Original Paper

Roundabout4 Suppresses Glioma-Induced Endothelial Cell Proliferation, Migration and Tube Formation *in Vitro* by Inhibiting VEGFR2-Mediated PI3K/AKT and FAK Signaling Pathways

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Key Words

Roundabout4 • Angiogenesis • Endothelial cell • Glioma • Vascular endothelial growth factor

Abstract

Background and Aims: Endothelial cell (EC) proliferation, migration, and tube formation are the critical steps for tumor angiogenesis, which is involved in the formation of new tumor blood vessels. Roundabout4 (Robo4), a new member of Robo proteins family, is specifically expressed in endothelial cells. This study aimed to investigate the effects of Robo4 on glioma-induced endothelial cell proliferation, migration and tube formation *in vitro*. **Methods and Results:** We found that Robo4 was endogenously expressed in Human Brain Microvascular Endothelial Cells (HBMECs), while Robo4 was significantly down-regulated in endothelial cells cultured in glioma conditioned medium. Robo4 over-expression remarkably suppressed glioma-induced endothelial cell proliferation, migration and tube formation *in vitro*. In addition, Robo4 influenced the glioma-induced angiogenesis via binding to its ligand Slit2. Further studies demonstrated that the knockdown of Robo4 up-regulated the phosphorylation of VEGFR2, PI3K, AKT and FAK in EC cultured in glioma conditioned medium. VEGFR2 inhibitor SU-1498, AKT inhibitor LY294002 and FAK inhibitor 14 (FAK inhibitor) blocked the Robo4 knockdown-mediated alteration in glioma angiogenesis *in vitro*. **Conclusion:** Our results proved that Robo4 suppressed glioma-induced endothelial cell proliferation, migration and tube formation *in vitro* by inhibiting VEGFR2-mediated activation of PI3K/AKT and FAK signaling pathways.

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Introduction

Glioma is one of the most highly angiogenic solid tumors, whose aggressive blood vessel formation contributes to the failure of conventional therapies such as surgery, radiation, and chemotherapy [1]. Recently, the role of angiogenesis in glioma growth and progression has gained increasing attentions. Anti-angiogenic therapies are considered as promising therapeutic strategies for gliomas [2-4].

Angiogenesis, described as the formation of new blood vessels from existing vessels, plays a critical role in the development and progression of glioma [5, 6]. This complex process includes proteolysis of the extracellular matrix, endothelial cell proliferation, migration, and differentiation as well as tube formation [7]. Glioma cells are able to produce and secrete several growth factors such as vascular endothelial growth factor (VEGF), interleukin (IL)-8 and basic fibroblast growth factor (bFGF), which acts on endothelial cells to regulate tumor angiogenesis [3, 8-10].

Roundabout (Robo) family is a class of highly conserved transmembrane proteins that contribute to synapse development [11, 12] and play crucial roles in the morphological differentiation of neurons [13]. Slit, the ligand of Robo, regulates neuronal cell proliferation and migration which is essential to the neural network formation [14]. This process is very similar to tumor angiogenesis. Robo4, a new member of Robo protein family, is expressed specifically in endothelium [15]. Kaur et al. reported that Robo4 could mediate angiogenesis in embryonic zebrafish through Rho GTPases signaling mechanisms [16]. Evidence also shows that Robo4 is essential for coordinated symmetric and directed sprouting of intersomitic vessels of zebrafish [17]. These findings proved that Robo4 could regulate angiogenesis. However, the role of Robo4 in glioma angiogenesis still remains unclear.

VEGF signaling pathway plays a crucial role in tumor angiogenesis, which is involved in the promotion of endothelial cell proliferation, migration, and capillary tube formation [18, 19]. VEGF could bind to two distinct receptor tyrosine kinases (RTKs), VEGFR1 (Flt-1) and VEGFR2 (KDR/Flk-1). VEGF mainly interacts with VEGFR2 in endothelial cells [20]. VEGFR2 could promote the activation of various pathways including phospholipase C gamma (PLC gamma) [21], extracellular signal-regulated kinases (ERKs), FAK (focal adhesion kinase), phosphatidylinositol 3-kinase (PI3K) and AKT [22, 23]. Recent studies have demonstrated that Robo4 could regulate the activation of VEGF signaling pathway. Report showed that Robo4 could maintain vessel integrity by inhibiting VEGF-induced vessel permeability increase and fixing barrier defects in mice [24]. In addition, Robo4-paxillin complex blocks the activation of small GTPase Arf6 and inhibits VEGF-165-stimulated vascular leak and retinal permeability increase [25]. Activation of Robo4 by Slit2 inhibits VEGF-165-induced lung endothelial cell migration and tube formation *in vitro* by blocking sarcoma oncogene/tyrosine kinase family kinase (Src) activation [26]. However, it remains unclear whether Robo4 has regulatory effects on VEGF signaling in glioma angiogenesis.

