Human placental multipotent mesenchymal stromal cells modulate placenta angiogenesis through Slit2-Robo signaling

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ABSTRACT

The objective of this study was to investigate whether human placental multipotent mesenchymal stromal cell (hPMSC)-derived Slit2 and endothelial cell Roundabout (Robo) receptors are involved in placental angiogenesis. The hPMSC-conditioned medium and human umbilical vein endothelial cells were studied for Slit2 and Robo receptor expression by immunoassay and RT-PCR. The effect of the conditioned medium of hPMSCs with or without Slit2 depletion on endothelial cells was investigated by in vitro angiogenesis using growth factor-reduced Matrigel. hPMSCs express Slit2 and both Robo1 and Robo4 are present in human umbilical vein endothelial cells. Human umbilical vein endothelial cells do not express Robo2 and Robo3. The hPMSC-conditioned medium and Slit2 recombinant protein significantly inhibit the endothelial cell migration, but not by the hPMSC-conditioned medium with Slit2 depletion. The hPMSC-conditioned medium and Slit2 significantly enhance endothelial tube formation with increased cumulated tube length, polygonal network number and vessel branching point number compared to endothelial cells alone. The tube formation is inhibited by the depletion of Slit2 from the conditioned medium, or following the expression of Robo1, Robo4, and both receptor knockdown using small interfering RNA. Furthermore, co-immunoprecipitation reveals Slit2 binds to Robo1 and Robo4. Robo1 interacts and forms a heterodimeric complex with Robo4. These results suggest the implication of both Robo receptors with Slit2 signaling, which is involved in endothelial cell angiogenesis. Slit2 in the conditioned medium of hPMSCs has functional effect on endothelial cells and may play a role in placental angiogenesis.

KEYWORDS

angiogenesis; endothelial cells; placental multipotent mesenchymal stromal cells; Robo; Slit2

Introduction

Slit proteins were initially described in the developing central nervous system of Drosophila as axonal repellents, which regulated the migration of neurons and axons by binding to cognate Roundabout (Robo) receptors. Slit has 3 isoforms (Slit1–3) and Robo has 4 (Robo1–4). Robo2 and Robo3 are abundantly expressed in the nervous system but undetectable in the vascular system. Slit–Robo signaling functions in a variety of developmental processes, such as kidney development, chemoattractants of vascular endothelial cells, leukocytes and cancer cell migration. Therefore, it is insightful to investigate the roles of Slit–Robo signaling in the placenta and their mechanisms.

Endothelial cells have been found to express Robo1 and Robo4, suggesting the involvement of Slit2–Robo signaling in vascular development. Previous studies on endothelial migration induced by Slit–Robo signaling however are inconsistent. Some reports suggest that Slit2 promotes angiogenesis in human umbilical vein endothelial cells (HUVECs) through Robo1,7,8 and others show that Slit2 inhibits migration of HUVECs through Robo4.2,9 Slit2 was shown to inhibit VEGF-induced microvascular endothelial cell migration, tube formation and endothelial cell permeability in a Robo4-dependent manner.10 Additionally, Robo1 was proposed to form a heterodimer with Robo4,11 and siRNA knockdown of Robo1 or Robo4 reduced the inhibitory effect of Slit on HUVEC migration and permeability.12 Thus, Robo1 and Robo4 have been suggested to co-express in endothelial cells, and regulate Slit2 responses differentially through signaling cascades.13,14

Placental angiogenesis starts from day 32 of gestation, but development of the vascular tree continues...
to term. Angiogenesis is a complex process that involves extracellular matrix alteration, endothelial cell proliferation, differentiation and migration, and vessel stabilization by pericytes and mural cells. We previously isolated human placental multipotent mesenchymal stromal cells (hPMSCs) from placentas. These cells were found to distribute in villous stroma, but their role in the placental villous microenvironment remains unknown. Multipotent mesenchymal stromal cells are known to produce various soluble growth factors and cytokines. We previously observed that hPMSCs expressed IL-6, IL-8 and HGF, which are involved in endothelial cell protection from oxidative stress and trophoblast migration. In the present study, we also observed that hPMSCs express Slit2. However, the role of Slit2 in the placental villous microenvironment has not been explored. Thus, we hypothesize that hPMSCs express Slit2, which may modulate endothelial cells in placental angiogenesis via Robo1/4 receptors.

