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Perspectives of SLIT/ROBO signaling in placental angiogenesis

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Summary.

A novel family of evolutionally conserved neuronal guidance cues, including ligands (i.e., Slit, netrin, epherin, and semaphorin) and their corresponding receptors (i.e., Robo, DCC/Unc5, Eph and plexin/neuropilin), has been identified to play a crucial role in axon pathfinding and branching as well as neuronal cell migration. The presence of commonalities in both neural and vascular developments has led to some exciting discoveries recently, which have extended the functions of these systems to vascular formation (vasculogenesis) and development (angiogenesis). Some of these ligands and receptors have been found to be expressed in the vasculature and surrounding tissues in physiological and pathological conditions. It is postulated that they regulate the formation and integrity of blood vessels. In particular, it has been shown that the Slit/Robo pair plays a novel role in angiogenesis during tumorigenesis and vascular formation during embryogenesis. Herein we summarize briefly the characteristics of this family of neuronal guidance molecules and discuss the extra-neural expression and function of the Slit/Robo pair in angiogenesis in physiological and pathological settings. We report expression of Robo1 protein in capillary endothelium and co-expression of Slit2 and Robo1 proteins in syncytiotrophoblast in healthy term human placental villi. These cellular expression patterns implicate that the Slit/Robo signaling plays an autocrine and/or paracrine role in angiogenesis and trophoblast functions. We also speculate a possible role of this system in pathophysiological placental angiogenesis.

Keywords

Slit; Robo; Angiogenesis; Placenta

Introduction

Angiogenesis is a tightly regulated multi-step process. It is initiated by endothelial cell activation upon local and/or systemic factor(s) to degrade extracellular matrix, followed by endothelial cells migration and proliferation, and eventually forms tube-like structures by recruitment of smooth muscle cells and pericytes (Folkman and Shing, 1992; Carmeliet, 2000). These processes are finely tuned by a balance between pro-angiogenic and anti-

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angiogenic factors, in which both can be originated from local and/or systemic sources. As a primary active site of physiological angiogenesis, the placenta is one of the richest sources of both pro-angiogenic and anti-angiogenic factors. For example, during the third trimester of both ovine and human pregnancy, at a time when maternal-fetal interface vascular growth, blood flow, and fetal weight increase exponentially, the fetal and maternal compartments produce numerous angiogenic factors, such as vascular endothelial growth factor (VEGF) (Senger et al., 1983; Keck et al., 1989; Leung et al., 1989), fibroblast growth factor 2 (FGF2) (Gospodarowicz et al., 1987), placental growth factor (Maglione et al., 1991), endocrine gland-derived-VEGF (LeCouter et al., 2001), transforming growth factor-β (Derynck et al., 1985), leptin (Zhang et al., 1994), and angiopoietins (Sato et al., 1995). It is noteworthy that this list is still expanding (Folkman, 2002). More recently, the Slit/Robo signaling system has been found to play a novel role in tumor angiogenesis (Wang et al., 2003; Bedell et al., 2005; Suchting et al., 2005) and vascular formation during embryogenesis (Kaur et al., 2006). Herein we are not intended to review the anatomical, cellular and molecular aspects of placental angiogenesis in detail, which can be found in previous elegant reviews elsewhere (Charnock-Jones et al., 2004; Kaufmann et al., 2004; Mayhew et al., 2004). The objective of this paper is to update the current information regarding Slit/Robo signaling in pathological and physiological angiogenesis as well as its implications in placental angiogenesis.

Placental angiogenesis

During embryogenesis, a class of primitive vascular progenitor cells evokes and eventually differentiates into all types of vascular cells for the formation of a tree-like vascular architecture throughout the body during *in utero* fetal development. The elaborate vascular system occupies all organs, which functions as the main transportation system via circulating blood for the delivery and exchange of nutrients, respiratory gases (oxygen and carbon dioxide), and metabolic wastes, etc. This primary task is obviously essential for the maintenance of normal functions of every single cell, tissue and organ. Circulation of bioactive materials also eliminates boundaries among different cells, tissues and organs, thereby coordinating all physiological processes systemically.

Fetal development *in utero* is a remarkable journey for early life of eutherians in the womb. During this period, fetus is supported, in essence, by her mother for growth and survival. The pre-implantation embryo uses the outer layer trophectoderm cells to make a unique organ, the placenta, for all kinds of communications with the mother. This ephemeral organ is extremely vascularized to facilitate the bidirectional mother-fetus exchanges that are linked directly to fetal/placental development, survival, perinatal/neonatal outcomes, and the mother's well-being during pregnancy and postpartum. The formation of placental vasculature occurs via vasculogenesis and angiogenesis. Vasculogenesis is a process of formation of new blood vessels via the vascular progenitor cells termed as angioblast, which occurs during organogenesis. Once placenta is formed, the expansion and growth of placental vasculature is primarily via angiogenesis, a process named for the new vessel formation from preexisting capillaries. Placental vascular growth begins in the process of placentation and continues throughout gestation. During the third trimester in parallel with rapid fetal growth, placental vasculature grows exponentially (Magness and Rosenfeld,

