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REVIEW

Slit/Robo Signaling Pathway in Cancer; a New Stand Point for Cancer Treatment

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Abstract

Angiogenesis and metastasis are two critical steps for cancer cells survival and migration. The microenvironment of tumor sphere induces new blood vessels formation for enhancing tumor mass. Preexisting capillaries and postcapillary venules in tumors bring about new blood vessels. ROBO1-ROBO4 are transmembrane receptors family which act as guidance molecules of the nervous system. The SLITs family is secreted glycoproteins that bind to these receptors. SLIT-ROBO signaling pathway plays an important role in neurogenesis and immune response. Linkage between ROBOs and their ligands (SLITs) induce chemorepllent signal for regulation of axon guidance and leukocyte cell migration, recent finding shows that it is also involved in endothelial cell migration and angiogenesis in various type of cancers. In this article we review recent finding of SLIT-ROBO pathway in angiogenesis and metastasis.

Keywords Slite · Robo · Tumor angiogenesis · Tumor markers

Introduction

Angiogenesis is a cellular process that induces the configuration of neovasculation that is a vital pathway in the developing embryo, wound healing, formation of granulation tissue and fundamental step in transition of benign state to a malignant one in tumors. Ischemic chronic wounds result from loss or defect of blood vessel formation, contrariwise in age-related macular degeneration create a local expansion of blood vessels.

Today, there are evidence of similarities between axonal migration and the formation of new vessels. For the first time Ramon y Cajal in the late 1800s indicated the presence of axon guidance molecules [1]. These molecules act to instruct the migration of axon in nervous system. Growth cones in axons move to their environment by reaction to repulsive and attractive signals. Recent studies have been shown the role of similar signaling molecules such as Neuropilin/Semaphorin, Ephrin/Eph recptor and Notch/Delta gene families in migration of both nerves and blood vessels and the fundamental effect of attractive and repulsive cues on formation of both vascular and nervous system. In addition, these molecules also function on the other organs, including immune system, mammary gland, heart, lung and kidney [2–4].

Another class of guidance molecules, Slits and their receptors (Robo) are a small group among the super families of axon guidance molecules [5]. These are also involved in the most invasive tumor cells and inflammation [6–8]. Expansion of tumor mass requires formation of new blood vessels in tumor microenvironment [9]. Indeed, there is an intimate crosstalk between tumor and endothelial cells for tumor development and metastasis. Tumor development requires the infiltration of endothelial cell into tumor mass as nourishing and oxygenation and metastasis requires the invasion of tumor cells into blood vessels. Fulfillment of the extensive studies indicate the role of angiogenic factors, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and cell adhesion molecules such as cadherins and integrins in this step [10].

Recent studies have uncovered a novel mechanism for angiogenesis and metastasis in cancer in the base of Slit/Robo signaling pathway. As we learn tumor angiogenesis is a necessary process for migratory properties of the most cancers,

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surprisingly inhibition of this pathway is an excellent method for cancer therapy.

Therefore, studying about Slit/Robo signaling pathway have been a major effort for the cancer researchers in over the last decade. Here, we explore the Robo/Slit signaling pathway and we indicate the dual role of this signaling pathway both as tumor suppressor and oncogene. We also show promising and potential therapeutic targets against angiogenesis and metastasis.

Slits and Roundabouts

SLIT1, SLIT2, and SLIT3 are three members of Slit family that act as guidance cues in neuronal system, angiogenesis and metastasis in most cancers [1]. Identified members of SLITs were originally detected in Drosophila through a genetic screening [11]. Slits are secreted glycoproteins with highly conserved domain across species [12] that their N-terminal contains four leucine-rich repeat (LRR) domains (D1-D4), followed by seven to nine domains which are EGF-like domains and a laminin G-like domain/Agrin-Perlecan-Laminin Slit (ALPS), their C-terminal domain contains of cysteine rich module [13]. Active N-terminal fragment is released by proteolitic cleavage in the EGF region [14]. SLITs attach to roundabout receptors (Robo) and induce signaling pathway which remain poorly understood. While the function of Slit2 is the regulation of tissue morphogenesis, proliferation and death, the function of SLIT1 is unknown. Studies have shown foot-print of Slit3 in endothelial proliferation and mobility, in addition to regulatory effect on the formation of vascular network [15].

