

Targeting the SLIT/ROBO pathway in tumor progression: molecular mechanisms and therapeutic perspectives

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Abstract: The SLITs (SLIT1, SLIT2, and SLIT3) are a family of secreted proteins that mediate positional interactions between cells and their environment during development by signaling through ROBO receptors (ROBO1, ROBO2, ROBO3, and ROBO4). The SLIT/ROBO signaling pathway has been shown to participate in axonal repulsion, axon guidance, and neuronal migration in the nervous system and the formation of the vascular system. However, the role of the SLIT/ROBO pathway has not been thoroughly clarified in tumor development. The SLIT/ROBO pathway can produce both beneficial and detrimental effects in the growth of malignant cells. It has been confirmed that SLIT/ROBO play contradictory roles in tumorigenesis. Here, we discuss the tumor promotion and tumor suppression roles of the SLIT/ROBO pathway in tumor growth, angiogenesis, migration, and the tumor microenvironment. Understanding these roles will help us develop more effective cancer therapies.

Keywords: migration, proliferation, SLIT/ROBO pathway, tumor, tumor microenvironment

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Introduction

The formation of tumors is modulated by multiple soluble and immobilized molecular factors including proteases, growth factors, extracellular matrix components, etc. Increasing evidence has shown that tumors can mimic embryonic development. Therefore, multiple molecular cytokines that affect embryonic development have been investigated in cancer progression. The SLIT/ROBO pathway is particularly involved in embryonic development.¹ Gara *et al.*² reviewed the roles of the SLIT/ROBO pathway in different types of cancer, molecular crosstalk, and the modulation of oncogenic signaling pathways. Huang *et al.*³ summarized the SLIT/ROBO pathway and its biological significance in gastrointestinal cancers. Recent studies showed that the SLIT/ROBO pathway can produce both beneficial and detrimental effects in the growth of malignant cells. It seems that SLIT/ROBO play contradictory roles in tumorigenesis. Therefore, in this brief review, the tumor promotion and tumor suppression roles of the SLIT/ROBO pathway and its biological significance in cancer will be summarized.

Overview of the SLIT/ROBO pathway

It has been shown that SLITs, ligands for Roundabout (ROBO), are required in preventing axons from recrossing the central nervous system midline in *Drosophila*.⁴ SLIT proteins are also identified as repulsion ligands for ROBO receptors in axon guidance in vertebrates including mammals.^{5,6} Thus far, three distinct SLIT genes (SLIT1, SLIT2, and SLIT3) and four distinct ROBO genes (ROBO1, ROBO2, ROBO3, and ROBO4) have been cloned in mammals.^{7–9}

SLITs (SLIT1–SLIT3), are expressed in the nervous system, which have been found in *Drosophila*,^{10–12} *Caenorhabditis elegans*,¹³ xenopus,¹³ mice,^{6,13} chickens,¹³ humans,¹³ and rats.^{14,15} The protein can also be expressed in tumor cells,¹⁶ leukocytes,⁷ luteal cells,¹⁷ and other cells. Structurally, SLIT protein, a secreted glycoprotein from the N terminus to C terminus, contains four leucine-rich repeats (LRRs: D1–D4), seven (in *Drosophila* SLIT) to nine (in vertebrate SLIT) epidermal growth factor (EGF) repeats, a laminin G-like module, and a cysteine rich knot

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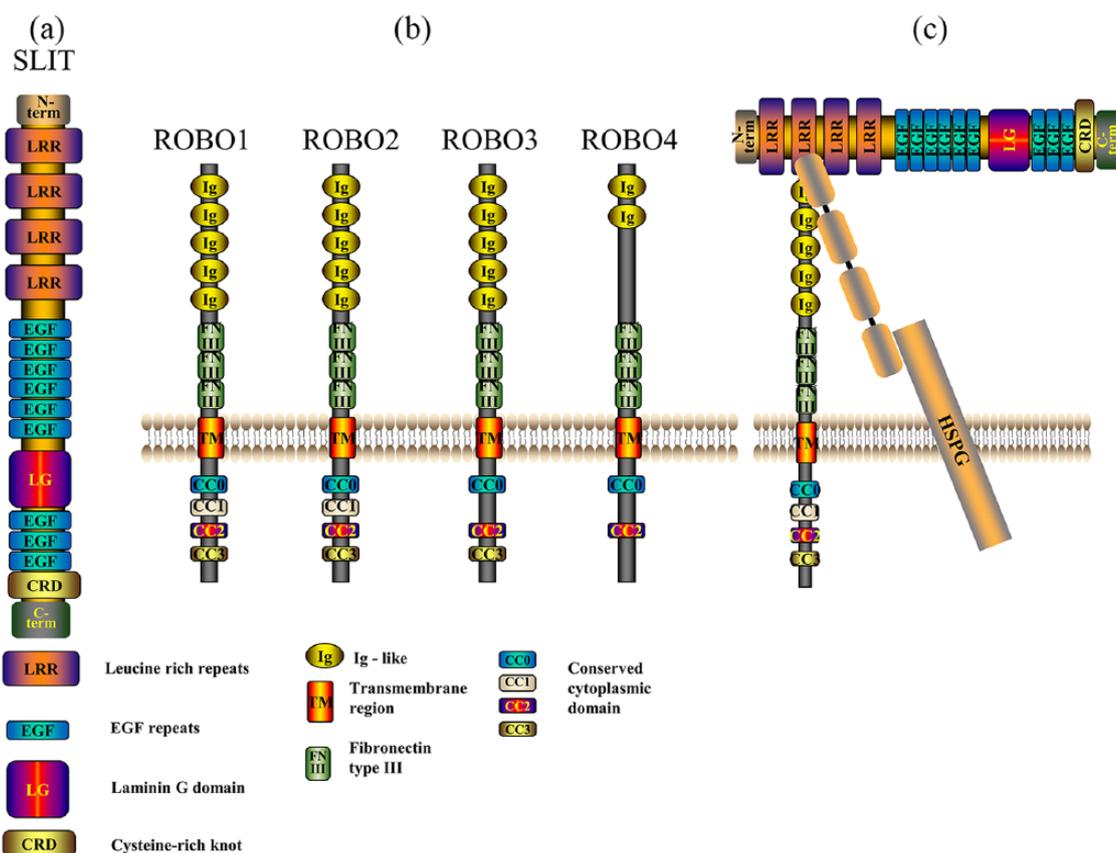