1989; Cross et al., 1994; Wheeler et al., 1995; Reynolds and Redmer, 2001; Leach et al., 2002; Borowicz et al., 2007). This is essential to accommodate the dramatic rise (up to 50- to 80-fold) in uterine blood flow as a maternal adaptation to pregnancy (Magness, 1996, 1999). The continuous and dramatic increase in fetoplacental and uteroplacental blood flows is responsible for the bidirectional fetal-maternal exchange of nutrient and oxygen supply to meet the progressively increasing demands of fetal growth and placental development and to exhaust metabolic wastes from fetus to mother (Magness, 1999; Reynolds and Redmer, 2001). It directly correlates with fetal growth, survival and neonatal birth weights. Insufficient blood flows lead to shortage of oxygen and nutrient supply that are causative of intrauterine growth restriction (IUGR) and higher prenatal/neonatal morbidity or even mortality (Magness and Rosenfeld, 1989; Morrow et al., 1989; Macara et al., 1996; Lang et al., 2003). From this point of view, it is very logic to hypothesize that, when pregnant women's food supply is not a concern, constrained nutrient/oxygen supply due to reduced uterine blood flow is the real "physiological" undernutrition cause of fetal programming of adult-onset diseases in the Barker hypothesis (Barker, 1997).

Slit/Robo Signaling System

The Slit/Robo system is a member of a conserved neuronal guidance cue family that also includes netrin/DCC/Unc5 (Freitas et al., 2008), ephrin/Eph (Cheng et al., 2002) and semaphorin/plexin/neuropilin (Neufeld and Kessler, 2008). In these systems, the former ones (i.e., Slit, netrin, epherin, and semaphorin) are secreted proteins that function as ligands; whereas the latter ones (i.e., Robo, DCC/Unc5, Eph, and plexin/neuropilin) are their corresponding receptors. Slit proteins are conserved from *Drosophila* to mammals. Mammals have at least three slit genes (slit 1, slit 2 and slit 3) (Itoh et al., 1998; Brose et al., 1999) that encode three Slit proteins with ~1500 amino acids. All Slit proteins have common domain structures, including four tandem leucine rich repeats (LRRs), nine epidermal growth factor (EGF)-like domains, a laminia G domain, and a C-terminal cysteine-rich domain (Brose et al., 1999) (Fig. 1A). Slit proteins can be proteolytically cleaved within the EGF-like region (between EGF-5 and -6) into Slit-N and Slit-C. Slit-N remains tightly bound to the cell membrane, whereas Slit-C is readily diffusible (Brose et al., 1999; Wang et al., 1999; Rothberg et al., 1990; Rothberg and Artavanis-Tsakonas, 1992; Nguyen Ba-Charvet et al., 2001; Chen et al., 2001). Both Slit and Slit-N bind to Robo at the second LRR region (Howitt et al., 2004; Morlot et al., 2007; Fukuhara et al., 2008). Slit may form noncovalent dimers via the fourth LRR, which may enhance their binding to Robo *in vitro* (Howitt et al., 2004).

Robo (roundabout) has been so named after characteristic defects in the nervous system of mutant flies. They are single-pass transmembrane proteins with ~1000 to 1600 amino acids (Brose et al., 1999) (Fig. 1B). Four Robo proteins, Robo1, 2, 3 and 4, have been found in mammals (Kidd et al., 1998a,b; Brose et al., 1999; Huminiecki et al., 2002; Park et al., 2003). Robo4 is also termed as magic roundabout (Huminiecki et al., 2002). Whether Robo4 functions as a Slit receptor needs further investigation (Park et al., 2003; Suchting et al., 2005; Legg et al., 2008; Sheldon et al., 2009); however, as being exclusively expressed in the vasculature Robo4 has been regarded as a specific vascular Slit receptor. The extracellular ligand binding domains of Robo1 to 3 are conserved from Drosophila

to human. They are composed of five immunoglobulin (Ig) and three fibronectin type III (FNIII) domains, a single transmembrane (TM) segment, and a cytoplasmic domain. Murine and human Robo4 genes encode only the first two Igs important for Slit binding and two FNIII domains (Howitt et al., 2004; Liu et al., 2004). In general, the cytoplasmic domains of Robo proteins have little homology (Dickson and Gilestro, 2006); however, four short conserved cytoplasmic sequence motifs (CC0, CC1, CC2 and CC3) are recognized among Robo proteins (Fig. 1B). These motifs are thought to serve as docking sites of interaction with various cytoplasmic proteins or kinases, which will be discussed below.