ROBOs are reseptors of IgCAM superfamily which are highly protected during evolution [16]. SAX-3 is a ROBO ortholog in *Caenorhabditis elegans*, three ROBO exists in chick and xenopus and four members of Robo (Robo1, Robo2, Robo3, and Robo4) appeared in mammals [17]. High levels of Robo2 and Robo3 are expressed in the nervous system but none of them is in the vascular system. Robo1 expression has been shown in both systems. Robo4 also called magic Robo which is specific receptor in endothelial cells. Robo receptor family are single-pass transmembrane receptors without autocatalytic or enzymatic activity, suggesting that signal transduction in these receptors mediated by scaffolding and downstream molecules [13].

ROBO1–3 has five immunoglobulin (IgG) domains (ectodomain) and three fibronectin domains (FNIII 1–3) in extracellular region but Robo4 has two and two respectively. Cytoplasmic domains in Robos contains four cc conserved intraspecies motifs,cc0-cc3, (cytoplasmic conserved) [13], although quite variable interspecies [18] these domans in ROBO1–2 are (cc0-cc3) but Robo3 lacks cc1 and ROBO4 has only two cc motifs (cc0,cc2) [19]. ROBO2 and ROBO3 are highly expressed in the nervous system but not in the vascular system [20]. ROBO1 has been shown to be expressed in both systems while ROBO4 is only expressed in vascular endothelial cells [21].

SLITs are able to form homodimer via LRR4 domain, although for binding to the ROBOs,the presence of LLR2 domain is essential. SLITs are also able to participate in additional heterophilic connection to some ECM molecules such as, Glypican, Netrin1, Neurexins, Syndecan and Ttpe IV Collagen. Heparan sulfate proteoglycans through connection to both Slit and Robo, play an important role in forming tertiary complex and stabilizing Slit-Robo interaction at the membrane [22, 23].

Slit-Robo Proteolytic Pathway

Proteolytic processing has been shown to be involved in Slit-Robo signaling pathway. Binding of ECM-immobilized Slit causes a change in the structure of the three-dimensional Robo1 [13]. After this change in Drosophila metalloprotease Kuzbanian (ADAM10 in mammals) cleaves at the conserved juxtamembrane region of Robo in human [24] and Drosophila [25]. This extracellular cleavage generates a free ectodomain. Then, intracellular cleavage by a γ-secretase leads to C-terminal fragment translocation to the nucleus [24]. There are several nuclear localization signals (NLSs) within these fragments of Robo1 that suggest the possibility of transcriptional role for the receptor, at least in cancer cells.

SLITs are also cleaved by an unknown protease and split into two different size of fragment: large N-terminal (Slit-N) and short C-terminal (Slit-C). These fragments are the same in Drosophila and vertebrate, suggesting that the cleavage site is conserved between the two species [5]. Different functions have been seen for various forms of Slit. Full length Slit (Slit-FL) and Slit-N act more as ligand that are associated with the cell surface, whereas Slit-C is mostly release into the extracellular space [5] . When Slit-FL and Slit-N binding to Robo receptors act as chemorepulsion, Slit-FL antagonizes the Slit-N-dependent induction of branching in sensory axons [26, 27]. Slit-C is able to bind to a new Slit receptor that is called Plexin A1.This connection induce growth cone collapse of spinal cord commissural axons in mouse [13, 28]. Slit-C also binds to the Dystroglycan (a scaffolding protein in basement membrane) [29]. Recently, researchesabout circulating Slit has been shown that Slit-2C actives PKA-dependent pathway and regulates adipose tissue thermogenesis and ameliorate glucose homeostasis [30].