**Results**

**Differential expression of Slit2, Robo1 and Robo4 in HUVECs and hPMSCs**

The mRNA encoding Robo1–4 of HUVECs was studied, and mRNAs of Robo1 and Robo4 were found in HUVECs (Fig. 1A). Slit2 mRNA was also observed in hPMSCs, while Slit3 mRNA expressed significantly less than Slit2 (Fig. 1C). Protein expression of Robo1 and Robo4 in HUVECs and that of Slit2 in hPMSCs were further confirmed by Western blot (Figs. 1B and D).

**Effect of Slit2 and hPMSC-conditioned medium on HUVEC migration**

Analyzing the conditioned medium of hPMSCs by Western blot and ELISA revealed that hPMSCs expressed Slit2. The Slit2 level in the hPMSC-conditioned medium significantly decreased after Slit2 depletion (Fig. 2A). When the HUVECs were exposed to Slit2 recombinant protein or the hPMSC-conditioned medium, decreased migration of HUVECs compared to HUVECs in the EGM control medium was observed. The inhibition effect of the hPMSC-conditioned medium on HUVEC migration was abrogated when Slit2 in hPMSC-conditioned medium was depleted by a blocking antibody (p < 0.01; Fig. 2B).

**Endothelial cell-hPMSC interactions mediated by Slit2–Robo1/4 in vitro**

To investigate the functional correlation of the endothelial cell–hPMSC interaction in vascularization, we investigated the effects of Slit2 and hPMSC-conditioned medium on formation of tube structures and endothelial cell-network stabilization formed by HUVECs on basement membrane-like growth factor-reduced Matrigel. HUVECs cultured in the EGM control medium were used as a control, and they were able to form characteristic tube structures (Fig. 3A). Culture medium containing either Slit2 recombinant protein or the hPMSC-conditioned medium significantly enhanced HUVEC elongation and formation of interconnecting cell networks. The tube structure was significantly inhibited in HUVECs cultured in medium containing hPMSC-conditioned medium with Slit2 depletion. These findings indicated that Slit2 significantly increased the ability of HUVECs to form tube structures (Figs. 3B–D). Quantification of the tube structures by measuring the cumulative tube length, polygonal network number and vessel branching point number was shown in Figures 3E–G. Thus, Slit2 expressed by hPMSCs supported tube formation of HUVECs in Matrigel assays.

To examine the role of Robo1 and Robo4 in angiogenesis, Robo1 and Robo4 genes were transiently knocked down in HUVECs using siRNAs. The expression of Robo1 or Robo4 was significantly reduced in Robo1- or Robo4-silenced endothelial cells, respectively. The expression of Robo4 in endothelial cells with Robo1 knockdown was not affected compared to that with non-silencing control siRNA transfection. Similarly, the expression of Robo1 did not alter in endothelial cells with Robo4 knockdown (Fig. 4A). *In vitro* angiogenesis assays using growth factor-reduced Matrigel, Robo1 knockdown HUVECs showed an impaired ability to form a tube network, as fewer branches were formed. Similarly, tube formation was significantly inhibited in Robo4 knockdown HUVECs compared to the controls (Fig. 4B). The tube formation was significantly increased in Robo1- or Robo4-silenced HUVECs cultured in the medium containing Slit2 recombinant protein or hPMSC-conditioned medium, but was significantly reduced in cells cultured in the medium containing an Slit2-depleted hPMSC-conditioned medium (Fig. 4B). Quantification of the tube structures by measuring the cumulative tube length, the polygonal network number (Figs. 4C and 4D) and vessel branching point number was shown (Fig. S1). However, even though the knockdown efficiencies of Robo4 and Robo1 varied slightly, we did find an
additive effects due to knockdown of both Robo1 and Robo4 on suppressing tube formation (Figs. 4A, 4C and 4D). There was nearly no tube formation in the HUVECs with or without medium containing Slit2 recombinant protein or hPMSC-conditioned medium. (Figs. 4B–D, Fig. S1). Based on these observations, we could not conclude which Robo receptor played a more important role in angiogenesis.