Tissue Distribution/Expression of Slit/Robo

Slits were originally identified as EGF-like proteins expressed by neurons and glial cells in the embryonic central nervous system (Nusslein-Volhard, 1984; Rothberg et al., 1988). Similarly, all Robo proteins were also initially identified in the nervous system (Kidd et al., 1998a,b). Slit/Robo molecules have later been detected in various non-neural tissues, including lung (Anselmo et al., 2003; Greenberg et al., 2004; Jones et al., 2008), kidney (Piper et al., 2000; Jones et al., 2008), heart (Qian et al., 2005, Santiago-Martinez et al., 2006), hindlimbs and muscle (Vargesson et al., 2001), mammary gland, skin (Dickinson et al., 2004) and ovary (Dickinson et al., 2008). The wide distribution of Slit/Robo signaling cues implicates that they may play conserved roles in the regulation of various non-neural cell types and the development of extra-neural tissues/organs. Indeed, Slit/Robo knock-out (KO) mice display defects in many organs. *Slit2, Dutt1/Robo1 and Robo2* null mice are embryonic lethal (Xian et al., 2001; Plump et al., 2002; Grieshammer et al., 2004). In addition to an axon-guidance defect in retinal ganglion cells and in several major axonal pathways in the forebrain (Bagri et al., 2002; Plump et al., 2002), Slit2-null mice also develop abnormal kidney due to supernumerary ureteric bud formation, similar to that seen in Robo2-null mice (Grieshammer et al., 2004). The majority mutant *Dutt1/Robo1* mice die at birth because of respiratory failure caused by delayed lung maturation; the surviving mice develop extensive bronchial epithelial abnormalities including hyperplasia (Xian et al., 2001). Slit3-null mice are significantly growth restricted and the majority display congenital diaphragmatic hernia (CDH) similar to a central (septum transversum) CDH syndrome in humans (Yuan et al., 2003, Liu et al., 2003). The causes of fetal growth restriction in these mice are currently not known; however, derangement of placental vasculature may, at least in part, be a potential factor. Abnormal kidney development also occurred in some of the Slit3-null mice (Liu et al., 2003). These findings suggest an important role of Slit/Robo in organogenesis. Robo4 mutant mice are viable and have normal developmental vascular patterning, suggesting that Robo4 is not required for vessel formation during embryogenesis but may be important in maintaining the vascular integrity by inhibiting VEGF-induced vascular leakage (Jones et al., 2008). In zebrafish, however, Robo4 is essential for coordinated symmetric and directed sprouting of intersomitic vessels (Bedell et al., 2005, Kaur et al., 2006). The phenotypic difference between mouse and fish Robo4-KOs indicates the possible redundancy of Robo receptors in mouse vascular development during embryogenesis.

Slit and Robo mRNAs have been detected in the vasculature, including Slit2 and Robo1, 2 and 4 in rat carotid arteries, thoracic aortas, abdominal aortas, and iliac arteries (Liu et

al., 2006). Slit2 mRNA is detected in human aortic smooth muscle cells (Liu et al., 2006) and aortic endothelial cells (Liu et al., 2006). Slit2 and Slit3 mRNAs are also found in rat endothelial cells (Wu et al., 2001). Slit1 is not expressed in vasculature (Liu et al., 2006). Except Robo3, all other three Robo proteins have been detected in many non-neural cell types, including endothelial cells (Wang et al., 2003; Liu et al., 2006) and smooth muscle cells (Liu et al., 2006). Among these Robo proteins, Robo4 has been isolated as an endothelium-specific receptor (Huminiecki et al., 2002; Park et al., 2003; Seth et al., 2005; Jones et al., 2008). In particular, Robo4 mRNA is detectable in primary endothelial cells isolated from different organs, including human umbilical cord vein endothelial cells (HUVEC) (Huminiecki et al., 2002; Park et al., 2003; Seth et al., 2005; Sheldon et al., 2009), human microvascular endothelial cells (Park et al., 2003; Seth et al., 2005; Jones et al., 2008), human aortic endothelial cells (Liu et al., 2006; Jones et al., 2008), and human dermal microvascular endothelial cells (Huminiecki et al., 2002). Interestingly, in mouse retinal vascular bed Robo4 is restricted in the stabilized or more mature endothelial cells of stalk endothelium, but not as expected in the endothelial tip cells where active angiogenesis takes place (Jones et al., 2008). The literature of Robo1 expression in HUVEC is not always consistent. Wang et al. (2003) first reported the presence of both Robo1 mRNA and protein in HUVEC (Wang et al., 2003). Shortly after, Seth et al. (2005) failed to detect Robo1 mRNA in HUVEC by RT-PCR (Seth et al., 2005). Recently, Sheldon et al. (2009) have detected Robo1 mRNA and protein in HUVEC (Sheldon et al., 2009). The discrepancies of Robo1 and Robo4 expression patterns in endothelial cells may, at least in part, contributes to the controversial finding that the Slit/Robo signaling plays dual roles in endothelial cell angiogenesis. Information on vascular Robo2 expression is currently limited; only one report found low levels Robo2 mRNA in human aortic endothelial cells (Liu et al., 2006). Regardless, these data show that differential expression of Robo proteins in the vasculature may play an important role in mediating Slit functions.

