho GTPase binding domain in-between them [30, 31]. In cortical neurons, R-Ras GAP activity of plexin-D1 inactivates R-Ras, leading to inhibition of cell migration and axon growth [32] (Figure 3B). In newborn neurons in the postnatal olfactory bulb, the formation of a filopodia-like lateral protrusion (FLP) is induced by downregulation of plexin-D1 and subsequent Rac1 (Ras-related C3 botulinum toxin substrate 1) activation, leading to microtubule polymerization and suppression of somal translocation, inhibiting neuronal migration [33].

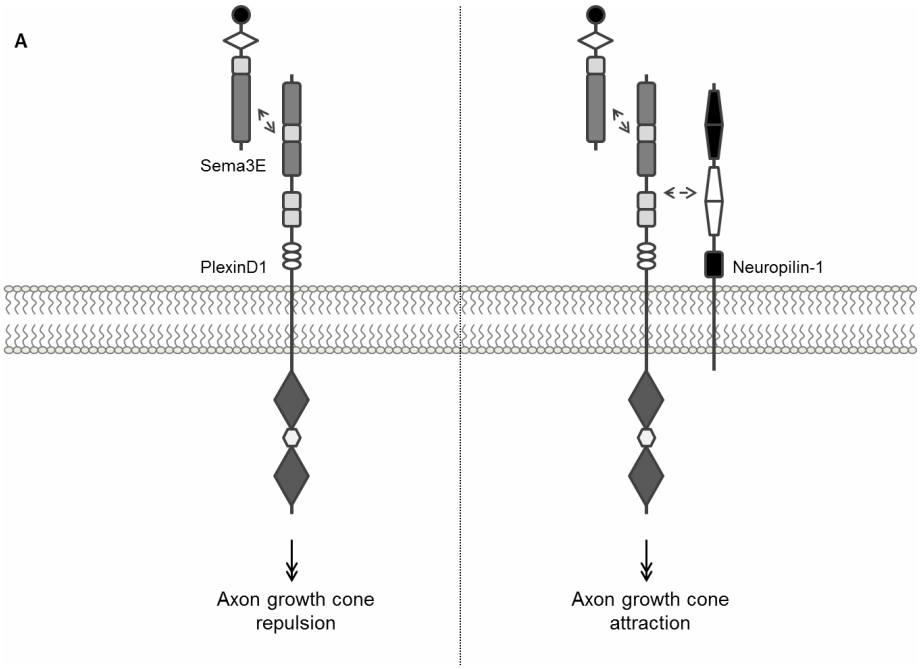

Figure 3

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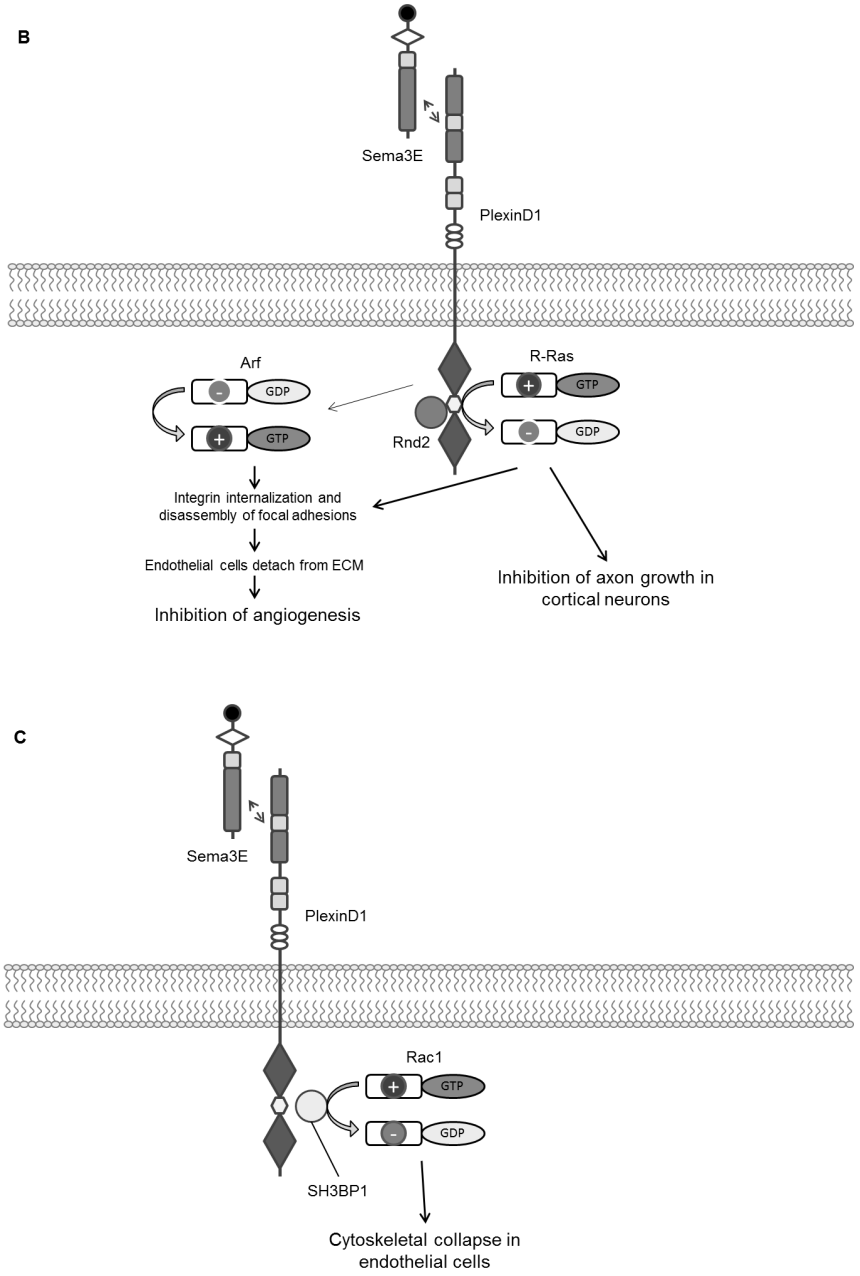