The function of Slit fragments in Drosophila is still unknown but a recent study has shown the binding of Slit-N to Dscam1 (Down syndrome cell adhesion molecule 1), independent of ROBOs, leads to dephosphorylation via the phosphatase RPTP69D (Ptp69D) in intracellular domain of the receptor which causes the regulation of axon collateral extension in Drosophila mechanosensory neurons [31].

Slit and Robo Expressions in Cancer

Cancer studies have proved that the Slit-Robo signaling pathway plays a fundamental role by high expression of both Robos and Slits in tumors [32–35]. Researchers indicate a novel mechanism in tumor angiogenesis. They demonstrated that tumor cells release Slit proteins to attract vascular endothelial cells and the mentioned cells consequently express Robo receptors in response to Slit secretion [34]. Ballard and Hinck [1] also have showed dual role of this signaling pathway in tumor angiogenesis and metastasis. Other literatures have demonstrated that these dual role of a guidance molecule is dependent on its accompanying molecules. On the other hand downregulation of Slit1–3 and Robo1,3 genes via hypermethylation within the promoter region in non-small cell lung, breast, ovarian, glioma, hepatocellular, and colorectal cancers and leukemia, in several studies reported that emphasizes a tumor suppressor role for Slit-Robo signaling pathway [1, 36]. However, some studies have shown upregulation of Slits in ductal carcinoma, prostate cancer, and lobular breast cancer that underline oncogenic role of in this pathway [1, 16]. Indeed, Slit/Robo signaling pathway can act as both of tumor cell promoting and suppressing.

Slit/Robo Signaling Effect in Angiogenesis Suppressing

Monomeric GTPase and proteins that involve in formation of cytoskeleton like actin and microtubule, have an important role in cell movement. Significant feature of a moving cell is polarity that means lack of molecular and shape uniformity in different parts of the cell. Binding of Epithelial Growth Factor (EGF) and Platelet Derived Growth Factor (PDGF) to their receptor promotes cell movement and proliferation in target cells. The downstream of Vascular Epithelial Growth Factor (VEGF) signaling pathway includes monomeric GTPase proteins like Rho, Rac and Cdc42. Activation of these GTPase proteins are essential for VEGF induced epithelial cell migration and permeability. ROBO4 counteracts the VEGF signaling by following mechanisms:

First, studies have shown that expression of Robo4 and interaction with Slit2 and MENA (effector of Slit/Robo signaling) in endothelial cells, triggers the inhibition of angiogenesis [37] suggesting that Slit2/Robo4 pathway can inhibits proangiogenesis signaling factors such as VEGF. As Picher reported, Slit2-Robo4 signaling pathway maintains the vessel integrity and the presence of this route lead to an overall survival in early stage non-small cell lung cancer [38]. (Fig. 1a).

Second, Jones et al. [39] have shown that treatment with Slit2 has no inhibitory effect in Robo4 deficient mice on angiogenesis and contrary to Robo1, Slit2 binding sites in Robo4 receptor are not conserved. This suggests that Slit2 affinity to Robo1 is more than Robo4. In deduction of these result, It's logical suggesting that Robo4 responsiveness to Slit2 has been mediated by Robo4-Robo1 heterodimerization [39, 40]. (Fig. 1b) Anti-Robo1 and Anti-Robo4 blocking antibody that inhibited interaction of Slit2 in a recent study confirms the implications of both the Robo1 and Robo4 [41]. Therefore interaction between Slit2 with Robo1-Robo4 heterodimer also enhances the vascular integrity [42].

Src family kinases are non-receptor tyrosine kinases that are actived by VEGFR2. Indeed, Src family are the VEGF signaling downstream to the VEGFR2 that are essential for Rac1 activation. VEGF induces endothelial cell migration through signal transduction related to Rac1 [43, 44]. The VEGF signaling downstream to VEGFR2 and upstream to Src family kinases (SFKs) has been counteracted by Slit2/Robo4 signaling pathway. In this case it is shown that Slit2 attachment to Robo4 leads to direct interaction of Robo4 cytoplasmic domain with the intracellular adaptor protein, called Paxillin, which makes a blocking cytoplasmic region that inhibits activation of GTPase ARF6 [45]. Blocking of ARF6, subsequently blocking Rac, inhibits VEGF signaling of inducing endothelial cell migration. (Fig. 1c).