Slit/Robo signaling and tumor development

The link between Slit/Robo signaling and tumor and cancer was first revealed in 1998 by the finding that the exon 2 of Robo1 was deleted in two lung tumor cell lines and one breast tumor cell line (Sundaresan et al., 1998). Subsequently, several studies have shown that Slit1, 2 and 3, and Robo1 promoters were hypermethylated (epigenetic inactivation) in different cancers, suggesting that these genes function as tumor suppressors (Dallol et al., 2002a,b, 2003; Dickinson et al., 2004). Robo1 methylation has been detected in less than 20% of breast tumors and clear renal cell carcinomas, but was rare in lung carcinomas (Dallol et al., 2002a,b). Robo1 expression is also significantly reduced in prostate tumors compared to normal prostate tissues (Latil et al., 2003). Furthermore, the Duut1/Robo1 heterozygous mice are more prone to develop lung adenocarcinomas and lymphoma, suggesting that, at least in the mouse, Robo1 may serve as a tumor suppressor gene (Xian et al., 2004). Slit2 could be a tumor suppressor gene as it is frequently inactivated in lung and breast cancers (Dallol et al., 2002a,b), and in gliomas (Dallol et al., 2003). Though less frequent than Slit2, Slit3 hypermethylation has also been detected in lung and breast cancers (Dickinson et al., 2004). In breast carcinoma cells, loss of Slits or Robo1 result in coordinated upregulation of the stromal cell-derived factor 1 and its receptor

CXCR4 (Marlow et al., 2008), a chemokine-receptor pair important in carcinogenesis and in neovascularization linked to tumor progression (Kryczek et al., 2007). Recombinant Slit2 inhibits medulloblastoma invasion by decreasing active $Cdc42^{GTP}$ formation, whereas it has no effect on glioma tumor (Werbowetski-Ogilvie et al., 2006).

In contrast, many studies also have shown that Slit/Robo expression is increased in tumors. For example, Slit2 and 3 expressions are increased in canine malignant mammary tumors (Tanno et al., 2006). Robo1 and Robo4 expressions are also elevated in colorectal cancer (Grone et al., 2006) as does Robo1 in hepatocellular carcinoma (Ito et al., 2006). Robo4 expression is significantly upregulated in cancer tissues from lung, kidney, liver, and metastatic melanoma (Seth et al., 2005). Wang et al. (2003) first reported that Slit/Robo signaling controls tumor-endothelial cell communication critical for tumor angiogenesis (Wang et al., 2003). Slit2 is expressed in a large number of solid tumors and tumor cell lines. Recombinant Slit2 protein attracts endothelial cells and promotes tube formation via a Robo1- mediated phosphatidylinositol 3-kinase dependent pathway. Neutralization of Robo1 reduces microvessel density and tumor mass grown from human malignant melanoma A375 cells *in vivo* (Wang et al., 2003). Thus, it is possible that the role of Slit/Robo signaling in tumor development is cell type- or tissue-specific. Nevertheless, these studies certainly extend the functional role of Slit/Robo to tumor development and tumor angiogenesis.

Slit/Robo signaling in angiogenesis

In the nervous system, Slit/Robo signaling can function as diffusible long-range cues or as cell membrane-associated short-range cues. It controls axon pathfinding and branching as well as cell migration by acting as repellents in some axons (Kidd et al., 1999; Li et al., 1999; De Bellard et al., 2003) or as attractants of branching and elongation of other axons (Wang et al., 1999; De Bellard et al., 2003). These findings reiterate the common theme that guidance cues often have dual roles (Dickson, 2002). It has also been shown that they function as repellents to regulate the migration of non-neural cell types including leukocytes (Wu et al., 2001), vascular smooth muscle cells (Liu et al., 2006) and their precursor cells (Kramer et al., 2001). Interestingly, the guiding signal of Slits switches from a repellent to an attractant during mesoderm migration (Kramer et al., 2001). These findings indicate the presence of fundamental conserved guidance machineries for all somatic cells (Wu et al., 2001; Rao et al., 2002). In the last few years, a critical role of Slit/Robo signaling in angiogenesis has been uncovered. Wang et al. (2003) first reported that Slit2, upon binding to HUVEC Robo1, functions as an attractant to promote the directional migration and vascular network formation *in vitro* (Wang et al., 2003). These cellular effects are inhibited by an anti-Robo1 antibody and are blocked by a soluble Robo1 extracellular fragment (RoboN). Their findings significantly extend the spectrum of Slit/Robo signaling to the area of tumor angiogenesis. Recently, Sheldon et al. (2009) have shown that Slit2 is able to promote HUVEC migration and tube-formation *in vitro*, possibly mediated by Robo1/Robo4 heterodimers (Sheldon et al., 2009). Another breakthrough that highlights Slit/Robo in angiogenesis is the identification of Robo4, the vascular-specific Slit receptor (Huminiecki et al., 2002; Park et al., 2003). Secreted soluble Robo4 is able to inhibit *in vivo* angiogenesis and the VEGF- and FGF2-stimulated endothelial cell proliferation and migration (Suchting et al., 2005). Knockdown or overexpression of Robo4 results in either lack of or misdirected