Figure 3

In endothelial cells, in addition to the inactivation of R-Ras by plexin-D1's GAP activity, plexin-D1 also promotes the activation of Arf6, both of which leads to β-integrin internalization and subsequent detachment of endothelial cells from the extracellular matrix (ECM), resulting in inhibition of angiogenesis [34, 35] (Figure 3B). GTPases that associate with the Rho GTPase binding domain include RhoJ and Rac1. Plexin-D1 activates small GTPase RhoJ, counteracting filopodia projections induced by vascular endothelial growth factor (VEGF) signaling, ensuring the directionality of retinal vasculature growth [36]. Another mechanism of cytoskeletal

Figure 3 continued..

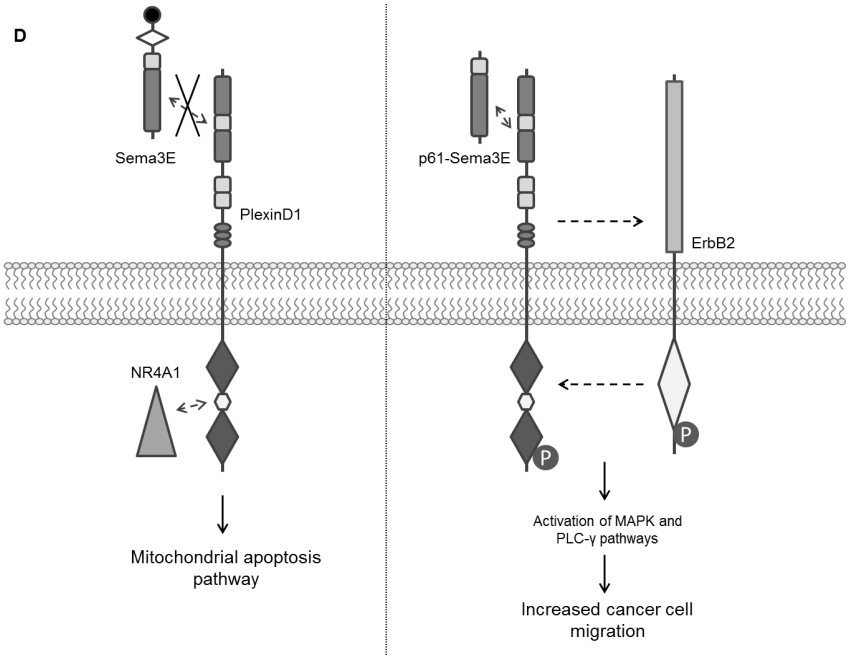

Figure 3. Major pathways of Sema3E/plexin-D1 signaling. (A) Left panel: In the absence of neuropilin-1, binding of Sema3E to plexin-D1 on neurons activates signaling that leads to repulsion or inhibition of axonal growth. Right panel: In the presence of neuropilin-1, the Sema3E/plexin-D1 complex can bind to neuropilin-1, converting the repelling signal to an attraction or growth signal. Hence, neuropilin-1 acts as a "gate" for plexin-D1 signaling. **(B)** In cortical neurons, the binding of Rnd2 to the GTPase-binding domain of plexin-D1 is required for stimulating the activity of the GAP domains, which activates R-Ras-GTPase, converting active R-Ras-GTP to inactive R-Ras-GDP, resulting in the inhibition of cell migration. In endothelial cells, in addition to the inactivation of R-Ras which leads to integrin inactivation, plexin-D1 activates Arf6 through recruiting phosphatidylinositol-4-phosphate 5-kinase and its lipid product, phosphatidylinositol 4,5-bis-phosphate, which binds GEP100, activating its guanine nucleotide exchange factor activity toward Arf6. This causes β-integrin inactivation and internalization, inhibiting endothelial cell adhesion to the extracellular matrix (ECM) as well as retracting filopodia in endothelial tip cells, thereby resulting in the inhibition of angiogenesis. **(C)** In endothelial cells, SH3-domain binding protein 1 (SH3BP1), a RhoGAP, interacts with plexin-D1 upon Sema3E binding to the receptor. Thereafter, SH3BP1 dissociates from plexin-D1, becomes activated, and inactivates Ras-related C3 botulinum toxin substrate 1 (Rac1) by converting Rac1-GTP to Rac1-GDP *via* its RhoGAP domain, leading to actin depolymerization and cell collapse. **(D)** Left panel: As observed in cancer cells, in the absence of Sema3E binding, the intracellular domain of plexin-D1 is able to bind to cytoplasmic Nuclear Receptor Subfamily 4 Group A Member 1 (NR4A1, an orphan nuclear receptor), activating the mitochondrial apoptosis pathway through the release of cytochrome c, an alternative mechanism for the activation of caspase-9. Plexin-D1's interaction with NR4A1 is independent of its GAP function. Binding of Sema3E to plexin-D1 abrogates the activation of this apoptosis pathway. Right panel: As observed in cancer cells, the binding of p61-Sema3E to plexin-D1 leads to the phosphorylation and transactivation of ErbB2/plexin-D1 complex, activating the MAPK and PLC-γ pathways that eventually lead to increased tumor cell migration and invasiveness.