SecinH3 is a pharmacological inhibitor of ARF6 activating proteins, GEFs [46, 47]. This drug is effective in arthritis and endotoxemia treatment and has enhancer effect in Robo4 function [47, 48]. ScinH3 also rescues ZO-1 expression. This protein involves in tight junction. Inflammatory cytokines, such as IL-1B and CCL2 that increase in tumors, cause leakage of endothelium by degradation of ZO-1 [49–52]. In some cancer such as breast cancer, degradation of ZO-1 provokes metastasis [53, 54]. Robust effects on SecinH3 by enhancing Robo4 signaling proved that high expression of Robo4 can inhibit angiogenesis, tumor growth and metastasis [55]. Enhancing Robo4 signaling also suppresses glioma-induced endothelial cells growth, migration and angiogenesis [56]. Studies have shown that Robo4 expression in ovarian cancer epithelial cells [57], hepatocellular carcinoma tissues [58] was lower than normal ovarian epithelium and normal liver tissues.

Interaction between Robo4 with UNC5B, a vascular receptor is another way that counteracts the VEGF signaling [59]. In this context, interaction between abovementioned receptors itself inhibit the VEGF induced pathological angiogenesis without Slit2 as the interaction partner. This independent interaction takes place in both of extracellular domain of Robo4 and UNC5B [60]. This may be trans contact (between two neighboring cells) or sis contact (in a single cell), therefore treatment with extracellular soluble Robo4 may be a promising course for inhibition of VEGF induced angiogenesis and vascular leak [59, 60]. (Fig. 1d).

Here, Slit2/Robo1-Robo4 signaling pathway and interaction between Robo4/UNC5B inhibit pathological angiogenesis and can be considered for treatment.