Under such backgrounds, this study aims to investigate the regulatory effects of Robo4 on glioma angiogenesis *in vitro* and thereby to reveal the possible mechanism.

Materials and Methods

Reagents and antibodies

Endothelial basal medium-2 (EBM-2) was purchased from Lonza (Walkersville, MD, USA). Fetal bovine serum (FBS) "Gold" and 10 mM HEPES were provided by PAA Laboratories GmbH Laboratories (Pasching, Austria). Penicillin-streptomycin, chemically defined lipid concentrate were offered by Invitrogen (Life Technologies, Carlsbad, CA, USA). Cultrex rat collagen-I was obtained from R&D Systems (Minneapolis, MN, USA). Human basic fibroblast growth factor (bFGF), hydrocortisone and ascorbic acid were supplied from Sigma-Aldrich (St Louis, MO, USA). High-glucose Dulbecco's modified Eagle medium (DMEM), Dulbecco's modified Eagle medium F12 mixed medium and FBS were bought from Gibco (Life Technologies, Carlsbad, CA, USA). N-terminal human Slit2 (Slit2N) and human VEGF were purchased from Peprotech (Rocky Hill, NJ, USA).

Rabbit polyclonal anti-Robo4 (Ab103674), anti-Slit2 (Ab7665), and mouse monoclonal anti-GAPDH (Ab8245) antibodies were provided by Abcam (Cambridge, MA, USA). Rabbit Monoclonal phospho-VEGF Receptor-2 (Tyr1175) (#2479), anti-PI3 Kinase p85 (#4257), anti-phospho-Akt (Ser473) (#4060), Polyclonal anti-VEGF Receptor-2 (#2472), anti-AKT (#9272), anti-phospho-FAK (Tyr397) (#3283), anti-FAK (#3285) and anti-Phospho-PI3 Kinase p85 (Tyr458)/p55 (Tyr199) (#4228) antibodies were obtained from Cell signaling technology (Beverly, MA, USA). Normal rabbit IgG and rabbit anti-VEGFA-neutralizing antibody (#MAB293) were provided by R&D Systems (R&D Systems, Minneapolis, MN, USA).

Recombinant Human VEGF-165 and N-terminal human Slit2 (Slit2N) were provided by Peprotech (Rocky Hill, NJ, USA). Cells were pretreated with Slit2N (10uM), VEGF-165 (25uM), or the combination of them for 24 hours. 1M PBS was used as the control. Specific inhibitors against VEGFR2 (SU-1498, #sc-355982) and FAK (FAK inhibitor 14, #sc-203950) were offered by Santa Cruz Biotechnology (Dallas, Texas, USA). Specific inhibitor against AKT (LY294002, L9901) was purchased from Cell signaling technology (Beverly, MA, USA). The concentrations used in this work were 10uM, 10uM and 5uM for SU-1498, LY294002, and FAK inhibitor 14, respectively. Cells were pre-treated with one of the inhibitors or DMSO as a control for 24 hours. All the other reagents were purchased from Sigma-Aldrich (St Louis, MO, USA) unless otherwise specified.

Patients and specimens

All human specimens were obtained from the Department of Neurosurgery, Shengjing Hospital of China Medical University (Shenyang, China). This study was approved by the institutional review board of Shengjing Hospital of China Medical University and each patient signed a consent form to participate this study. Glioma specimens were divided into two groups: grade I-II glioma group (n=3) and grade III-IV glioma group (n=3) according to the WHO classification of tumors in the central nervous system. Human brain tissues obtained from surgeries of epilepsy were used as negative control (n=3).

Cell culture

The Human Brain Microvascular Endothelial Cell line (HBMECs) was provided by Dr. Couraud (Institut Cochin, Paris, France). The cells (passage 30-35) were cultured in culture flasks coated with 150 µg/ml of cultrex rat collagen I. The culture medium contained endothelial basal medium (EBM-2) supplemented with 5% FBS "Gold", 1% penicillin-streptomycin, 1% chemically defined lipid concentrate, 1 ng/ml bFGF, 1.4 µM hydrocortisone, 5 µg/ml ascorbic acid and 10 mM HEPES. Cells were maintained in a humidified incubator at 37 °C and 5% CO₂ and the medium was refreshed every 48 hours.

Human glioblastoma cell lines U87MG, A172 and U118MG were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Glioma conditioned medium were collected from the indicated human glioma cells grown in 100-mm-diameter Petri dishes. Tumor conditioned medium was prepared from the glioblastoma cell culture grown to near confluency. After washing the incubator twice with serum free medium, cells were incubated in serum free EBM-2 medium in a humidified incubator at 37 °C and 5% CO₂ for 24 hours. The supernatant was harvested, centrifuged at 2000g at 4°C for 10 minutes and supplemented with 5% FBS "Gold", 1% penicillin-streptomycin, 1% chemically defined lipid concentrate, 1ng/ml bFGF, 1.4 µM hydrocortisone, 5µg/ml ascorbic acid and 10 mM HEPES, EGF and hydrocortisone prior to use.