collapse in endothelial cells is attributed to the inactivation of Rac1 by the binding and dissociation of SH3-domain binding protein 1 (SH3BP1), a RhoGAP, to plexin-D1 [37] (Figure 3C). In thymocytes, the cytoplasmic domain of plexin-D1 does not seem to have intrinsic GAP activity - signaling upon Sema3E binding is instead mediated by TAGAP (a guanosine triphosphatase (GTPase)-activating protein) which binds to cytoplasmic plexin-D1, thereby activating RhoA, leading to cytoskeletal reorganization. In the absence of Sema3E binding to plexin-D1,

the cytoplasmic domain of plexin-D1 recruits Cdc42 GAP, which inactivates Cdc42. Upon Sema3E binding, Cdc42 GAP is released, enabling the activation of Cdc42, enabling chemokine-mediated migration [38].

Various downstream signaling pathways have been reported to be activated upon Sema3E/plexin-D1 signaling, and these include the mitogen-activated protein kinases/extracellular signal-regulated kinases (MAPK/ERK), phosphatidylinositide 3-kinases/Protein kinase B (PI3K/AKT), and phosphoinositide phospholipase C-γ (PLC-γ) pathways. In neuroblastic cells, Sema3E activates the Ras-MAPK signaling pathway which involves extracellular Ca^{2+} influx, leading to neurite outgrowth [39]. In ovarian endometrioid cancer cells, Sema3E/plexin-D1 activation induces the nuclear translocation of Snail1, mediated by the activation of PI3K and MAPK pathways, leading to epithelial-to-mesenchymal transition (EMT) [40]. In melanoma and lung cancer cells, activation of plexin-D1 by the 61 kDa form of Sema3E cross-activates the ErbB2/HER2 receptor, which activates MAPK and PLC-γ pathways, leading to increased cell migration and invasion [41] (Figure 3D, right panel). In breast cancer cells, plexin-D1 has been described as an independence receptor: in the absence of Sema3E binding, plexin-D1 could activate the mitochondrial apoptosis pathway *via* interaction with NR4A1 [42] (Figure 3D, left panel).

The activation of PI3K signaling pathway could be dependent on the presence of neuropilin-1. In subicular neurons, VEGFR2 (KDR/Flk1) associated with the plexin-D1/neuropilin-1 receptor complex becomes tyrosine-phosphorylated upon Sema3E binding, leading to activation of the PI3K/Akt pathway that is required for axonal growth [43]. In gonadotropin-releasing hormone (gnRH) neurons, the PI3K signaling is also activated, ensuring cell survival [44]. Although the evidence is unclear, it is also likely that in other cells and conditions, the presence of neuropilin-1 leads to the activation of the PI3K pathway, while the PI3K pathway is inhibited in its absence.

2. Functions in normal development

Sema3E and its receptor plexin-D1 have been shown to be major regulators in embryonic development, particularly in somite and vascular patterning, nervous system development, cardiovascular development, and skeletal development.

2.1. Roles of plexin-D1 in embryonic development

The importance of the role of plexin-D1 signaling in embryonic development can be inferred from studies performed on plexin-D1 knockout mice. Although viable mice are born, they die within 24 hours after birth. These mice display severe structural cardiovascular defects, abnormal intersomitic vessel patterning, splitting of the vertebral bodies, as well as abnormal rib bone patterning [20, 45]. In agreement with the above studies, a Cre-mediated knockout of plexin-D1 in Tie2-expressing precursors (vascular endothelial cell lineage) in mice also generated myocardial, vascular, and skeletal defects [46]. Plexin-D1 also plays a crucial role in embryonic skeletogenesis, as reflected by major axial skeletal patterning defects in plexin-D1 knockout mice that includes the splitting of the vertebral body and shortening of rib bones [47].

2.2. Roles of Sema3E in embryonic development

Sema3E-knockout mice display similar morphological defects during embryonic development as those of plexin-D1 knockout mice; however, Sema-3E knockout mice carry out a self-correcting remodeling process, which enables these mice to survive into adulthood [8, 48]. Sema3E-knockout mice also express defects in intersomitic vessel formation and patterning. Expression of Sema3E in developing somites provides a repulsive signal for adjacent intersomitic vessels expressing plexin-D1, thereby restricting blood vessel growth in the intersomitic space [8]. In a recent study comparing the functions of Sema3E and Sema3D in endothelial cell repulsion and vascular patterning, it was found that although both molecules can exert the same effect on endothelial cells, they do so *via* distinct molecular pathways. For Sema3E, the receptor plexin-D1, but not neuropilin-1, was required for endothelial repulsion. It was also demonstrated that unlike Sema3D, Sema3E does not use the PI3K/Akt pathway for endothelial guidance and cytoskeletal reorganization [49].