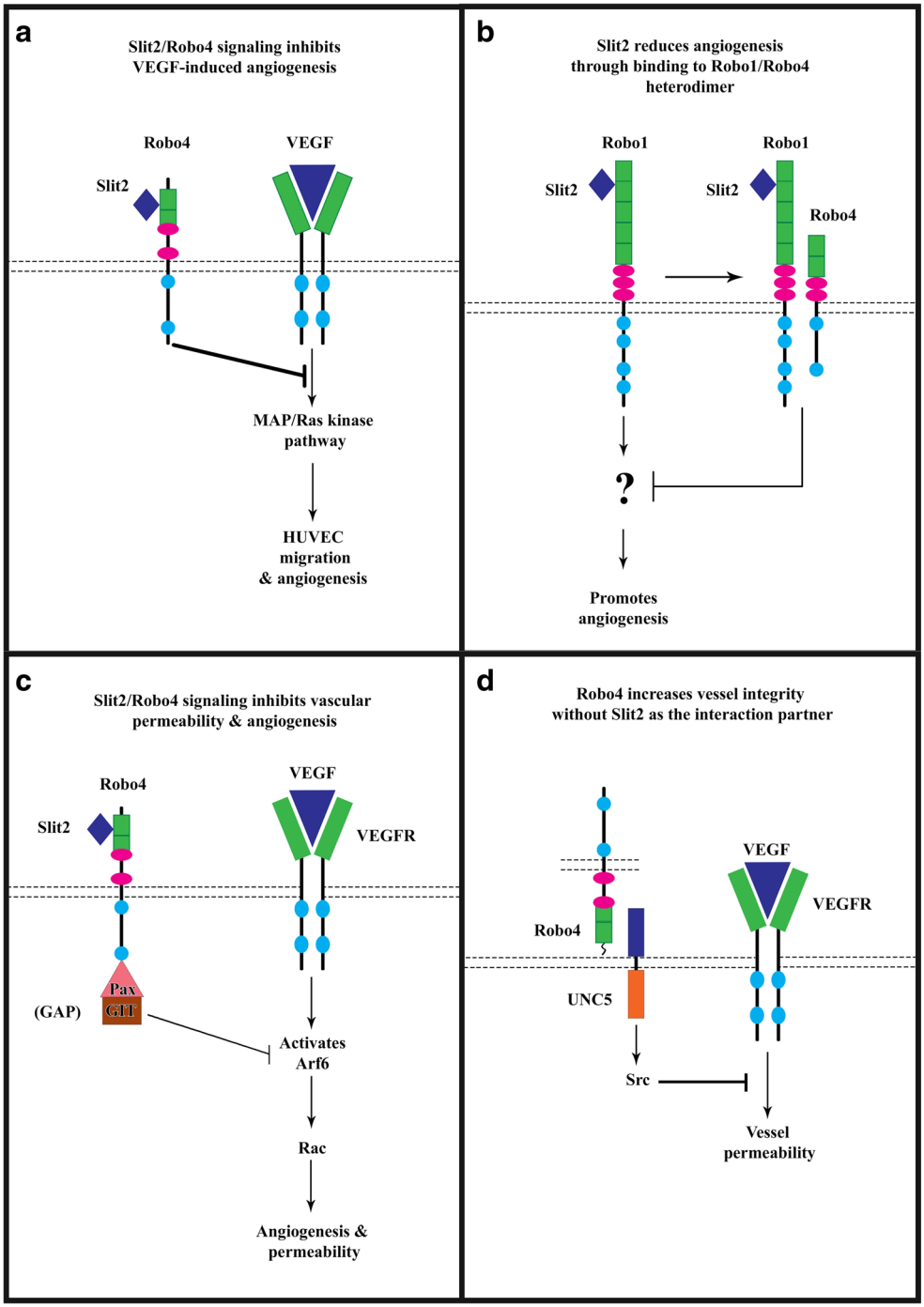

Fig. 1 The effect of Slit/Robo signaling pathway on angiogenesis: **a** Slit2-Robo4 signaling pathway maintains the vessel integrity by inhibiting MAPK/ERK kinase pathway. **b** Interaction between Slit2 with Robo1-Robo4 heterodimer reduces the angiogenesis that mediated by Slit2/Robo1 signaling pathway. **c** Blocking of ARF6 and subsequently Rac mediated Slit2/Robo4 inhibits angiogenesis and permeability. **d** Trans or sis contact between extradomain of Robo4/UNC5 inhibit the VEGF induced pathological angiogenesis without Slit2

Slit/Robo Signaling Effect in Angiogenesis Promoting

As we discussed Robo4 signaling enhances vascular integrity via counteracting the VEGF signaling pathway. Contrary to this policy, some studies show evidence that Robo4 signaling enhances the pathological angiogenesis. Slit2 expression also was observed on the angiogenesis sites in various type of cancer. Transfection of human A375 melanoma cells for a high level of Slit2 expression induced by soluble Robo-1 or blocking of Slit/Robo signaling with antibody have shown angiogenesis and tumor growth inhibitory effect respectively [61].

Robo4 plays an essential role to induce filopodia formation through the cytoplasmic actin, a fundamental event of cell migration, angiogenesis and metastasis. Angioblasts that result from Robo4 deficient embryos show low activity of CDC42 [42]. Studies results also indicate that both

extracellular and intracellular domains of Robo4 are essential for CDC42 activation [20].

CDC42, Rac, Rho are members of G-proteins super family that have GTPase activity. These monomeric GTPase that induce polymerization of different actins are essential for cell movement. CDC42 is responsible for cell polarity and also induces actin polymerization that result in filopodia formation through Arp2/3 activation. Rac also recruits Arp2/3 for actin polymerization that result in lamellipodia formation. Both of these GTPases act in formation of cell leading edge. Rho is another member of this family that has at least two effects: 1.Stress fiber formation (non-branched actin) 2.Myosin activation for contraction.