Figure 1. Structure of the SLIT/ROBO protein family. (a) Structure of SLIT protein, a secreted glycoprotein from the N terminus to C terminus, contains four leucine-rich repeats (LRRs, D1–D4), seven to nine epidermal growth factor (EGF) repeats, a laminin G-like module and a cysteine-rich knot. (b) The ROBO family contain five immunoglobulin (Ig) domains, three fibronectin type III modules in the extracellular region, one transmembrane region and one intracellular conserved cytoplasmic domain including CC0, CC1, CC2, and CC3. (c) SLIT D2 binds on its concave surface to the Ig1 domain of ROBO through electrostatic and hydrophobic contact regions such as the heparan sulfate proteoglycan (HSPG) syndecan.

(Figure 1).^{12,18} The LRRs are sufficient for SLIT interaction with the receptor ROBO.¹⁹ Proteolytic processing of the human SLIT-2 protein gives rise to an N-terminal fragment (SLIT-N) and a C-terminal fragment (SLIT-C).^{5,6} SLIT-N contains all four LRRs and five of the EGF repeats (amino acids 1–1117) and SLIT-C contains the rest of the protein.⁵ Both the full-length protein and the fragments of SLIT can be secreted extracellularly.^{5,10–12} Studies have confirmed that SLIT2 is a large glycoprotein of approximately 200 kD that is typically processed into a 55–60 kD diffusible C-terminal fragment (SLIT2-C), a 140 kD N-terminal fragment (SLIT2-N), and a number of unknown fragments that are more tightly associated with cells. All SLIT fragments show different functional activities *in vivo*.²⁰

The ROBO family, which contains four members (ROBO1, ROBO2, ROBO3/Rig-1, and ROBO4/

Magic), is a class of conserved transmembrane receptor proteins (Figure 1). They are mainly expressed in the nervous system but can also be expressed elsewhere such as in vascular endothelial cells and muscle cells.¹⁶ ROBO1, ROBO2, and ROBO3, which have been identified in organisms including *Drosophila*,²¹ *C. elegans*,²² zebrafish,²³ human,²¹ and mouse,²⁴ contain five Ig-like (immunoglobulin-like) domains, three fibronectin type III modules in the extracellular region, one transmembrane region, and one intracellular conserved cytoplasmic domain including CC0, CC1, CC2, and CC3.^{21,22} ROBO4 contains only two Ig-like functional areas and three fibronectin type III modules in the extracellular region, and one transmembrane region and one intracellular region including CC0 and CC2 small areas only.^{8,9}

The SLIT-ROBO interaction is mediated by the second LRR domain of SLIT (D2) and the first

two N-terminal Ig domains of ROBO,¹⁹ heparin sulfate (HS) disaccharide units are required to support SLIT-ROBO signaling.²⁵ SLIT D2 binds on its concave surface to the Ig1 domain of ROBO through electrostatic and hydrophobic contact regions.¹⁹ By binding to their receptor ROBOs, SLITs activate intracellular signal transduction pathways in order to mediate biological effects on cells.²⁶

In the nervous system, SLIT/ROBO signaling has been shown to be involved in axonal repulsion,^{4,27} neuronal migration,²⁸ and axon guidance.²⁹ SLITs and ROBOs also have an evolutionarily conserved role in preventing axons from migrating to inappropriate locations during the assembly of the nervous system.²⁰ The SLIT/ROBO signal from the floor plate repels longitudinal axons away from the ventral midline and maintains straight longitudinal growth.³⁰ In addition, SLIT proteins have also been shown to stimulate branching and elongation of sensory axons and cortical dendrites *in vitro*.⁵ It was reported that SLIT1 protein promotes neurite outgrowth and elongation when added to both adult rat dorsal root ganglion (DRG) and cultured DRG, where SLIT1/ROBO2 mRNA and protein were detected.³¹ SLIT/ROBO signaling also contributes to the patterning of both the peripheral and central branches of sensory neurons with distinct positive branching and negative guidance actions, respectively.³²

SLITs and ROBOs are also expressed in non-neuronal tissues, such as mouse lung and kidney.³³ Several SLITs and ROBOs have also been found to be aberrantly expressed during the development of ovarian, endometrial, cervical, and prostate cancer in the reproductive system.³⁴ The SLIT/ROBO pathway in tumors have gradually received more attention. However, the roles of SLIT/ROBO pathway in tumors should be further discussed.

SLIT/ROBO pathway as an oncogene in tumor progression

In the last decade, studies have demonstrated the dual roles of SLITs and ROBOs as axon guidance cues in the developing nervous system, where they both attract and repel neuronal migration. This bifunctionality is also observed in cancers as both oncogenes and tumor suppressor genes. The expression of SLIT and ROBO is altered in a wide variety of cancer types, identifying them as potential therapeutic targets³⁵ (Table 1).