intersomitic vessels (Bedell et al., 2005). In KO mice, Robo4 is shown to be important for the maintenance of vascular integrity by inhibiting abnormal angiogenesis and endothelial hyperpermeability (Jones et al., 2008). Accordingly, it is reasonable to infer that Robo4 regulates vessel growth to a proper path by either attracting or repulsing endothelial cell function.

Although available evidence points to an angiogenic role of Slit/Robo signaling, it is still in debate whether Slit/Robo signaling functions as attractant or repellent in angiogenesis. In general, it is postulated that whether Slit acts as an attractive or a repulsive cue depends on which Robo(s) it binds, the cellular context of the target cells, and/or other environmental factors (Autiero et al., 2005; Weinstein, 2005; Song et al., 1997; Song et al., 1998). It is speculated that Slit binding to Robo1 may initiates attractant responses, while Slit binding to Robo4 may initiate repellent responses in angiogenesis (Park et al., 2003; Autiero et al., 2005; Acevedo et al., 2008). In addition, Robo1 has been shown to form homodimers (Morlot et al., 2007) or heterodimers with other Robo proteins (Liu et al., 2004; Camurri et al., 2005). It is possible that Robo1 and Robo4 may interact on vascular sprouts or that perhaps, Robo4 modulates the response to Robo1. Recently, Sheldon et al. (2009) have shown that Robo1 forms heterodimers with Robo4 in HUVEC and the dimerization is important to mediate Slit2-stimulated HUVEC migration (Sheldon et al., 2009). In all, investigations on deciphering the role of this neuronal signaling system in angiogenesis have just begun to gain momentum. Obviously, more extensive investigations are needed for a comprehensive understanding of the mechanisms that Slit/Robo signaling utilizes to regulate both physiological and pathological angiogenesis.

Slit/Robo initiated intracellular signaling

The majority of the knowledge gathered to date on the Slit-initiated intracellular signaling upon binding to Robo has been generated from the studies in *Drosophila* neuron. Upon Slit stimulation, activated Abelson kinase (Abl) phosphorylates a tyrosine residue in CC1 of Robo1, thereby downregulates its activity; whereas the substrate of Abl Enabled (Ena) binds to CC2 that in turn stabilizes Robo function. Thus, Abl and Ena directly regulates Slit/Robo signal transduction in an opposite way (Bashaw et al., 2000). Slit also increases the interaction of Slit-Robo GAPs (srGAPs, a novel family of Rho GTPase activating proteins) with the CC3 motif of a Robo protein. This interaction leads to activation of RhoA and inhibition of Cdc42; both are members of the Rho GTPase family that is important for regulating actin cytoskeleton and cell structure (Wong et al., 2001). This shift in the balance of GTPase activation may lead to growth cone collapse and repulsion. These concepts have been supported by genetic evidence in *Drosophila* that downregulation of Cdc42 also regulates axon repulsion at the midline (Fritz and VanBerkum, 2002). Another GAP member vilse/CrossGAP also binds to Robo, which will inactivate Rac (Lundstrom et al., 2004; Hu et al., 2005). Slit also stimulates the recruitment of the SH2-SH3 adaptor proteins to Robo, such as the Dreadlocks (Dock in *Drosophila,* the homologue of mammalian Nck) and p21-activated serine-threonine kinase (Pak)-an effector of Rac and Cdc42 signaling. This in turn results in Rac1 activation and axon repulsion (Fan et al., 2003). Furthermore, on Slit stimulation, Dock also recruits an activator of Rho GTPase, the guanine nucleotide exchange factor son of sevenless (Sos) to Robo, which then activates Rac during midline repulsion

(Yang and Bashaw, 2006). Because Rac acts as a positive regulator for axon outgrowth (Luo, 2000), it is possible that Rac may have different or even opposite effects on actin cytoskeleton, depending on the cellular context in which it is activated and its overall level of activity (Fan et al., 2003). It is noteworthy that the cytoplasmic domains of Robo are solely responsible for mediating the biological responses to Slit stimulation (Seeger and Beattie, 1999). Binding of the aforementioned signaling proteins to CC motifs of Robo results in either activation or suppression of downstream signaling pathways, and transmits growth and differentiation responses to Slit (Rhee et al., 2002). It is important to note that not all Robos contains all four CC motifs (Fig. 1B). Their diversified cytoplasmic domains may be engaged in binding of different sets of partners, thereby leading to distinct growth responses (Dickson and Gilestro, 2006).