Researchers show that in nervous system recruitment of guanine nucleotide exchange factor (GEFs) actives CDC42 and Rac, result in cell movement and recruitment of GTPase activating proteins (GAPs) inactivates CDC42-Rac, and inhibits cell movement [42]. Slit2 has high binding affinity to Robo1 and recently, it was uncovered that interaction between Slit2-Robo1 suppress CDC42 activation [62] because this interaction induces Rho GTPase activating proteins (srGAPs) to attach to cc3 domain in Robo1,this connection means the promotion of intrinsic GTPase activity of CDC42 in cell leading edge [63].

Consequently, asymetric actin polymerization result in the cell movement away from Slit2 source, induced by repulsive signaling [64–66].

Different amount of actin polymerization on cell proximal side and cell distal side bring about repulsion mechanism mediated by Slit2. Proximal side that has less actin than distal side moves in the opposite direction of Slit2 source [20]. Therefore Slit2-Robo1 pathway is a repulsion signaling in nervous system. How Robo4 makes an attracting signal in endothelial cell is a question to be addressed.

VILSE is a similar protein to srGAP that binds to Robo4 and it is possible that heterodimerization of Robo1-Robo4 prevents VILSE binding to Robo4 [20] . To understand the question raised up in the above paragraph, researchers focus on protein(s) that connect with Robo4 intracellular domains specially proteins that involved in actin polymerization. Proteins that involved in this pathway include the Wiskott-Aldrich syndrome protein (WASP), neuronal WASP proteins (NWASP), WASP-interacting protein (WIP) and syndapin [67]. Interaction of CDC42-GTP with these proteins is essential for the formation of filopodia and cell migration. These connections mediated by SH3 domain-containing proteins. One such protein is insulin receptor substrate protein 53 (IRSP53). IRSP53 has a CRIB motif that interaction between this motif and CDC42 removes an autoinhibitory effect of IRSP53 that allows the attachment of MENA to the IRSP53 SH3 domain [68]. Then, IRSP53-MENA complex binds to intracellular cc2 domain of Robo4 and mediates actin nucleation resulting in filopodia formation. So, Robo4 promotes cell movement in endothelial cells via interacting with Slit2/Robo1 complex.

Slit-Robo Signaling Pathway and Metastasis

Organization of distinct tissue in multicellular organisms require to two cellular interactions: between neighboring cells, extracellular matrix and cells. Cell adhesion molecules (CAMs) are transmembrane proteins that are attached together between two neighbor cells. Cadherins are the most important type of CAMs that mediated cell-cell contact by trans-interaction. Cytoplasmic domains of classic cadherins (E, M, P) are related with adaptor proteins like β-catenin, p120-catenin and α-catenin [69, 70]. Perturbation between these cadherins with adaptor proteins reduces cell-cell adhesion. Loss of cell-cell contact because of this and downregulation of one type of cadherin (e.g; E-cadherin) or switching of the expression among these family members (e.g; switching from E-cadherin to N-cadherin) have an important role in tumor progression and metastasis [71, 72].

Abelson (Abl) is a key cytoplasmic protein kinase that influences both actin and microtubule. In Drosophila, phosphorylation of Robo in CC1 domain by Abl inhibits Robo activity [13, 73] but another study has been shown a positive effect via Abl-interacting protein, Capulet [74] on repulsive midline guidance. More recent study argues that different domains of Abl are responsible for repulsive and attractive axon guidance [75].

In nervous system detected phosphorylation of β-catenin by Abl was resulted from formation of a multimeric protein complex between Robo2, N-cadherin and Abl with Slit1 engagement in this complex. Formation of this tertiary complex depends on a scaffold that mediated by Abl effector, Cables, lead to loss of N-cadherin-mediated cell adhesion [76]. (Fig. 2e) Connection between Slit2 and Robo1 has been shown to have different effect in various cancers. This connection has been shown to lead in both upregulation and downregulation of E-cadherin expression in different type of cancers. Recently, some studies have shown upregulation of Slit-Robo signaling pathway and subsequent induction of E-cadherin degradation via ubiquitin ligase Hakai in proteasome in colorectal cancer [16, 77]. (Fig. 2f) In contrary, loss of normal Slit-Robo signaling that lead to increase of E-cadherin/β-catenin complex through AKT/GSK3β/Snail pathway was shown in breast and lung cancers [16, 78, 79]. (Fig. 2g).