To demonstrate the importance of Sema3E in early vascular and heart development, Meadows *et al*. (2012) showed that Sema3E was a key molecule required for the proper formation and patterning of the paired dorsal aortae [50]. Sema3E was also expressed by the lateral plate mesoderm, defining dorsal aortae boundaries and creating lateral avascular zones *via* strong repulsion of endothelial cells [50]. Despite severe defects in dorsal aortae patterning, Sema3E knockout mice can survive through adulthood because of a self-correction process where aberrantly branched aortic vessels remodel into normal, single aortae *via* cellular rearrangements [48].

In neural and brain development, it was first established that Sema3E (then known as mouse SemaH) could induce the collapse of axonal growth cones in the dorsal root ganglia, and that expression of the protein in different areas of the brain varied widely throughout development. Sema3E binding or signaling *via* neuropilins was involved in the process [51]. It was later found that Sema3E exerts its repellent or attractant activity on growing axons in the forebrain *via* a special gating mechanism. In the presence of plexin-D1 alone, Sema3E acts as a repellent; in the presence of both neuropilin-1 and plexin-D1, Sema3E acts as an attractant [29] (Figure 3C). Sema3E has been demonstrated to regulate the migration of Cajal-Retzius (CR) cells during development of the cerebral cortex in mice. Sema3E/plexin-D1 signaling negatively regulates the migration of cortical hem CR cells by decreasing CXCL12/CXCR4 signaling and increasing ADF/Cofilin activation, thereby enhancing motogenic control and enabling CR cells to populate the cortical surface [52].

Sema3E also plays a prominent role in the development of the retina, even through adulthood [1, 53, 54]. Notably, as has been described for other class 3 semaphorins, there is crosstalk between semaphorins and neurotrophins in the regulation of axonal growth cones [55-57]. Similarly, it was found that Sema3E's collapsing effect on retinal ganglion cell (RGC) axons could be antagonized by bone-derived neurotrophic factor (BDNF) [58].

In a study which investigated the association between nerve and vessel growth during development of the mouse whisker pad, it was found that nerves and blood vessels form independently, instead of *via* a "one-patterns-the-other" model that had been widely believed to be the case [59]. Importantly, the study found that Sema3E/plexin-D1 repellent signaling towards nerves and vessels was the dominant mechanism behind this neurovascular growth pattern. Sema3E originates from the follicle, maintaining the organization of the outer ring of vessels, while the selective downregulation of plexin-D1 on neurons allows them to maintain their inner ring position [59]. An important takeaway is that these studies have primarily been carried out using knockout mice and *ex vivo* cultures; and therefore, how Sema3E proteolytic processing and neuropilin co-receptor binding affect development is unknown. The complexity of how Sema3E post-translational cleavage and neuropilin/plexin-D1 interactions affect signaling in cancer underscores the need for a more thorough understanding of these events and which particular pathways they influence during development.

3. Potential roles of Sema3E in cancer

Given the extensive evidence of Sema3E's involvement in developmental and cellular processes that are also utilized in the cancer context, such as angiogenesis, axon and neuron growth, immune cell migration, and wound healing, it is plausible to hypothesize that Sema3E is involved in cancer progression *via* the regulation of these processes. Indeed, over the past decade, many studies have reported the different roles and effects of Sema3E in several different cancers, most notably on how it affects cancer metastasis. A summary of the major findings of Sema3E's involvement in cancer is summarized in Table 1.

3.1. Tumor growth and metastasis

Sema3E's association with cancer was initially identified by studying mouse breast cancer cell lines. Sema3E, then known as mouse SemaH, had significantly increased mRNA levels in metastatic mouse breast cancer cell lines compared to non-metastatic cell lines [60]. Subsequently, this finding was validated by the observation of

Table 1. Summary of major findings of Sema3E's involvement in cancer.

Cancer Type	Effects	Mechanism(s) involved
Breast cancer	Sema3E is associated with metastatic cancer cells and promotes metastasis to the lung [17, 60]	p61-Sema3E was directly responsible for this effect [17]
Colon cancer	Positive correlation between Sema3E levels and metastatic disease [41, 61]	N/A
Breast cancer	Sema3E promoted extravasation of cancer cells but suppressed tumor growth [41]	p61-Sema3E/plexin-D1 binding transactivates ErbB2, activating MAPK and PLC-γ that promote cancer cell migration [41]
Ovarian cancer	Positive correlation between Sema3E levels and high grade ovarian carcinoma; Sema3E promoted metastasis in mouse model [40]	p61-Sema3E/plexin-D1 binding activates EMT, leading to increased cancer cell migration [40]
Tongue cancer	Positive correlation between Sema3E levels and lymph node metastasis; association between high Sema3E levels and poor survival [64]	N/A
Gastric cancer	Positive correlation between Sema3E levels and metastasis; association between high Sema3E levels and poor survival in intestinal type cancer [65]	Overexpression of Sema3E activates ERK pathway that leads to increased cell proliferation [65]
Pancreatic cancer	Positive correlation between Sema3E levels and tumor progression; association between high Sema3E levels and poor survival in adenocarcinoma [66]	Overexpression of Sema3E activates ERK pathway that leads to increased cell proliferation [66]
Breast cancer	Sema3E knockdown or plexin-D1 overexpression induced increased cell death [42]	Unliganded plexin-D1 promotes apoptosis *via* interacting with NR4A1 to activate mitochondrial apoptosis pathways [42]
Prostate cancer	Sema3E overexpression decreases invasion and adhesion [70]	Coexpression of neuropilin-1 may affect Sema3E-plexin-D1 signaling
Melanoma	Negative correlation between Sema3E levels and tumor progression [62]	Overexpression of Sema3E decreases metastasis in mouse model [62]