In endothelial cells, recent studies have revealed the downstream intracellular signaling pathways that transduce the angiogenic responses of Slit/Robo signaling. Wang et al. (2003) reported that activation of phosphatidylinositol 3-kinase is involved in the endothelial cell responses to Slit2 (Wang et al., 2003). In addition, Slit2-induced endothelial cell migration is derived by the activation of Wiskott-Aldrich syndrome protein (WASP), neural Wiskott-Aldrich syndrome protein (N-WASP) and WASP-interacting protein complex that is important in actin-nucleating and endothelial cell migration (Sheldon et al., 2009). In zebrafish embryonic vasculature, Robo4 mediates the attractive signaling of Slit through activation of a cytosolic signaling cascade involving Cdc42 and Rac1 Rho GTPase in endothelial cells (Kaur et al., 2006). In other studies, Slit2 or Robo4 inhibits the VEGF or FGF2 induced HUVEC migration, possibly via the Ras-Raf-MEK-extracellular signal-regulated protein kinase (ERK2/1) signaling pathway (Seth et al., 2005). Activation of Robo4 by Slit2 inhibits the VEGF-induced angiogenic responses through inactivation of the Src family kinase (Jones et al., 2008). Since the novel role of Slit/Robo in angiogenesis has just begun to be recognized, more studies are necessary to dissect a complete spectrum of the intracellular signaling pathways activated by Slit via binding to Robo and their pro-angiogenic or anti-angiogenic effects in endothelial cells.

Slit/Robo Signaling and Placental Angiogenesis

Placenta is perhaps the most active site of physiological angiogenesis. It serves as the best model for research toward uncovering the molecular mechanisms of this complex process. To date, virtually little is known about the expression and function of the Slit/Robo signaling system in the placenta. Robo4 mRNA has been shown to be expressed in placental arteriole and venule (Huminiecki et al., 2002; Seth et al., 2005). Robo4 protein has been also detected in trophoblasts undergoing pseudo-vasculogenesis (Seth et al., 2005). Slit3 mRNA has been detected in placental tissue (Dickinson et al., 2004). These are the only pieces of evidence to date about Slit/Robo expressions in the placenta. We have recently analyzed the expression of Slit/Robo system in healthy term human placentas using immunohistochemical staining. As shown in Fig. 2, Slit2 protein is localized in the syncytiotrophoblast of the placental villi (open arrowheads). Similar to Slit2, Robo1 protein is also detectable in the syncytiotrophoblast (open arrowheads) of the placental villi and in the trophoblast capillary endothelium (solid arrowheads). These interesting results may have implicated a paracrine role of Slit2 produced by the trophoblast cells in regulating

endothelial cell functions and angiogenesis by binding to Robo1 in endothelial cells. The trophoblast co-expression of Slit2 and Robo1 also implicates a potential autocrine role of Slit2 in regulating trophoblast functions such as differentiation, invasion, transport, and hormone secretion, etc. It is also possibly that other Slits and Robo proteins may be expressed in the trophoblast and capillary endothelium. They may play an integral role in regulating trophoblast functions and placental angiogenesis (Fig. 3). However, these hypotheses await further investigation.

Although the function of Slit/Robo in the placenta is currently unknown, the available Slit and Robo KO mice models might have offered excellent opportunities for indentifying the role of this system in placental biology. It would be of great interest to investigate the placental phenotypes of mice bearing mutant Slit/Robo genes as these studies will decipher the functional outcomes of genetic deletion of Slit/Robo in placental development, particularly vasculature formation via angiogenesis and trophoblast differentiation and invasion. Interestingly, Robo4 expression in human dermal microvascular endothelial cells is inducible by hypoxia (Huminiecki et al., 2002), a condition readily present in the placentae in the first trimester during pregnancy (Jaffe et al., 1997; Burton et al., 1999) and in many pathologic conditions like preeclampsia (James et al., 2006; Myatt, 2006). Hypoxia has been considered as the major physiological regulator for placental development (Genbacev et al., 1997). Thus, the co-expression of Slit and Robo at the sites of active angiogenesis and the possible regulation of Slit/Robo signaling by hypoxia in the placenta strongly implicate a role of this system in placental angiogenesis.