Heterodimerization of Robo1 and Robo4

The Robo receptors were known to form homo- and heterodimers, which were important for their functions. Slit2 could modulate the expression of Robo1 and Robo4 receptor in endothelial cells (Fig. 5A). We further demonstrated that the expression levels of Robo1 and Robo4 receptors in endothelial cells decreased with increasing doses of Slit2 from 0.5 to 2 μg/ml (Fig. 5A). To study heterodimerization, endogenous Robo1 was immunoprecipitated from HUVEC lysates with or without Slit2 treatment. Robo1 and Robo4 Western blots showed that endogenous Robo1 co-immunoprecipitated with Robo4, suggesting that these proteins interacted in HUVECs. Furthermore, immunoprecipitates followed by Slit2 immunoblot also revealed the interaction between Slit2 and Robo receptors (Fig. 5B).

Figure 1. Determination of the levels of Slit2, Roundabout (Robo)1 and Robo4 in human umbilical vein endothelial cells (HUVECs) and human placental multipotent mesenchymal stromal cells (hPMSCs). The mRNA and protein levels of Slit2, Robo1 and Robo4 were examined 24 hours after cell plating by RT-PCR (A, C) and Western blotting (B, D). The mRNA (A) and protein (B) expression of Robo1 and Robo4 expressions were detected in HUVECs. Both mRNA (C) and protein (D) levels of Slit2 were observed in hPMSCs. 1, 2: different strains of cells in HUVECs (A, B) or hPMSCs (C, D).
Discussion

This study revealed that the Slit2 secreted by hPMSCs was an important modulator for Robo1/Robo4 mediated endothelial cell migration and angiogenesis in vitro. Robo2 and Robo3 mRNA was not detected in endothelial cells, whereas Robo1 and Robo4 mRNA exhibited relatively high levels of expression. Robo1 and Robo4 are the cognate receptors of endothelial cells for Slit2. Slit2

Figure 2. Slit2 was expressed by human placental multipotent mesenchymal stromal cells (hPMSCs) and the Slit2 effect on HUVEC migration. (A) The Slit2 levels in the hPMSC-conditioned medium before and after Slit2 depletion by immunoprecipitation was assessed by Western blot (upper panel) and enzyme-linked immunosorbent assay (lower panel). CTL: hPMSC-conditioned medium without Slit2 depletion. Data are mean ± SD of 3 independent experiments. (B) The HUVEC migration ability was significantly reduced by EGM with 2% FBS (CTL) containing 1 μg/ml Slit2 (Slit2), or CTL mixed with a hPMSC-conditioned medium containing 2% FBS (1:1 ratio; CM), but the inhibition was abrogated by Slit2 depletion from the conditioned medium (CTL mixed with hPMSC-conditioned medium with 2% FBS after Slit2 depletion, 1:1 ratio; CM-Slit2 depletion). CTL: EGM with 2% FBS. Data are mean ± SD of 3 independent experiments.

Figure 3. In vitro angiogenesis: The effect of Slit2 on the formation of tube structures by human umbilical vein endothelial cells (HUVECs). Slit2 increased the angiogenesis in HUVECs. HUVECs (1 × 10⁴ cells/well) were plated on growth factor-reduced Matrigel and cultured in 50 μl of (A) EGM with 2% FBS (CTL), (B) CTL containing 1 μg/ml Slit2 (Slit2), (C) CTL mixed with hPMSC conditioned medium with 2% FBS (1:1 ratio; CM) or (D) CTL mixed with hPMSC-conditioned medium with 2% FBS after Slit2 depletion (1:1 ratio; CM-Slit2 depletion). HUVECs formed characteristic tube structures. Cell elongation and interconnecting cell networks were observed. Slit2 significantly increased the ability of HUVECs to form tube structures. Representative data of 5 different experiments are shown. Quantification of the tube structures by measuring (E) the cumulative tube length, (F) the polygonal network number and (G) vessel branching point number formed by HUVECs in different culture conditions. A significant inhibition of tube structure formation was observed when Slit2 was depleted from the hPMSC conditioned medium. Error bar: SD. Scale bar: 100 μm.
binds to the heterodimer receptor formed by Robo1 and Robo4. Co-culture of hPMSCs with endothelium on a growth factor-reduced Matrigel, the Slit2 or an hPMSC-conditioned medium containing Slit2 could enhance endothelial cells forming more tube networks than endothelial cells alone. Tube formation was significantly reduced in endothelial cells cultured with the Slit2-depleted hPMSC-conditioned medium or in endothelial cells transfected with siRNA for Robo1, Robo4 or both. These observations indicated that hPMSC-derived Slit2 was involved in tube formation. Robo1 cooperated with Robo4 to modulate signaling pathways downstream of Slit2 and mediate angiogenesis in vitro.