Epithelial-mesenchymal transition (EMT) is accompanied by low expression of E-cadherin results in loss of cell-cell contact. For example, in hereditary diffuse gastric cancer loss of E-cadherin function plays an important role in epithelial-malignant carcinoma transition. Many studies emphasize on the relationship between Slit-Robo signaling pathway and Wnt downstream signaling axis to modulate E-cadherin-dependent cell adhesion [78]. However, it remains unclear whether abovementioned pathway advocates or attenuates cell-cell contact and malignant transformation.

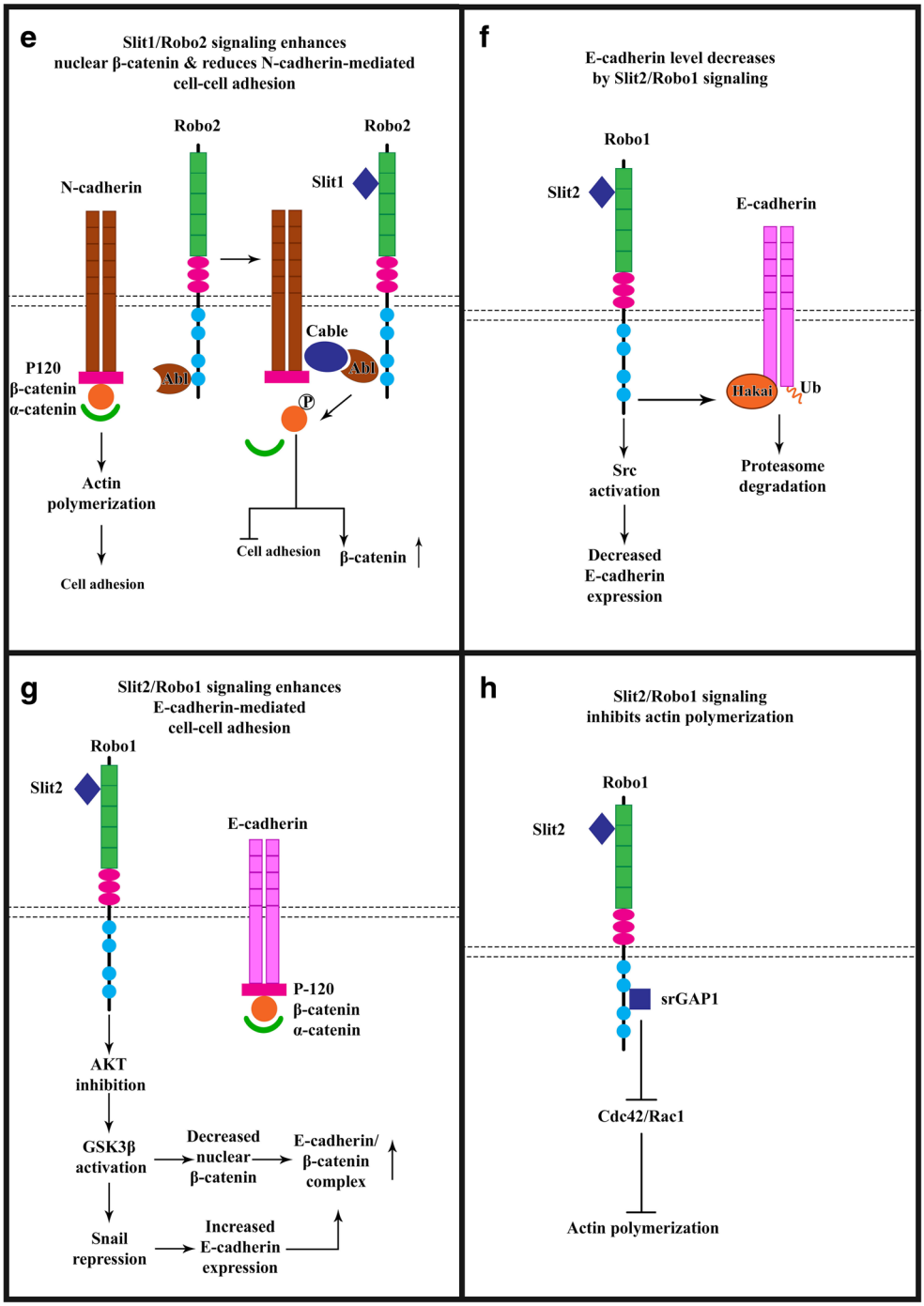

Fig. 2 Loss of cell-cell contact has an important role in tumor progression and metastasis. **e** Formation of tertiary complex including Abl, Cables and P120 lead to loss of N-cadherin-mediated cell adhesion. **f** Upregulation of Slit2-Robo1 signaling pathway lead to E-cadherin degradation via ubiquitin ligase Hakai in proteasome. **g** Slit2-Robo1 signaling increases formation of E-cadherin/β-catenin complex through AKT/GSK3β/Snail pathway. **h** Slit2-Robo1 signaling leads to srGAP1 recruitment and Cdc42 inactivation which subsequently inhibits actin polymerization

Interaction between ECM and cells that mediate by adhesion receptors including integrins, coordinates cell functions to regulate cell adhesion, cell growth, proliferation and gene expression. Integrin from one side link to Proteoglycans, Collagen, Laminin, Fibronectin and other components in ECM and in other side link to Paxillin, Tallin, Vinculin and α-actinin to form adhesion plaque that finally is connected to actin stress fibers in cytoskeleton. Slit-Robo signaling pathway has a functional role in cell motility through effect on actin polymerization and intercellular junctions. Major players in signaling downstream of Slit-Robo are cytoplasmic kinases, monomeric GTPase and proteins that involve in formation of cytoskeleton like actin and microtubule. Monomeric GTPase belong to Rho family including RhoA, Rac1 and cdc42 are key regulators of tissue barriers integrity [16].