Previous studies have shown that high levels of SLITs and ROBOs are expressed in many types of tumors and SLIT/ROBO signaling has a positive effect on tumor growth. For instance, by means of microarray analysis and real-time polymerase chain reaction (PCR), Groene *et al.*³⁶ showed that the expression of ROBO1 and ROBO4 was significantly upregulated in colorectal carcinoma compared with normal tissue, while SLIT2 showed no differential expression between colorectal carcinoma and normal tissue. ROBO1 expression was mainly in tumor cells, whereas ROBO4 was located primarily in the endothelial cells of tumor vessels.³⁶ Zhou *et al.*³⁷ demonstrated that the expression of SLIT2 and ROBO1 was significantly associated with an increased metastatic risk and poorer overall survival in colorectal carcinoma patients. ROBO1 and SLIT2 mRNAs were detected in breast cancer cell lines and breast cancer tissues, and ROBO1 expression was elevated in breast carcinoma compared with normal breast mammary epithelial cells.^{38,39} There was also a striking increase in SLIT1 expression in prostate tumors.⁴⁰ Owing to the overexpression of ROBO1 in hepatocellular carcinoma and the shedding of ROBO1 into serum in humans, this receptor of SLITs was thought to be a potential new serological marker for hepatocellular carcinoma.⁴¹ In addition, SLIT2 and ROBO1 were overexpressed in human mucoepidermoid carcinoma Mc3 cells.⁴²

SLIT2/ROBO1 pathway promoted the Mc3 cells proliferation and the treatment of Mc3 cells with the monoclonal antibody R5 which can interrupt the SLIT2/ROBO1 pathway caused significantly suppressed cell growth and proliferation and markedly lowered the expression of PCNA.⁴² Furthermore, SLIT2 expression was correlated with the loss of basement membrane in the samples of human skin squamous cell carcinoma at different stages of disease progression. The SLIT2-Tg mice were found to develop significantly more skin tumors than wild-type mice, the skin tumors that occurred in SLIT2-Tg mice were significantly larger than those in the wild-type mice after 7,12-dimethylbenz[a]anthracene initiation until the end of the experiment. SLIT2 also could promote the invasive ability of the squamous cell carcinoma cell line A431 and this effect could be significantly repressed by the antibody R5.⁴³

Tumor metastasis, the process of tumor cells migrating to other distant organs, invading blood and lymphatic vessels and leading to secondary

Table 1. Oncogenic and tumor-suppressive role of SLIT/ROBO signaling in human cancers.

Tumor type	SLIT/ROBO pathway status	Function	References
Pancreatic cancer	ROBO3↑	Oncogenic	Han <i>et al.</i> ⁴⁵
Pancreatic cancer	SLIT2↓	Tumor-suppressor	Goehrig <i>et al.</i> ⁴⁸
Lung cancer	SLIT3↓	Tumor-suppressor	Zhang <i>et al.</i> ⁴⁹
Lung cancer	USP33 ↓	Tumor-suppressor	Wen <i>et al.</i> ⁵⁰
Colorectal cancer (CRC)	SLIT2↓; USP33 ↓	Tumor-suppressor	Huang <i>et al.</i> ⁵¹
Colorectal cancer	ROBO1 ↑; ROBO4 ↑.	Oncogenic	Groene <i>et al.</i> ³⁶
Colorectal cancer	SLIT2 ↑; ROBO1↑	Oncogenic	Zhou <i>et al.</i> ³⁷
Colorectal cancer	SLIT2↑; ROBO2↑	Oncogenic	Sanz-Pamplona <i>et al.</i> ⁵²
Breast cancer	srGAP3↓	Tumor-suppressor	Lahoz and Hall ⁵³
Mucoepidermoid carcinoma	SLIT2 ↑; ROBO1↑	Oncogenic	Han <i>et al.</i> ⁴²
Ovarian cancer	SLIT2↓; SLIT3↓; ROBO1↓ ROBO2↓; ROBO4↓	Tumor-suppressor	Dickinson <i>et al.</i> ⁵⁴
Oral squamous cell carcinoma	SLIT2↓	Tumor-suppressor	Bauer <i>et al.</i> ⁵⁵
Nasopharyngeal cancer	ROBO1↑	Oncogenic	Alajez <i>et al.</i> ⁵⁶
Hepatocellular cancer	SLIT1, SLIT2, and SLIT3 genes were methylated	Tumor-suppressor	Zheng <i>et al.</i> ⁵⁷
Hepatocellular cancer	ROBO1↑; ROBO2↑; ROBO4↓; SLIT3↓	Oncogenic	Avci <i>et al.</i> ⁴¹
Cervical cancer	SLIT1 ↓; SLIT2 ↓; SLIT3 ↓ ROBO1 ↓; ROBO3 ↓	Tumor-suppressor	Narayan <i>et al.</i> ⁵⁸
Gliomas	SLIT2 ↓	Tumor-suppressor	Dallol <i>et al.</i> ⁵⁹
Prostate cancer	SLIT1 ↑; ROBO1 ↓	Oncogenic	Latil <i>et al.</i> ⁴⁰

tumor formation, is important for cancer development. Recent studies indicated that the SLIT/ROBO signaling pathway plays a role in promoting tumor cell migration and, thus, promotes tumor metastasis. SLIT2 functions as a potent chemoattractant for breast cancer cells, inducing migration of cells expressing ROBO1. Furthermore, the SLIT2/ROBO1 signal was shown to upregulate MMP-9 to enhance breast cancer cell invasion.³⁹ Low mRNA expression of the ROBO2 was associated with poor patient survival, whereas high mRNA expression of ROBO3, a known inhibitor of ROBO2 signaling, demonstrated an appropriate reciprocal inverse association with poor survival in pancreatic cancer.⁴⁴ ROBO3 expression is upregulated in pancreatic cancer tissue samples and pancreatic cancer cell lines. Overexpression of ROBO3, which was associated with activated Wnt/β-catenin and GSK-3β, and other markers indicating epithelial-mesenchymal

transition (EMT), promotes pancreatic cancer cell growth, invasion, and metastasis *in vitro* and in mouse xenograft tumor models. MiR-383 was also identified as a suppressor of ROBO3, and its expression was inversely correlated with ROBO3.⁴⁵ To further explore the SLIT/ROBO signaling in tumor progression, Yang *et al.*⁴⁶ intercrossed SLIT2 transgenic mice with a nonmetastatic RIP1-Tag2 mouse tumor model. They found that transgenic overexpression of SLIT2 significantly enhanced tumor lymph angiogenesis and subsequently promoted mesenteric lymph node metastasis of pancreatic islet tumors. SLIT2 was expressed minimally in normal and hyperplastic mucosa, moderately in dysplastic mucosa, and highly in neoplastic mucosa obtained from hamster buccal pouch in a multi-stage model of 7,12-dimethyl-1,2-benzanthracene-induced squamous cell carcinoma, and increased SLIT2 expression was associated with higher tumor angiogenesis.⁴⁷