Figure 4. Robo1 and Robo4 receptors were involved in angiogenesis in human umbilical vein endothelial cells (HUVECs). Robo1, Robo4, and both receptor knockdown (KD) significantly inhibited angiogenesis. (A) The Robo1- (Robo1 KD) and Robo4-silenced (Robo4 KD) in HUVECs were shown by Western blot. (B) In vitro angiogenesis was evaluated 4 hours after Robo1, Robo4 and both receptor knockdown in HUVECs. The cells were plated on growth factor-reduced Matrigel, treated with EGM with 2% FBS (CTL), CTL containing 1 μg/ml Slit2 (Slit2), CTL mixed with hPMSC-conditioned medium with 2% FBS (1:1 ratio; CM) or CTL mixed with hPMSC-conditioned medium with 2% FBS after Slit2 depletion (1:1 ratio; CM-Slit2 depletion). Quantitative analysis showed that the cumulative tube length (C), polygonal network number (D) in various culture conditions were increased significantly with 1 μg/ml Slit2 and CM treatment, and the enhanced effects were blocked significantly after Robo1, Robo4 and both receptor knockdown. HUVECs in CTL were transfected with non-silencing control siRNA. Error bar: SD. Scale bar: 100 μm.
Mesenchymal stem cells have been reported to cooperate with endothelial cells to enhance and stabilize vascular network formation through secreting angiogenic growth factors with or without the need of a direct cell to cell contact. We previously showed that hPMSCs have a phenotype similar to that of bone marrow mesenchymal stem cells. Here, we also observed that the hPMSC-conditioned medium could stabilize endothelial cell vascularization with increasing cumulative tube length, polygonal network number and vessel branching point number. Analysis of hPMSC-secreted proteins revealed Slit2 mediated these responses, extending our knowledge of vascular endothelial growth factor, bFGF, and hepatocyte growth factor secreted by stem cells associated with angiogenesis.

Our data have shown that Slit2-Robo receptor signaling was involved in angiogenesis. Slit2 released from tumor cells has been found to affect Robo1-dependent tumor angiogenesis. By binding to Robo4, Slit2 can stabilize the vasculature and inhibit VEGF-induced endothelial cell migration and permeability in vitro, and prevent pathologic angiogenesis in mouse retina. Vessel maturation during angiogenesis requires recruitment of mural cells and development of surrounding matrix to stabilize endothelial cells and inhibit endothelial cell migration. We thus suggested that Slit2 could modulate placenta angiogenesis and maintain vessel integrity via Slit2-Robo1/Robo4 interaction. We observed that hPMSs from placental villous stroma expressed Slit2, which inhibited endothelial cell migration to stabilize tube formation, as demonstrated by the effects of Slit2 recombinant protein or the hPMS-conditioned medium on cell elongation and tube network formation. Additionally, pericytes and vascular smooth muscle cells constitutively expressed Slit2 and/or Robo1 and Robo4, raising the possibility of Slit2-based autocrine and paracrine influence on vascularization. These findings supported the hypothesis that hPMS-derived Slit2 might contribute to placenta angiogenesis in a similar communication network involved mural cells and endothelial cells. Nonetheless, there were contradictory results showing that binding of Slit2 to Robo1/4 receptor induced actin cytoskeleton movements, filopodia formation, and led to endothelial cell migration.

Our results revealed Slit2 modulation of Robo1 and Robo4 receptor expression in endothelial cells. The expression levels of Robo1 and Robo4 receptors decreased as Slit2 treatment concentration increased as shown in Western blot. The interaction between Robo1 and Robo4 with or without 1 μg/mL Slit2 for 5 to 15 minutes was examined by co-immunoprecipitation (IP) in HUVEC lysate. The HUVEC Robo1 was immunoprecipitated from HUVEC lysate and probed by an antibody against Robo1, Robo4 or Slit2. Western blot (WB) revealed that endogenous Robo1 co-immunoprecipitates with Robo4. Slit2 interacted with the Robo1/4 receptor complex. The lower panel showed the input of Slit2, Robo1, Robo4 and α-tubulin.