Sema3E overexpression in human breast cancer cell lines and patient samples [17]. Ectopic expression of the protein in breast cancer cells promoted lung metastasis *in vivo*, as well as enhanced migration of endothelial and neuroblastic cells *in vitro*. Importantly, these effects were found to be induced by the 61 kDa furin-processed truncated form of the protein [17].

In human colon carcinoma, Sema3E expression levels were higher in tumors associated with metastatic disease compared to non-metastatic tumors [41], and this finding was also confirmed in a recent study [61]. Levels of both Sema3E and plexin-D1 were higher in metastases than primary tumors, providing the first line of evidence that indicated that Sema3E/plexin-D1 signaling correlated positively with metastasis. The same study found that in melanoma, Sema3E expression correlated strongly with invasive and metastatic disease [41], contrary to the findings from a previous report that also studied Sema3E expression in melanoma [62].

Overexpression of Sema3E in lung, colon, and breast cancer cell lines promoted metastases to the lung in subcutaneous xenograft mouse models, and the 61 kDa form of Sema3E (p61-Sema3E) binding to plexin-D1 was found to be primarily responsible for this effect. However, surprisingly,

Sema3E overexpression resulted in suppressed tumor growth; the authors postulated that this was probably caused by reduced vascularity attributed to Sema3E's anti-angiogenic effects. It was also found that p61-Sema3E binding to plexin-D1 transactivated the receptor tyrosine kinase (RTK) ErbB2, which activated MAPK and PLC-γ signaling cascades, resulting in migration of cancer cells [41] (Figure 3D).

In human ovarian endometrioid carcinoma (OEC), Sema3E was again implicated in tumor progression - Sema3E levels correlated with increasing tumor grade, while plexin-D1 levels were relatively constant amongst low- and high-grade tumors. p61-Sema3E, but not full-length Sema3E, was found to enhance cell migration *in vitro via* plexin-D1 binding, while overexpression of Sema3E in OEC cells enhanced metastasis in a xenograft mouse model. It was also found that the binding of Sema3E to plexin-D1 induced the translocation of transcriptional repressor Snail1 into the nucleus, leading to increased epithelial-to-mesenchymal transition (EMT), which enhanced cell motility. This was likely mediated by the activation of PI3K and ERK/MAPK pathways [40]. However, interestingly, in a 3D spheroid model of ovarian cancer using the HEY ovarian cancer cell line, induction of EMT downregulated Sema3E by more than 10-fold, suggesting that Sema3E may instead inhibit EMT in ovarian cancer, although the observation is limited to this particular cell line [63].

In a study of Sema3E expression levels in squamous cell carcinoma of the tongue, Sema3E levels correlated positively with lymph node metastasis, and high Sema3E expression was also associated with poor survival [64]. Similarly, Sema3E was found to be correlated with metastasis in gastric cancer, and high Sema3E levels were associated with poor survival in intestinal type gastric cancer [65]. Overexpression and knockdown of Sema3E in gastric cancer cells promoted and attenuated tumor growth respectively, proving that Sema3E was directly responsible in promoting cancer cell proliferation, through activating the ERK pathway, as the study revealed [65]. Our own study on Sema3E's effects in pancreatic cancer yielded very similar findings - we found that Sema3E levels correlated with tumor progression and was associated with poor survival in pancreatic adenocarcinoma [66]. Overexpression and knockdown of Sema3E in pancreatic cancer cell lines likewise enhanced and inhibited cancer cell growth and migration, respectively. Additionally, we found that Sema3E was expressed in the nuclei of pancreatic adenocarcinoma cells, which was surprising, given that Sema3E is a secreted protein [66]. We are currently investigating the potential roles of Sema3E in the nucleus.

Although it is typical for class 3 semaphorins to be subjected to processing by furin and furin-like proprotein convertases in order to yield a functional and active form of the protein [16, 67, 68], in the case of Sema3E, furin-based processing produces a truncated protein with pro-metastatic properties as compared to the full-length protein [17]. This was further investigated in a study where the properties of a lab-designed uncleavable form of Sema3E were compared alongside those of the cleaved p61-Sema3E form. Despite being able to bind to plexin-D1, full-length uncleavable Sema3E was unable to induce the phosphorylation of ErbB2 that activates a signaling cascade which leads to metastasis, unlike the 61 kDa form. Moreover, uncleaved Sema3E was unable to activate MAPK/ERK signaling. Uncleaved Sema3E was also a potent anti-angiogenic factor; it promoted the rapid disassembly of focal adhesion kinase (FAK) complexes and internalization of β-integrins on endothelial cells, triggering a rapid "collapsing" response. When administered *in vivo*, uncleavable Sema3E resulted in reduced tumor growth, angiogenesis and metastasis, pointing towards its potential use as a therapeutic molecule [69].