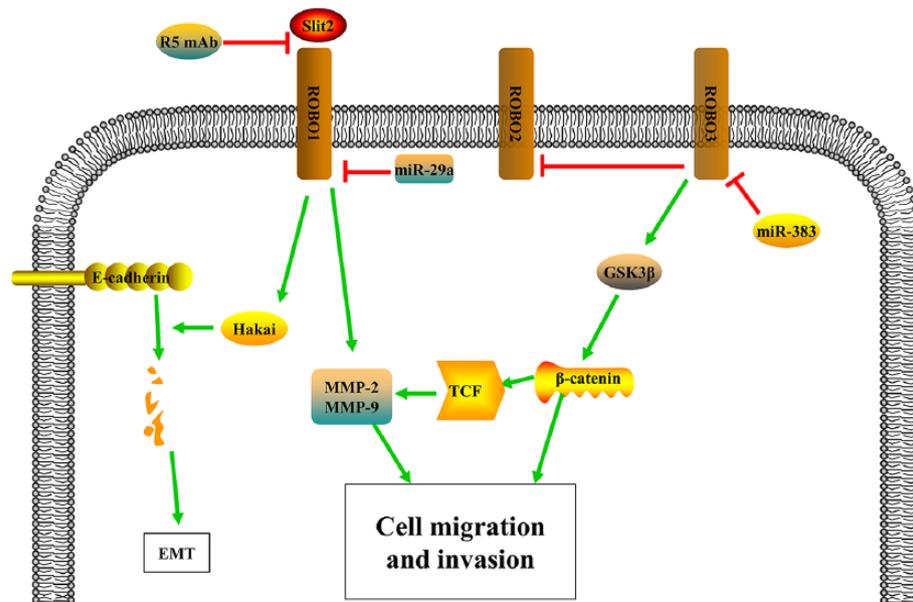


Figure 2. Mechanisms of the SLIT/ROBO pathway as an oncogene in cancer. The SLIT2/ROBO1 signal upregulates MMP-2 and MMP-9, thus promotes cell migration and invasion. SLIT2/ROBO1 signal recruits a ubiquitin ligase Hakai for E-cadherin ubiquitination and lysosomal degradation and thus promotes the epithelial-mesenchymal transition (EMT). The monoclonal antibody R5, which can interrupt the SLIT2/ROBO1 pathway, causes significantly suppressed cell growth and proliferation. ROBO3 promotes cancer cell growth, invasion and metastasis, which is associated with activated Wnt pathway components, β -catenin and GSK-3 β , and other markers indicating the EMT, and miR-383 functions as a suppressor of ROBO3.

Importantly, interruption of the SLIT2–ROBO1 interaction using R5 inhibited tumor angiogenesis and growth in an *in vivo* model. Therefore, targeting SLIT/ROBO signaling may offer a novel approach for oral cancer therapy (Figure 2).

As is well known, the EMT is one of the initiating steps that play a key role during tumor invasion and metastasis. In colorectal epithelial carcinoma cells, recombinant SLIT2 inducing ROBO1 expression recruited a ubiquitin ligase Hakai for E-cadherin ubiquitination and lysosomal degradation, thus promote the EMT, tumor growth, and liver metastasis. Moreover, this effect can be attenuated by knockdown of Hakai.³⁷

Therefore, the SLIT2, ROBO1, and ROBO3 may function as oncogenes that promote cancer proliferation and metastasis which may provide potential target structures for the antitumorigenic and anti-angiogenic therapy of these special carcinomas.

SLIT/ROBO pathway as tumor suppressor genes

Compared with several studies on promoting tumor progression, SLITs and ROBOs are tumor

suppressor genes in some special tumors. Here we further elucidate the anticancer function of the SLIT/ROBO pathway.

It has been shown that SLIT/ROBO pathway genes are frequently inactivated by promoter region's hypermethylation, resulting in down-regulated gene expression in many human cancers.^{58,60–62} SLIT2 was methylated in 71% (5/7) of glioma cell lines and in 59% (37/63) of other tumors and the SLIT2 expression was downregulated in methylated gliomas tumor samples, which indicated that SLIT2 was frequently inactivated by promoter region CpG island hypermethylation in gliomas and might be a good candidate for a glioma tumor suppressor gene.⁵⁹ SLIT2 expression was reduced in CRC tissues because of hypermethylation of the SLIT2 gene in CRC cells, and SLIT2 could inhibit CRC cell migration that required USP33 by deubiquitinating and stabilizing ROBO1.⁵¹ Another study also showed that USP33 was downregulated in lung cancer patients and that low expression of USP33 was associated with poor prognosis, which may be associated with reduced protein stability of ROBO1 in lung cancer cells.⁵⁰