Perspectives

The role of Slit/Robo signaling system in neuronal axon pathfinding is currently well-defined (Dickson and Gilestro, 2006). The newly discovered role of this system in angiogenesis has certainly added the paradigm of the regulation of endothelial and vascular functions. Although our knowledge on Slit/Robo signaling in angiogenesis is still limited, it is becoming increasingly clear that this system plays a key role in determining how vessels are patterned. Studies are warranted to address important questions as to how Slit/Robo induces or suppresses endothelial cell migration, differentiation and/or proliferation. Because the role of the Slit/Robo signaling in placental angiogenesis is currently completely unknown, it would be of great interest to investigate the expression and function of the Slit/Robo signaling in the placenta, especially in its vasculature. Furthermore, controversial findings showing a dual role of Robo1 and Robo4 in endothelial cell angiogenesis merit further investigations for clarifying the specific functions of Slit/Robo in the placenta. In addition, since both *in vivo* and *in vitro* studies have shown a regulatory role of Slit/Robo in VEGF/FGF2 functions, it is also of importance to investigate how Slit/Robo signaling integrates with other angiogenic factors in regulating angiogenesis, especially in the placenta. Investigations of the role of this system in the placenta in both physiological and pathophysiological settings will advance the understanding of angiogenesis, which will offer a promise for better management of high risk pregnancies such as preeclampsia and IUGR and potential disease interventions. Because of its endothelial specific expression, Robo4 and its downstream signaling pathways evoked by Slit stimulation might have potential for the development of diagnostic markers and/or for defining therapeutic targets for

pregnancy complications such as preeclampsia and IUGR with characteristics of endothelial dysfunctions.

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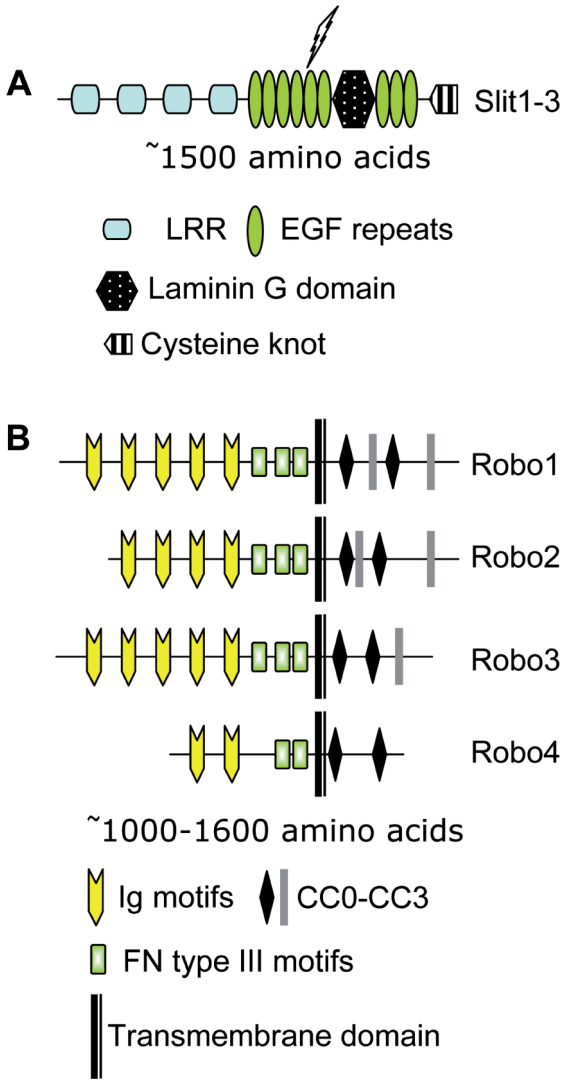

Fig. 1.

Domain structures of Slit and Robo. **A.** Primary structures of mammalian Slits contain leucine rich repeats (LRRs), EGF repeats, a laminin G domain, and a C-terminal cysteine-rich domain. **B.** Mammalian Robo proteins are constituted by immunoglobulin (Ig) motifs, fibronectin type III (FNIII) domains, a single transmembrane (TM) segment, and conserved cytoplasmic motifs (CC0-CC3).