Interaction between Slit2 and Robo1 has been reported which leads to srGAP1 recruitment and inactivates Cdc42 that subsequently inhibits actin polymerization and cell migration

in colorectal cancer [80]. This connection also inhibits glioma cell migration in the brain [56]. (Fig. 2h).

Our literature review indicates that Robo4 has a key role in angiogenesis and anti-angiogenesis. Indeed, it is a double edge sword. Slits also have been indicated to enhance and reduce tumor angiogenesis and metastasis by promoting or preventing cellular attachment and migration [77, 79].

Conclusion

By the discovery of Slit/Robo signaling pathway as key regulators in a wide variety of developmental and pathological processes, it is considered as a possible treatment target. Convergent points in this review indicate that Robo4 has a dual behavior. Robo4 signaling pathway inhibits angiogenesis via both VEGF signaling and interaction with UNC5B. This signaling pathway also enhances tumor angiogenesis via both Slit2/Robo1-Robo4 signaling pathway and Robo4 interaction with an unknown ligand. Slit2 also has a dual feature that may be as promoting or preventing of tumor angiogenesis.

So, Slit/Robo signaling pathway may be a useful therapeutic target in various type of cancer but this could be as double edge sward because this signaling axis could result in opposite outcome, including oncogenic behavior. Thus, success in this way will require a broader knowledge about this disparate signaling pathway. Furthermore, designing of drugs with elaborate delivery mechanisms which attack cancer cells but spare normal cells will be safe and effective by preventing the adverse side effects. The members of this signaling pathway are also interesting as clinical biomarkers and excellent targets for immunotherapy.

Compliance with Ethical Standards

Conflict of Interests The authors declare that there is no conflict of interests from the publication of this article.

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