SLITs and ROBOs inhibit cancer cell proliferation and invasion

Basal SLIT2, SLIT3, ROBO1, ROBO2, and ROBO4 expression level was lower in primary cultures of ovarian cancer epithelial cells when compared to normal OSE and in poorly differentiated SKOV-3 cells compared with the more differentiated PEO-14 cells. Furthermore, blocking SLIT/ROBO activity reduced apoptosis in both PEO-14 and SKOV-3 tumor cells.⁵⁴ SLIT/ROBO signaling was shown to decrease the proliferative rate and increase the apoptotic rate of the oral squamous cell carcinoma line Tb through regulating Fas-FasL proteins, and this effect could be interrupted by R5 which could neutralize the binding of ROBO1 to SLIT2.⁶³

The overall expression of SLIT3 is low in lung tumor tissues compared with normal tissues. Silencing of SLIT3 induced EMT by downregulation of E-cadherin and upregulation of vimentin, and enhanced MMP2 and MMP9 expression, thus promoting proliferation, migration, and invasion of A549 cells.⁴⁹ In addition, treatment with SLIT3 led to strong inhibition of migration in malignant melanoma cells, and downregulation of AP-1 activity and target gene expression contributed to the negative regulation of migration.⁶⁴ In pancreatic cancer, SLIT2 mRNA expression was reduced in human pancreatic ductal adenocarcinoma (PDAC) and correlated with lymphatic metastasis. SLIT2 inhibited directed migration and invasion of PDAC cells *in vitro*. To further address the effects of SLIT2 on PDAC growth and progression *in vivo*, MiaPaCa2-SLIT2 cells were grown as orthotopic xenografts and it was found that SLIT2 and ROBO1 inhibited invasion, metastasis, and angiogenesis of PDAC xenografts *in vivo*. Recombinant human SLIT2 dose-dependently decreased migration of oral squamous cell carcinoma (OSCC) cells PCI52-PC, which might be associated with an interaction of P-cadherin with ROBO3, a high affinity receptor of SLIT2. Downregulation of ROBO3 expression *via* siRNA neutralized SLIT2 induced migration block in PCI52-PC cells. This phenomenon demonstrated that the effect of SLIT2 on P-cadherin expressing OSCC cells was supposedly *via* modulation of ROBO3 interaction.⁵⁵

SLIT2/ROBO1 pathway interrupts the HGF/c-MET mediating cancer progression

The hepatocyte growth factor (HGF) and its receptor, the transmembrane tyrosine kinase c-MET,

promote cell proliferation, survival, motility, and play a crucial role both in tumor progression.⁶⁵ The shRNA-mediated depletion of SLIT2 or ectopic expression of a soluble decoy ROBO enhance HGF-induced migration, matrix invasion, accompany with the upregulation of Cdc-42 and the downregulation of Rac-1 activities. Accordingly, autocrine overexpression or exogenous administration of SLIT2 prevent HGF-induced motile responses, reduce Cdc-42 activation, and stimulation of Rac-1.⁶⁶ In addition, medulloblastoma invasion was inhibited after recombinant SLIT2 protein treatment, which was accompanied with downregulation of activated Cdc42.⁶⁷

SLIT2/ROBO1 pathway prevents the CXCL12/CXCR4 induced cancer progression

Upregulation of CXCR4 is associated with poor prognosis in breast cancer and pancreatic cancer. Marlow *et al.* showed that loss of SLITs (SLIT2, SLIT3) or their ROBO1 receptor in murine mammary gland or human breast carcinoma cells resulted in coordinate upregulation of the CXCL12 and CXCR4 signaling axis, which was accompanied by hyperplastic changes in cells and desmoplastic alterations in the surrounding stroma. Furthermore, SLIT overexpression downregulated CXCR4 and dominantly suppressed tumor growth in a xenograft model.⁶⁸ SLIT2 treatment inhibited CXCL12/CXCR4-induced breast cancer cell metastasis. SLIT2 inhibited CXCL12-induced tyrosine phosphorylation of focal adhesion components such as RAFTK/Pyk2 at residues 580 and 881, focal adhesion kinase at residue 576, and Paxillin. It was also found that SLIT2 inhibited CXCL12-induced phosphatidylinositol 3-kinase (PI3K), p44/42 MAPK, and MMP-2 and MMP-9 activities, but it did not have an effect on JNK and p38 MAPK activities.⁶⁹ These studies classified SLITs as negative regulators of the CXCL12/CXCR4 pathway and identified a molecular signature in hyperplastic breast lesions that signified inappropriate upregulation of key prometastatic genes.⁶⁸

In addition, there were some studies that revealed that SLIT/ROBO signaling might not play an important role in regulating human cancer cell proliferation and migration. Dai *et al.* found that three major members (SLIT2, SLIT3, and ROBO1) of the SLIT/ROBO family were widely expressed in the human normal and malignant ovarian tissues and in OVCAR-3 and SKOV-3 cells.⁷⁰ However, recombinant human SLIT2 did