A more recent study of Sema3E in breast cancer has revealed a novel mechanism for Sema3E/plexin-D1 signaling in cancer growth and metastasis [42]. The authors first found a positive correlation of Sema3E levels with progressively worse and invasive/metastatic breast cancer, although plexin-D1 levels remained relatively constant regardless of tumor progression [42]. Unlike previous studies which have focused on knocking down plexin-D1 to study Sema3E/plexin-D1 signaling, this study took the unconventional approach by overexpressing plexin-D1 instead. The authors found that unliganded plexin-D1 induced caspase-based cellular apoptosis, which was inhibited in the presence of Sema3E (but not Sema4A, another

ligand of plexin-D1), therefore pointing to the function of plexin-D1 as a dependence receptor. Importantly, unliganded plexin-D1 was found to induce apoptosis *via* interacting with cytoplasmic nuclear receptor subfamily 4 group A member 1 (NR4A1) protein, which activates the caspase-9 apoptosome [42].

While the majority of studies have shown that Sema3E promotes cancer metastasis, there are also some reports that indicate the opposite, that Sema3E slows down the rate of cancer progression or inhibits metastasis. A study of Sema3E overexpression in prostate cancer cell lines found that Sema3E inhibited cell invasion, and that exogenous transferrin could reverse these effects by promoting the binding of insulin growth factor (IGF-1) to the IGF-1 receptor [70]. This suggests that signaling pathways activated by IGF-1, such as the MAPK/ERK or PI3K/AKT/mTOR pathways, may be involved in Sema3E's regulation of cancer metastasis in prostate cancer. A recent study of the growth inhibitory effects of the flavonoid fisetin on a few cancer cell lines revealed that SEMA3E gene was significantly upregulated upon addition of fisetin to the cells, suggesting that Sema3E may induce growth inhibitory effects on cancer cells [71].

In human melanoma, plexin-D1 levels were found to be positively associated with higher grade and metastatic melanomas, while Sema3E was inversely correlated with tumor progression [62]. In gastric cancer, it was found that Sema3E was downregulated in patient samples, and that Sema3E levels were inversely correlated with tumor volume, lymphatic invasion, and gastric cancer progression [72]. However, the same study evaluated Sema3E expression in prostate, mammary, ovarian, and uterine cancer, and found, in agreement with previous reports [17, 40-42, 61], that Sema3E was overexpressed in these cancers. Hence, it is probable that Sema3E is differentially expressed in different types of cancers. As to Sema3E's role in tumor growth, a few studies have reported that Sema3E inhibits tumor growth. Cassaza *et al*. found that both the full-length uncleavable form as well as the furin-cleaved p61 form of Sema3E inhibited tumor growth *in vivo* (but not *in vitro*), presumably *via* Sema3E's paracrine effects on inhibiting tumor-induced angiogenesis [69]. The same tumor growth inhibitory effects were observed in a study of the overexpression of full-length uncleavable Sema3E in glioblastoma cell lines, *in vitro* and *in vivo* [73]. Sema3E overexpression in gastric cancer cell lines yielded the same result - reduced cell proliferation *in vitro* due to less G1 to S phase transition as well as promotion of apoptosis.

A study of the expression of class 3 semaphorins and their receptors in cancer cell lines may provide some clues on how receptor expression is related to the effects of these semaphorins [74]. Sema3E was overexpressed in the breast cancer cell line MDA-MB-231 and the now-verified melanoma cell line [75, 76] MDA-MB-435, and had significant growth-suppressing effects and slight growth-promoting effects on these cells, respectively. MDA-MB-231 expresses both receptors for Sema3E - plexin-D1 and neuropilin-1 at high levels, while MDA-MB-435 expresses only low levels of plexin-D1, and does not express neuropilin-1 [74]. The inference from this is that expression of both receptors can activate Sema3E's effects on suppressing cell growth, while expression of plexin-D1 alone activates Sema3E's growth-promoting effect. This is in agreement with Luchino *et al.*'s study on Sema3E's effects on breast cancer cells [42]; in the presence of plexin-D1 alone without high expression of Sema3E, cellular apoptosis is activated; however, when Sema3E was expressed at high levels, cell growth was observed instead (Figure 3D). However, this study did not analyze the level of expression of neuropilin-1; hence the effects of the presence of neuropilin-1 on Sema3E's ability to control cancer cell growth still remain to be uncovered.

The contrasting observations of Sema3E's roles from different studies and in different cancer types reflect the complex biology of Sema3E in the cancer context. However, these dissimilar results can be attributed to: how the cleaved 61 kDa vs. uncleaved full-length Sema3E alters the function of the plexin-D1 receptor, the presence or absence of the neuropilin co-receptor, the different signaling pathways elicited by the binding or non-binding of Sema3E to plexin-D1, as well as many other potential signaling pathways that may interact downstream of Sema3E/plexin-D1 signaling. Other factors to consider are Sema3E's autocrine effects on cancer cells or paracrine effects on non-tumor cells in the tumor

microenvironment, such as endothelial and immune cells, and the expression levels of plexin-D1 and neuropilin-1, on these cells, as mentioned in the above paragraph. Further and more detailed investigation into how Sema3E is regulated in the cancer context is clearly needed, and may provide the key to understanding and exploiting the use of Sema3E as a therapeutic target or prognostic marker for certain cancers.