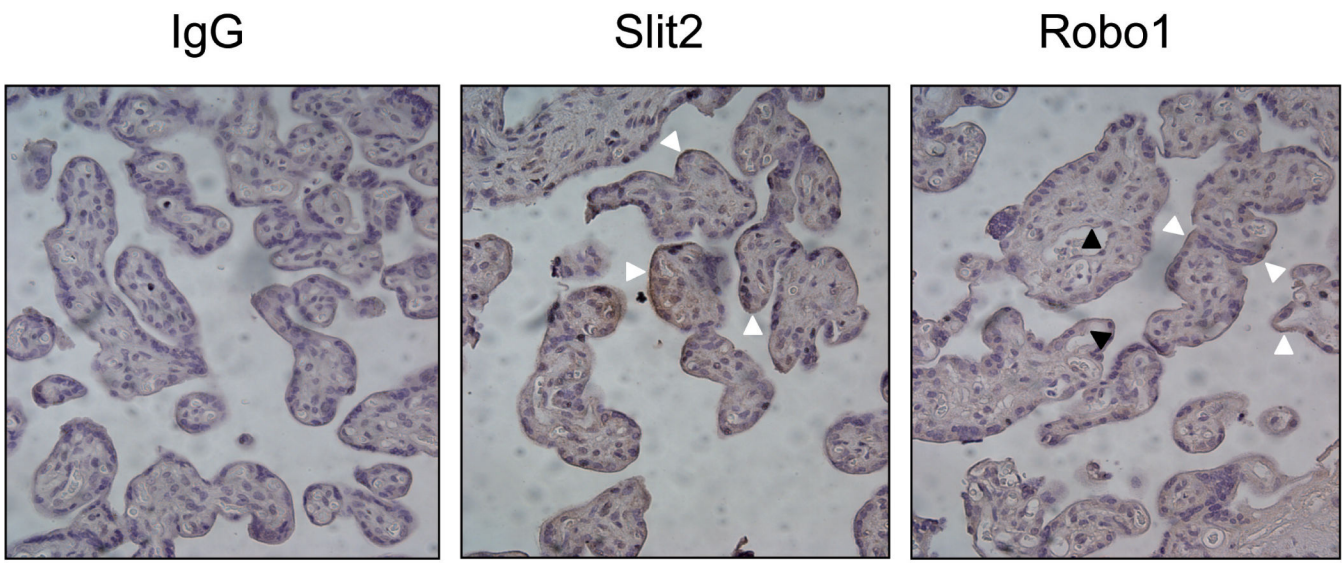

Fig. 2.

Slit2 and Robol protein expression in healthy term human placental tissues. The placental tissues were collected from the University of California San Diego (UCSD) Medical Center Hospital with an approval from UCSD Institutional Review Board. The tissue segments were fixed with 3.7% paraformadehyde and then paraffin embedded. Sections (6-μm) were cut and used for immunohistochemically (Liao et al., 2005) localizing Slit2 and Robol proteins with anti-Slit2 monoclonal antibody (10 μg/ml) or anti-Robol monoclonal antibody (10 μg/ml) with the SuperPicture kit from Zymed Laboratories, Inc. (Invitrogen). Negative controls ran in parallel with anti-mouse IgG (10 μg/ml). Open arrowhead denotes syncytiotrophoblast and solid arrowhead denotes capillary endothelium.

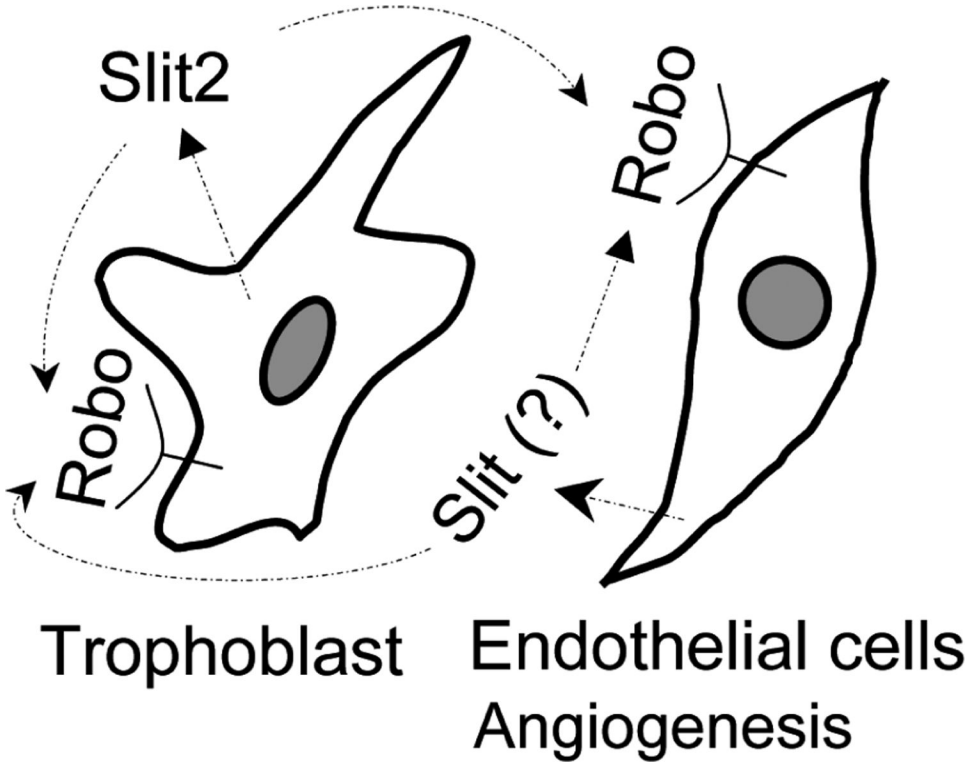

Fig. 3.

Proposed paracrine/autocrine roles of Slit/Robo in trophoblast and capillary endothelial cells in the placenta. Trophoblast produced Slit2, possibly other Slits, regulates placental angiogenesis through binding to Robo1 and/or Robo4 on the endothelial cells, whereas they also regulate trophoblast functions via binding to Robo proteins.