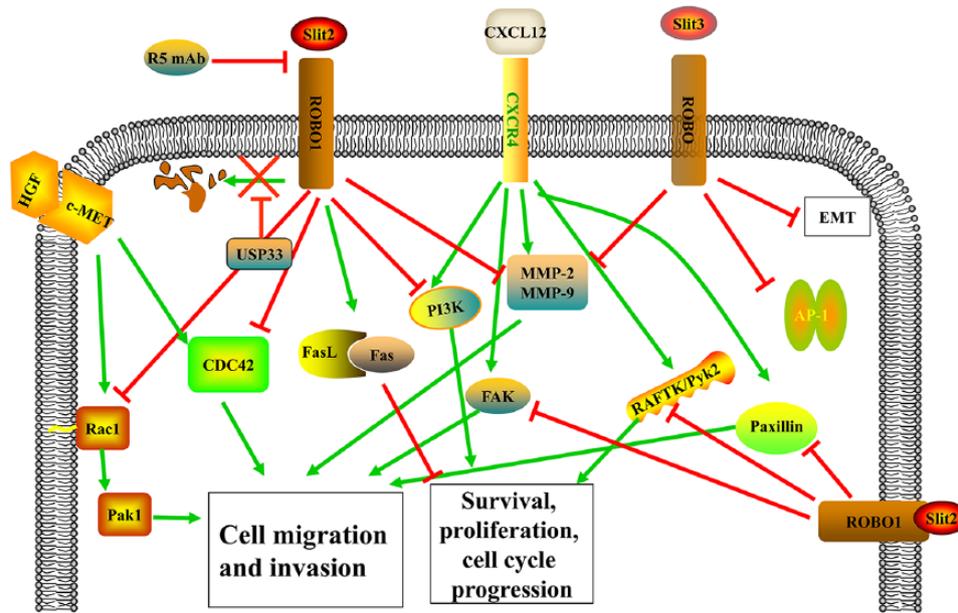


Figure 3. Mechanisms of the SLIT/ROBO pathway as a tumor-suppressor gene in tumor progression. SLIT2/ROBO1 signal decreases the proliferative rate and increases the apoptotic rate through regulating Fas-FasL proteins and PI3K/AKT pathway. USP33 can inhibit the lysosomal degradation of ROBO1, and SLIT3 leads to strong inhibition of migration through downregulation of AP-1 activity and targeting vimentin, MMP2, and MMP9. SLIT2/ROBO1 signal prevents hepatocyte growth factor (HGF)-induced motile responses, reduces Cdc-42 activation, and stimulates Rac-1 activities and, thus, inhibits cell proliferation, survival, and motility. SLIT2/ROBO1 functions as negative regulators of CXCL12/CXCR4 pathway through inhibiting CXCL12-induced tyrosine phosphorylation of focal adhesion components such as RAFTK/Pyk2, FAK, and Paxillin.

not significantly affect SKOV-3 cell migration, and OVCAR-3 and SKOV-3 cell proliferation. SLIT2 also did not induce ERK1/2 and AKT1 phosphorylation in OVCAR-3 and SKOV-3 cells. Sanz-Pamplona *et al.* also demonstrated that no significant association between SLIT2 or SLIT3 level of expression and prognosis was found in colon cancer.⁵²

In short, these inconsistent results showed the complexity of the role of the SLIT/ROBO signaling pathway in tumor proliferation. More thorough and careful studies are needed to clarify the precise role of this pathway (Figure 3).

Downstream and crosstalk of the SLIT/ROBO pathway

SLIT/ROBO signaling is important for tumor development, but its mechanisms in tumors remain unclear. There are several molecules that have been confirmed to be downstream factors of the SLIT/ROBO pathway. A study in *Drosophila* provided evidence that both the Abelson (Abl) tyrosine kinase and its substrate Enabled (Ena), which binds directly to ROBO's cytoplasmic domain, played

opposing roles in ROBO signal transduction. Abl and Ena may bind CC2, which was partly responsible for ROBO repulsion in the midline, whereas Abl antagonized ROBO-mediated repulsion by phosphorylating a tyrosine residue in CC1.⁷¹ According to their work, Wong *et al.* showed SLIT bound ROBO (ROBO1) through the CC3 motif and the C-terminal sequence of ROBO to interact with SLIT-ROBO GTPase activating proteins (srGAPs), a new member of the Rho GTPase activating proteins (GAPs). They further showed that srGAPs inactivated Cdc42, resulting in reconstruction of cytoskeletal proteins *via* regulation of actin polymerization to inhibit cell migration in the mammalian nervous system. Ubiquitin-specific protease 33 (USP33)/VDU1 was also shown to be involved in ROBO1-USP33 interaction and participate in SLIT/ROBO signaling in cancer cell migration.⁷² Another study also showed that srGAP1 is an important downstream molecule of Slit2 signaling in CRC, and mediates the antimigration function of Slit2 by inhibiting Cdc42.⁷³ SrGAP2 protein expression is reduced or absent in a subset of primary osteosarcoma samples, srGAP2 and other axon guidance proteins likely play a role in osteosarcoma metastasis.⁷⁴

The intracellular signal transduction pathway that includes Abelson kinase, Enabled protein, GAPs, and the Rho family of small GTPases may also play a role in endothelial cells. In addition, PI3K is important for endothelial cell responses to SLIT2, but the mechanism remains unknown.⁷⁵ Apart from this, it was shown that cellular protrusive activity was inhibited *via* a SLIT2–ROBO4–paxillin–GIT1 network.⁷¹

The study led by Zhang and Zhou demonstrated that downregulation of ROBO1 using small interfering RNA inhibited mesenchymal stem cell (MSC) proliferation.⁷⁶ In addition, four miRNAs (miR), including miR-218, miR-29a, miR-146, and miR-148, inhibited the protein expression of ROBO1 in the MSCs, with miR-29a having the most marked effect. ROBO1 was identified as a novel target of miR-29a with a luciferase reporter assay. Overexpression of miR-29a suppressed the protein expression levels of ROBO1 and SLIT2 and inhibited the viability and proliferation of the MSCs. By contrast, overexpression of ROBO1 partly rescued these inhibitory effects of miR-29a on the MSCs. These results indicated that the miR29a/ROBO1 axis was crucial for the regulation of MSC viability and proliferation, suggesting that miR29a may serve as a potential clinical target for MSC expansion and stem cell transplantation. In addition, miR-218 suppressed nasopharyngeal cancer progression through downregulating the SLIT2–ROBO1 pathway in a negative feedback loop manner.⁵⁶

The role of SLIT/ROBO in the tumor microenvironment

The tumor microenvironment is composed of tumor cells, stromal cells, and other cellular components as well as extracellular matrix and various molecules that mediate intercellular interactions. These components form a complex tumor microenvironment that prominently affects the occurrence, growth, invasion, metastasis, and drug resistance.⁷⁷ Here we further investigate the role of SLIT/ROBO pathway in the tumor microenvironment.