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Our results revealed Slit2 modulation of Robo1 and Robo4 receptor expression in endothelial cells. The expression levels of Robo1 and Robo4 receptors decreased as Slit2 concentrations were increased from 0.5 to 2 μg/mL. This finding could be explained by the observation that microRNA-218 encoded intronically in Slit2 gene and shared the same transcript as Slit2, targeted the untranslated region of the Robo receptor and inhibited its protein translation. HUVECs treated by Slit2 significantly increased the intracellular levels of Slit2 and microRNA-218, revealing a positive regulation of Slit2 expression. The microRNA-218 negatively regulated the expression of Robo1 and Robo2. This suggested potentially there was a reciprocal modulation between Slit2 and microRNA-218 on Robo expression. Conceivably, increasing Slit2 protein expression drove synthesis of microRNA-218, which could then suppress the expression of Robo receptor and Slit2-Robo signaling. A study on zebrafish heart tube formation also observed Slit2 stimulation suppressed Robo1 and Robo2 expression and inhibited endocardial migration.
co-immunoprecipitation experiments, the interaction between Slit2 and Robo1/Robo4 was not altered. Thus, the optimal levels of Slit2 and Robo expression involved in angiogenesis will need further investigation. Additionally, Robo4 can be co-immunoprecipitated with Robo1 in cultured endothelial cells with or without Slit2 stimulation. In agreement with previous reports, this finding suggests that Robo1–Robo4 heterodimerization responds to Slit2 binding, leading to Robo1 and/or Robo4 downstream signaling and cellular responses.

A variety of signaling factors are known to orchestrate placenta angiogenesis, including VEGF, PDGF and Ang/Tie 2. Here we found that Slit2–Robo signaling might also play an important role. Slit2 is expressed by hPMSCs and therefore may act in a paracrine fashion through interacting with Robo receptors expressed by endothelial cells. The present data extend our understanding of hPMSC functions in villous development, particularly in placenta vascularization.

Materials and methods

hPMSC isolation and culture

Placental tissue was obtained after informed consent of the women, and all experiments were approved by the Institutional Review Board of Mackay Memorial Hospital, Taipei. hPMSCs were isolated from clinically normal human term placentas (37 to 40 weeks of gestation) collected after cesarean section as previously described. Briefly, about 100 g of tissue from central placental cotyledons was minced. The tissue was trypsinized (0.25% trypsin-EDTA solution; Invitrogen, Carlsbad, CA) and treated with 10 U/ml DNAse I (Sigma-Aldrich, St. Louis, MO) in Dulbecco’s modified Eagle’s medium (DMEM; Gibco-BRL, Grand Island, NY, USA) at 37°C for 5 min 3 times, and finally filtered through a cell strainer (BD Biosciences, San Diego, CA). The supernatants were centrifuged and the mononuclear cells in the supernatants were recovered by Percoll density gradient fractionation (1.073 g/ml, Sigma-Aldrich). The cell cultures were maintained in DMEM (Gibco-BRL) with 10% FBS (HyClone, Logan, UT) at 37°C. The hPMSCs which were initially isolated from tissue and plated on culture dishes were defined as passage 0. The cells were used for experiments at passage 4 to 5.

The cell phenotype of the hPMSCs was characterized by a panel of phycoerythrin- or fluorescein isothiocyanate-conjugated antibodies using standard fluorescence-activated cell sorting analysis and CellQuest software as previously described. The hPMSCs isolated from term placentas were found to express CD13, CD29, CD44, CD49b, CD54, CD73, CD90, CD105, CD166 and SSEA4, independent of gestational age. The cells are negative for CD14, CD34, CD45, HLA-DR, but are multipotent with the ability to differentiate into osteocytes, adipocytes and endothelial cells (not shown).

HUEVCs collected as previously described were cultured using an endothelial cell growth medium (EGM kit with SupplementPack (PromoCell GmbH, Heidelberg, Germany). The cells were used for experiments at passage 4 to 6.