3.2. Angiogenesis

In addition to its direct action on cancer cells, Sema3E/plexin-D1 signaling has been implicated in other processes that are known to affect tumor growth and metastasis, specifically angiogenesis and immunomodulation. Unfortunately, the effects of Sema3E and plexin-D1 on these processes in cancer have not been specifically addressed. Therefore, this discussion will focus on how Sema3E and plexin-D1 affect angiogenesis in the context of other diseases, and how these results can be related back to cancer.

In a study to elucidate the role of Sema3E/plexin-D1 signaling in post-natal angiogenesis, it was found that in ischemic limbs of mice Sema3E could directly suppress the pro-angiogenic VEGF signaling pathway leading to inhibition of endothelial cell growth and tube formation. Disruption of this pathway partially restored blood flow [77]. In a model of tumor-induced angiogenesis, Sema3E could inhibit angiogenesis in angioreactors containing VEGF and basic fibroblast growth factor (bFGF) - two factors commonly found to be secreted by cancer cells. In addition, treatment of plexin-D1-expressing endothelial cell lines with Sema3E induced cell detachment from the ECM *via* the internalization and inactivation of β1 integrins. Sema3E also caused cytoskeletal collapse in COS-7 cells that were transfected with plexin-D1, inducing the activation of Arf6, thereby decreasing β1 integrin-dependent cell adhesion [34]. A follow-up study on the detailed mechanisms involved is illustrated in Figure 3A [35].

The anti-angiogenic properties of Sema3E/plexin-D1 signaling has been shown to be involved in diseases related to ischemia, with administration of exogenous Sema3E having been shown to have therapeutic benefits. In a mouse model of ischemic retinopathy, intravitreal administration of Sema3E was able to suppress extra-retinal vascular outgrowth and restore the directionality of proper vascular growth towards the ischemic retina [36]. Likewise, in a clinical study of diabetic retinopathy, Sema3E levels were lower in the patient group than the control group, and also correlated negatively with VEGF levels [78]. These indicate that Sema3E may be a potential therapeutic molecule in the inhibition of extraretinal neovasculatization [78]. In a mouse model of oxygen-induced retinopathy (OIR), retinoic acid receptor-related orphan receptor α (RORα) was found to transcriptionally suppress Sema3E expression in retinal ganglion cells. Genetic deficiency of RORα leads to reduction of pathological neovascularization in OIR and induced expression of Sema3E, while shRNA-mediated downregulation of Sema3E could reverse inhibitory vascular effects of RORα deficiency by promoting pathological neovascularization [79].

Another application of using Sema3E as a therapeutic molecule was shown for choroidal neovascularization (CNV), where intravitreal injection of Sema3E in a mouse model decreased CNV size, while this effect was abolished in plexin-D1-KO mice, therefore demonstrating the therapeutic potential in stimulating Sema3E/plexin-D1 signaling in CNV [80]. On the other hand, another application of targeting Sema3E is in ischemic stroke, where it was found that Sema3E suppresses angiogenesis, and that inhibition of Sema3E-plexin-D1 signaling can enhance neovascularization and improve the integrity of the blood-brain barrier [81].

Sema3E also plays a role in the regulation of vascular smooth muscle cells (VSMCs). In a carotid ligation model, Sema3E expression was found to be decreased during neointimal formation, a common feature of atherosclerosis. Overexpression of Sema3E suppressed neointimal formation by inhibiting migration and proliferation of VSMCs *via* binding and signaling through plexin-D1. Sema3E also suppressed the expression of platelet-derived growth factor-B (PDGF-B) in endothelial cells [82]. On the other hand, there are contradictory findings with regard to Sema3E's role in neointimal formation, particularly in a large-cohort clinical study of metabolic syndrome. In this study, increased serum Sema3E levels

were found in the disease group compared to controls, and Sema3E was also found to be positively correlated with carotid intima-media thickness, indicating that it is associated with carotid atherosclerosis [83].

The anti-angiogenic effect of Sema3E may be dependent on the protein isoform present. Although earlier reports found that p61-Sema3E can stimulate SVEC4-10 endothelial cell migration and neurite outgrowth in PC12-E2 cells [17, 39], another study has reported the anti-angiogenic effects of both full length and p61-Sema3E in both *in vitro* and *in vivo* assays [41]. This subject remains controversial to this date, and further investigation of detailed mechanisms involved will be the key to understanding the difference in functions between the two protein isoforms.