SLIT/ROBO pathway in tumor angiogenesis

The SLIT/ROBO signal is critical for axon guidance and neuronal precursor cell migration in the nervous system. Evidence suggested that classical neuronal guidance cues also regulated vascular development.⁷⁸ Expression of ROBO1 and

ROBO4 has been observed in vascular endothelial cells. They may serve different functions in SLIT/ROBO signaling due to differential expression in various phenotypes of endothelial cells, ROBO1 induced long and thin actin fibers, whereas ROBO4 induced short and thick actin bundles along with membrane ruffles.⁷⁹ SLIT3, which was also observed in endothelial cells and vascular smooth muscle cells, promoted angiogenesis but decreased neurogenesis.⁸⁰ Here, we focus on SLIT/ROBO signaling in angiogenesis *via* SLIT/ROBO1 and SLIT/ROBO4. The SLIT2 was expressed in a broad spectrum of tumor cell lines and interacted with human umbilical vein endothelial cells (HUVECs) and tumor-associated endothelial cells in the presence of ROBO1. Tumor cells appeared to secrete the SLIT2 protein, which formed a gradient field for the attraction of endothelial cells through interaction with ROBO1 on endothelial cell surface. Thus, endothelial cells migrated toward tumor and formed new blood vessels.¹⁶ The SLIT/ROBO signaling inhibited tumor angiogenesis and growth in a model of chemically induced squamous cell carcinoma when blocked by R5, a monoclonal antibody against the first immunoglobulin domain of ROBO1.⁴⁷ The observation that ROBO1 was highly expressed in the early postnatal days of C57BL/6J mice correlated with superficial vascular and deep vascular plexus formation, indicating that ROBO1 might participate in retinal neovascularization.⁸¹ Thus, SLIT/ROBO1 signaling may promote angiogenesis in tumors.

ROBO4 is a vascular-specific receptor and mainly expressed in active angiogenesis, especially tumor vessels.^{8,9,82} Previous studies showed that ROBO4 inhibited endothelial cell migration through interaction with SLIT2, suggesting that ROBO4 might negatively regulate new vessel formation. This point was supported by several studies focusing on HUVECs, microvascular endothelial cells,^{83–85} and an animal model of ocular angiogenesis.⁸⁶ In addition, Acevedo *et al.* suggested that SLIT2 could both positively and negatively regulate angiogenesis by binding to ROBO1 and ROBO4, respectively, and activation of ROBO4 blocked vascular endothelial growth factor (VEGF)-induced angiogenesis and vascular permeability.⁸⁷ However, a study by Huang *et al.* suggested that ROBO4 expressed much in fibrovascular membranes (FVMs), and loss of ROBO4 disturbed tube formation.⁸⁸ Knockdown or overexpression of ROBO4 impaired intersomitic vessels formation in zebrafish showed contradictory functions

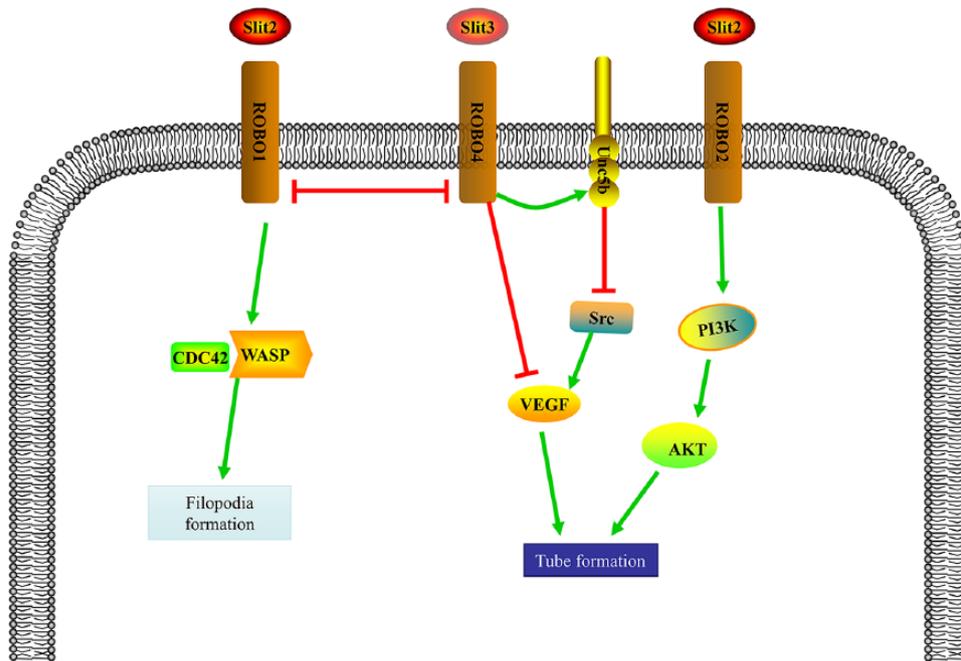


Figure 4. Mechanisms of the SLIT/ROBO pathway in tumor angiogenesis. SLIT2 and SLIT3 lead to bidirectional adjusting of angiogenesis. Through modulating the activity of ROBO1, ROBO2, ROBO4, Src, and vascular endothelial growth factor (VEGF) pathway, endothelial cell behaviors, including tube formation and filopodia formation, could be regulated.

of ROBO4 in angiogenesis.⁸⁹ Furthermore, using siRNA knockdown of both ROBO1 and ROBO4 decreased endothelial motility and disrupted tube formation.⁷⁹

Taking these studies together, it is suggested that SLIT/ROBO1 and SLIT/ROBO4 signaling might interact with each other and cooperate in tumor angiogenesis (Figure 4).