Preparation of the conditioned medium

hPMSCs were grown in DMEM (Gibco-BRL) with 10% FBS (HyClone) until 80% confluent. The culture medium was removed and cell layers were washed and incubated with endothelial cell basal medium (PromoCell) with 2% FBS (Hyclone) for 2 days. Conditioned medium was collected, centrifuged at 2300 × g for 5 min, passed through a 0.22 µm filter, and stored at −80°C for use in subsequent experiments.

To remove Slit2 from the conditioned medium, the medium was incubated with 2.4 µg/ml of anti-Slit2 polyclonal antibody (GeneTex, Hsinchu, Taiwan) for 4 to 6 h at 4°C with constant rotation. Then, 100 µl of protein G-agarose beads (50% slurry; Thermo Scientific, Rockford, Illinois, USA) were added and incubated overnight at 4°C with rotation. Conditioned medium containing agarose beads were centrifuged at 800 × g for 3 min and the supernatant was collected and used immediately. To verify that Slit2 had been removed from the conditioned medium, an aliquot of the conditioned medium stripped of Slit2 through immunoprecipitation was tested for Slit2 concentration by an enzyme-linked immunosorbent assay.

Enzyme-linked immunosorbent assay (ELISA)

ELISA was used to assess the Slit2 concentration in the hPMSC supernatant as per the manufacturer’s instructions (MyBioSource, San Diego, California, USA).

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA (1 µg) of isolated HUVECs and hPMSCs was extracted using the TRIzol reagent (Life Technologies, Carlsbad, CA) according to the manufacturer’s protocol, and cDNA was synthesized using oligodeoxynucleotidemin (Promega Corporation, Madison, WI) and a Superscript II reverse transcriptase (Invitrogen). The cDNA was
amplified via PCR for 35 cycles at 95°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec. 18s rRNA was used as an internal control. PCR products were checked by 2% agarose gel (Amresco, Solon, OH) electrophoresis. The primers used were shown in Table 1.

**Western blot**

Total protein (30 μg) was separated by 8% sodium dodecyl sulfate-polyacrylamide gel and transferred on to an Immobilon polyvinylidene difluoride membrane (Amersham Biosciences, Piscataway, NJ). We conducted immunoblot analysis using antibodies against Slit2 (1:1000; GeneTex), Robo1 (1:1000; GeneTex), Robo4 (1:1000; Santa Cruz Biotechnology, Dallas, TX), α-Tubulin (1:5000; Millipore, Temecula, CA) overnight at 4°C, and followed by incubation with the appropriate horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. The proteins were visualized by chemiluminescence detection kit (Millipore).

**Transwell migration**

HUVECs (1.0 × 10⁴ cells/well) were added to the upper chamber of a Transwell® device (Costar) and allowed to migrate for 24 h in EGM (PromoCell GmbH) with 2% FBS containing either Slit2 (1 μg/ml, Peprotech, Rocky Hill, NJ), EGM (PromoCell) mixed with hPMSC conditioned medium with or without Slit2 depletion (1:1 ratio) in the lower chamber. Trans-membrane migrated cells were stained by DAPI (Sigma-Aldrich) and quantified under microscopy (magnification 50; Axiovert 200; Carl Zeiss MicroImaging) equipped with Image-Pro Plus software (Media Cybernetics).

**Transfection with Robo1 siRNAs or Robo4 siRNAs**

The HUVECs (5 × 10⁵) were seeded in a 6-cm dish with EGM (PromoCell GmbH) containing 2% FBS (HyClone) for overnight. The HUVECs were then transfected with the siRobo1 (SMART pool; Dharmacon, Lafayette, CO), siRobo4 (SMART pool; Dharmacon), or both (Table 1) and non-silencing control siRNA (Invitrogen) using a jetPRIME (Polyplus, New York, NY) transfection reagent. After 48 h of transfection, the levels of Robo1 and Robo4 were confirmed by Western blotting analysis.