3.3. Inflammation and regulation of immune cells

Plexin-D1 has a wide range of expression on immune cells including Tregs, dendritic cells, macrophages, and B cells among others [84]. At the same time, Sema3E expression, although more limited, has also been shown to be present in macrophages, thymocytes, dendritic cells, and T cells, with particularly high transcription in TH2 type T cells [84-87]. How Sema3E/plexin-D1 signaling relates to cancer is of particular interest due to the immunosuppressive environment encompassing the tumor. Similar to angiogenesis, previous studies have not focused specifically on the tumor microenvironment, but much can be gleaned from studies of Sema3E and plexin-D1 in other immune-related functions. In particular, a study reporting the role of Sema3E in immune cell migration showed that Sema3E/plexin-D1 signaling could regulate the migration of thymocytes into the medulla during thymus development [87]. Plexin-D1 was expressed specifically in double positive (DP) thymocytes while soluble Sema3E was expressed in a high-to-low concentration gradient from the medulla to the cortex. The binding of Sema3E to plexin-D1 on DP thymocytes inhibits the response of CCR9 to CCL25 ligand in the cortex, enabling DP thymocytes to migrate efficiently into the medulla [87]. The same group found in a later study that unliganded plexin-D1 could induce the clustering of integrins on the cell membrane in patches, while the binding of Sema3E to plexin-D1 inactivates these integrins, enabling the detachment of thymocytes [88]. In a further study to elucidate this mechanism, Sema3E-plexin-D1 signaling was found to be mediated by TAGAP, as mentioned in section 1.4 [38]. Knockdown of TAGAP in mice resulted in disruption of Sema3E-plexin-D1 signaling, leading to defective thymocyte migration and hence formation of ectopic medullary structures in the cortex. This is of translational significance as single nucleotide polymorphisms (SNPs) of *TAGAP* gene have been associated with multiple autoimmune diseases [38].

Sema3E has been found to be involved in inflammation in atherosclerosis [84]. In an $ApoE^{-/-}$ mouse model of atherosclerosis, both Sema3E and plexin-D1 were expressed in macrophages of atherosclerotic lesions. Conversely, in mouse models of atherosclerosis regression, Sema3E mRNA expression was downregulated in plaque macrophages, which coincided with reduced numbers of inflammatory M1 macrophages and increased numbers of reparative M2 macrophages. In deciphering the mechanisms behind the retention of macrophages in plaques, *in vitro* experiments revealed that Sema3E inhibited the migration of macrophages in response to CCL19, *via* disrupting the Rho GTPase signaling pathway, resulting in reorganization of the actin cytoskeleton and thereby inhibiting cell migration [84].

Sema3E has also recently been implicated in dietary obesity-associated inflammation [89]. In a diet-induced mouse model of obesity, Sema3E was mainly expressed in adipocytes while plexin-D1 was highly expressed in infiltrating macrophages and adipocytes. Blocking Sema3E binding to plexin-D1 or genetically disrupting Sema3E led to decreased infiltration of macrophages into adipose tissue and also improved insulin resistance and glucose tolerance. Conversely, overexpressing Sema3E elicited an opposite effect. *In vitro* studies showed that Sema3E could act as a chemoattractant to induce macrophage migration *via* binding to plexin-D1. Moreover, it was found that the upregulation of Sema3E was induced by the upregulation of p53 in response to reactive oxygen species and DNA damage in adipose tissue [89].

On the other hand, Sema3E is instead found to inhibit Th2/Th17 inflammation in allergic conditions, including asthma. Sema3E was able to inhibit the proliferation and migratory effects

of the inflammatory cytokine platelet-derived growth factor (PDGF)-BB26 in human airway smooth muscle cells (HASMCs), *via* suppressing PDGF-induced Rac1 GTPase activity and phosphorylation of PI3K/Akt and MAPK/ERK1/2 [90]. Moreover, knockout of Sema3E in mice leads to Th2/Th17 inflammation and worsens airway hyperresponsiveness [91]. In another study, Sema3E was found to inhibit migration of neutrophils induced by CXCL8/IL-8 *in vitro*, while *in vivo*, allergen exposure in the airway led to greater neutrophil migration and inflammation in Sema3E knockout mice compared to wild type mice [92]. In another mouse model of asthma induced by exposure to house dust mites, Sema3E expression was found to be decreased - administration of exogenous Sema3E protected mice from asthma by reducing eosinophilic inflammation and serum IgE, as well as suppressing Th2/Th17 responses [93, 94].

CONCLUSIONS

The discovery of the many other functions of semaphorins beyond their role in axonal growth guidance has ignited much interest in research on the different forms of semaphorins, particularly in how they regulate various developmental and disease states, as well as the precise mechanisms and signaling pathways involved. More recently, given emerging evidence of the significant roles various semaphorins play in the progression of cancer, the focus of research has shifted towards the study of how semaphorins may affect cancer-related processes, such as angiogenesis and metastasis. Sema3E, in particular, has been widely studied in a number of cancers, which primarily implicate Sema3E in cancer cell invasion and metastasis. However, given some contrasting pieces of information about the roles of Sema3E in tumor growth and metastasis, more extensive research is needed to elucidate and explain the precise behavior of Sema3E in cancer cells and in other cells of the tumor microenvironment. Given that Sema3E is also involved in immune cell migration and inflammation, it is logical to postulate that Sema3E could influence the behavior of tumor-infiltrating immune cells. Moreover, given the accumulating evidence on the contribution of perineural invasion to cancer invasion, as summarized in Amit *et al.*'s review [95], it is plausible that Sema3E may also exert its axon guidance effects on nerves in the tumor microenvironment. In all, a better understanding of the role of Sema3E in cancer and other diseases will help to evaluate its value as a potential diagnostic or prognostic marker, or as a therapeutic target.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest associated with this study.

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