SLIT/ROBO signaling in inflammation

SLIT/ROBO signaling influences neuronal migration, axon guidance, and also functions in a similar way in directing inflammatory cells discrepantly. SLIT2 is involved in inflammation modulating in several ways. SLIT2/ROBO1 selectively impaired directional migration of neutrophils and T cells toward chemoattractant especially CXCL12/CXCR4-induced chemotaxis^{90,91} and neutrophil recruitment,⁹² and also mediated inhibition of Langerhans cell migration resulting in suppression of contact hypersensitivity responses.⁹³ However, during lung inflammation, SLIT2/ROBO1 led to enhancement of eotaxin-induced eosinophil chemotaxis, and exaggeration of allergic airway inflammation.⁹⁴ Furthermore, through SLIT2/ROBO4 interaction, SLIT2 could regulate endothelial

related inflammation by inhibiting vascular leak and stabilizing the vasculature by downregulating lipopolysaccharide or VEGF.^{85,95} In patients with acute kidney injury (AKI), higher levels of plasma ROBO4 suggested a link between endothelial dysregulation and onset of AKI.⁹⁶ SLIT3 took a role in increasing the spontaneous and chemoattractant-induced migration of primary monocytes by inducing a chemokinetic effect.⁹⁷

SLIT/ROBO particles have shown their potentials as markers or targets in treating inflammatory diseases such as periodontitis, AKI, and crescentic glomerulonephritis (Figure 5).^{96,98}

SLIT/ROBO plays a dual role in tumor neural invasion

Perineural invasion (PNI) is considered as an alternative route for the metastatic spread of pancreatic cancer cells; however, the molecular changes leading to PNI are still poorly understood. Andreas *et al.* showed that disrupting SLIT2–ROBO signaling in PDAC might enhance metastasis and PDAC cells to neural invasion. It has been found that a reduction in SLIT2–ROBO pathway activity existed in PDAC and restoring the SLIT2 expression in SLIT2-deficient PDAC

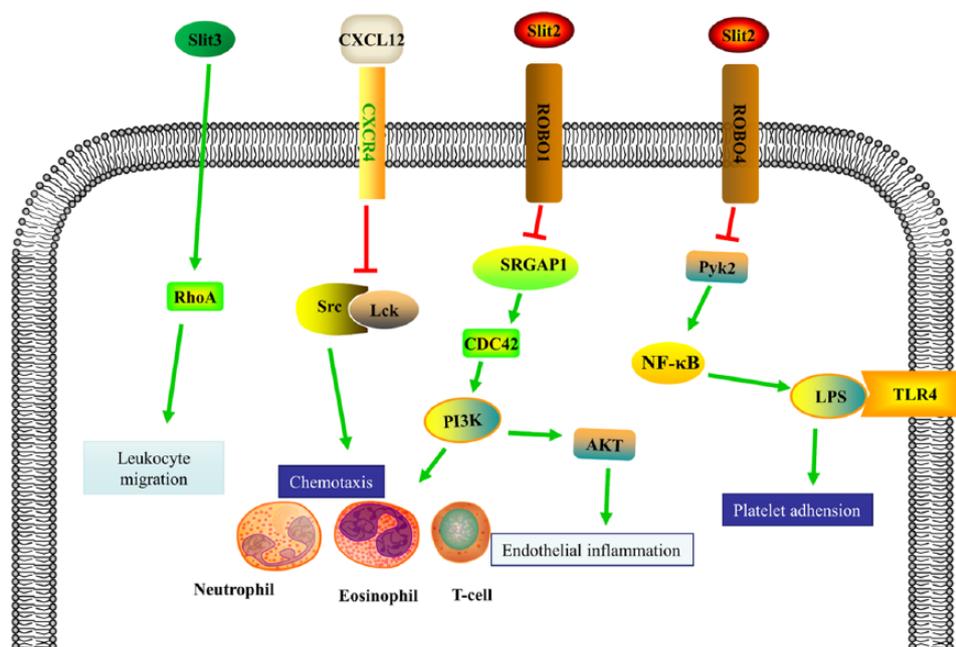


Figure 5. Mechanisms of the SLIT/ROBO pathway in tumor inflammation process. SLIT2 is involved in inflammation modulated by ROBO1, ROBO4, and AKT pathway. The downstream pathways of ROBO1 and ROBO4 including PI3K and nuclear factor (NF)- κ B pathways involve in chemotaxis of inflammatory cells (T cells, Langerhans cells, neutrophils, and eosinophils) and angio-associated components (endothelial cells, platelets). SLIT3 regulates CXCL12/CXCR4 and RhoA in modulating inflammation, chiefly monitoring inflammatory migration and chemotaxis.

cells inhibited their bidirectional chemoattraction with neural cells.⁴⁸ However, another group showed that SLIT2 was expressed by cancer-associated fibroblasts (CAFs), increasing neurite outgrowth from dorsal root ganglia neurons as well as from Schwann cell migration or proliferation. Inhibition of SLIT2/ROBO signaling disrupted this stromal/neural connection.⁹⁹

The controversial effects of SLIT/ROBO on tumor neural invasion reflect its complicated role in tumor progression and need to further be explored.

Conclusion

There is a close relationship between abnormal signaling and tumor development. To develop effective cancer therapeutics, it is useful to understand how SLIT/ROBO signaling affects tumor formation and angiogenesis and the mechanisms by which SLIT/ROBO signaling exerts these effects. To develop an effective therapeutic approach for cancer treatment, additional studies are required, also taking into account the

important regulation mediated by SLIT/ROBO pathway.

Author Contributions

Zhengdong Jiang and Gang Liang conceived the concept for this paper. All authors (Ying Xiao, Tao Qin, and Xin Chen) collected the available literature. Erxi Wu, Qingyong Ma, and Zheng Wang revised the manuscript critically. Zhengdong Jiang and Gang Liang contributed equally to this work.

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Conflict of interest statement

The authors declare that there is no conflict of interest.

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