**Co-immunoprecipitation assay**

To demonstrate the homo- or heterodimerization of Robo1 and Robo4, HUVECs treated with or without 1 μg/ml Slit2 recombinant protein (Peprotech) for 5 to 15 min were extracted in 1 × CHAPS lysis buffer (20 mM Tris, 136.8 mM NaCl pH7.5, 1% CHAPS) and centrifuged at 10,000 × g for 15 min at 4°C to obtain cell lysates. Cell lysates (150 μg) were precleared with 40 μl protein G beads (Thermo Fisher Scientific). Cell lysates were incubated with an anti-Robo1 or anti-Robo4 (R&D) antibody overnight at 4°C, followed by an addition of 40 μl protein G beads (Thermo Fisher Scientific) for 3 h at 4°C. Protein G beads (Thermo Fisher Scientific) were collected by centrifugation at 3,000 × g for 5 min at 4°C, and the immunoprecipitates were washed twice with 1 × CHAPS lysis buffer and once with PBS. The protein complexes were subjected to immunoblot with specific primary anti-Slit2 (GeneTex), Robo1 (GeneTex), and Robo4 (Abcam, Table 1. The primer sequences used for RT-PCR and siRNA.

| Gene   | RT-PCR Forward (5’-3’) | RT-PCR Reverse (5’-3’) |
|--------|------------------------|------------------------|
| Slit1  | TGGCCTTCCCTGACCTCAGGGTGTG | GTTCCCTTGACCTCAGGTCACC |
| Slit2  | CAGATCAAAGCAAGAAATTCCGGTTG | GAACATCTTATGCTGCACATTTT |
| Slit3  | CCTCTGTCAGATGAGGCCAAGTGCCGTGGCCTGGTACAGCTCCAGTG | GGAGGGCAACUAAUGCAUA |
| Robo1  | CAGCCATGCTCATCTTGGAATTCATT | GAGGGGCAACUAAUGCAUA |
| Robo2  | GATCAGATTGGTTGCTCAAGGTCG | GTAAATCCCTCCTTACAGGCA |
| Robo3  | CAGCCGTGACCTGTTAGCAGC | TCCATCCTGGCAAGAACTTCATTT |
| Robo4  | GAGGGCAGCUUAGUACAGCAGC | TCTCGAGGGGCGAGCAGACAG |
| 18s rRNA | TAGAGCTTAATCAATGCCCAGGG | GGGCCTTGAAGAAAGTCTCTGATT |

**siRNA**

| Gene   | SMART pool (5’-3’) |
|--------|-------------------|
| Robo1  | GAAUCAGACUGGUUAGUU |
| Robo2  | GAGGGGCAACUAAUGCAUA |
| Robo4  | CCUCAGAAGUUCAGGGCAAU |

Table 1. The primer sequences used for RT-PCR and siRNA.
Cambridge, UK) antibodies, and subsequently hybridized with the respective secondary antibody.

In vitro angiogenesis

The μ-slide angiogenesis (ibidi GmbH, München, Germany) was coated with 10 μl per well of growth factor-reduced Matrigel (BD Biosciences). Slides were incubated at 37°C for 30 min. The HUVECs were plated at a number of 1 × 10⁴ cells/well. HUVECs were cultured in 50 μl of EGM/2% FBS, EGM/2% FBS with 1 μg/ml Slit2 (Peprotech), EGM/2% FBS mixed with hPMSCs conditioned medium (1:1 ratio) or EGM/2% FBS mixed with hPMSCs conditioned medium after Slit2 depletion (1:1 ratio) at 37°C with 5% CO₂ for 4 h and monitored by a microscope (Zeiss). For studying the role of Robo receptors in angiogenesis, HUVECs were transfected by Robo1 siRNA, Robo4 siRNA (Dharmacon), both siRNA or non-silencing siRNA control (Invitrogen) and incubated as described. Tube formation was quantified by counting the sum of the tube length or the tube formation structures by measuring the polygonal network and vessel branching point number in each well. The total length of the tubes was calculated as the sum of the length of the individual branches including several tube-like structures merged together or branched. Results are represented as total tube length (pixels) or a number for 6 random photographic fields per experimental condition (magnification 50; Axiovert 200; Carl Zeiss MicroImaging).

Statistics

The data are presented as means ± standard deviation. At least 3 independent experiments were carried out for each experiment. Differences were assessed using the Student’s t test. A p value of <0.05 is considered significant.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Funding

This work was supported by a grant from Mackay Memorial Hospital (MMH-E 103001) and Taiwan’s Ministry of Science and Technology (MOST-104-2314-B-195-010-MY3 to C.-P Chen).

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