Our previous data from EC proliferation, migration and tube formation assays found that no significant difference in the viability, migration and tube formation of ECs was found among different kinds of glioma conditioned medium (Fig. 3C). But the expression level of Robo4 in EC was lowest in U87MG glioma conditioned medium group (Fig. 3A, B). Therefore, U87MG glioma conditioned medium were selected as the optimum glioma conditioned medium in the subsequent experiments.

Vectors and constructs

Total RNA from endothelial cells was extracted using Trizol reagent following the manufacturer's protocol (Invitrogen, Carlsbad, USA). 0.5 µg of total RNA was used to synthesize the first-strand cDNA with the reverse transcription (RT) system (Promega, USA). Full-length human Robo4 (Gene ID: 54538) was amplified by PCR using the specific primers (Invitrogen, Carlsbad, USA) containing Hind III and KpnI restriction sites. The amplified product was cloned into the pIRES2-EGFP expression vectors by standard procedures and confirmed by restriction digestion and DNA sequencing. An empty vector, pIRES2-EGFP, was used as the control.

The short hairpin RNA (shRNA) against human Robo4 gene was constructed into U6/GFP/Neo plasmid vector (GenePharma, Shanghai, China). A plasmid carrying a non-targeting control sequence was used as a transfection control.

Establishment of stable Robo4 over-expression and knockdown in endothelial cell lines

The pIRES2-Robo4-EGFP and shRNA vectors were transfected into HBMECs using Lipofectamine LTX Reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. The HBMECs were also transfected with pIRES2-EGFP and shRNA of non-targeting control sequence as negative controls. The stably transfected cells were selected by the culture medium containing 0.4 mg/ml G418. After approximately four weeks, G418-resistant cell clones were established. Robo4 protein expression and knockdown efficiency were assessed by Western blot analysis.

Enzyme-linked immunosorbent assay (ELISA)

Concentrations of slit2 and VEGF in the conditioned medium were measured using Human VEGF ELISA kit (R&D Systems, Minneapolis, MN USA). Concentrations of slit2 in the conditioned medium were measured using Human Slit2 ELISA kit (Cusabio Biotech Co, Wuhan, China). Conditioned medium was collected after 24 hours. Measurements were conducted according to the instruction provided by the manufacturer. Serials dilution of human recombinant VEGF and Slit2 was included in each assay to obtain a standard curve.

Cell proliferation assay

ECs were seeded in 96-well plates at the density of 2000 cells/well. After being washed twice with serum free medium, ECs cells were incubated in serum free EBM-2 medium or glioma conditioned medium in a humidified incubator at 37 °C and 5% CO₂ for 24 hours. Cell viability rate was assayed using the Cell Counting Kit-8 (CCK-8, Beyotime Institute of Biotechnology, Jiangsu, China) according to the instruction provided by the manufacturer. 10 µL CCK-8 was added into each well and cells were incubated for another 2 hours in a humidified incubator. Optical density value was measured at 450 nm. Cell viability was expressed as the percentage of that of the control (untreated) cells. Five replicated wells were set up in each group and three independently repeated experiments were performed.

Cell migration assay

ECs migration *in vitro* was assayed using a Transwell chamber (Costar, Corning, NY, USA) with polycarbonic membrane (6.5 mm in diameter, 8 µm pore size). ECs were trypsinized and suspended into single cells with serum-free medium at the density of 5×10^5 cells/ml. 100 µl of the cell suspension was added to the upper chamber, and 600 µl of EBM-2 medium (or glioma Conditioned medium) supplemented with 10% FBS was added to the lower chamber. Cells were incubated for 24 hours at 37°C. Non-migrating cells on the top surface of membrane were removed with cotton swabs. Cells that migrated to the lower surface of the membrane were fixed with methanol and glacial acetic acid at the ratio of 3:1, stained with 20% Giemsa solution for 30 minutes at 37°C and were washed twice with phosphate buffer saline (PBS). Then stained cells were observed under an inverted microscope to count and average the cell numbers within five randomly chosen fields.

Tube formation Assay

We used standard Matrigel assay to evaluate *in vitro* angiogenesis activity by quantifying the tube formation, as previously described [27]. 48-well culture plates were coated with 200 µl of Matrigel (BD Biosciences, Bedford, MA, USA) per well and then allowed to polymerize for 30 minutes at 37°C. Confluent cells were suspended in fresh EBM-2 medium (or glioma conditioned medium) at the density of 1.5×10^5 cells/ml. 100 µl of the cell suspension were seeded in wells of a 48-well culture plate polymerized Matrigel and incubated at 37°C for 24 hours. Each culture was photographed at $\times 100$ magnification using Olympus DP71 immunofluorescence microscopy (Olympus, Tokyo, Japan); total tubule length and number of tubule branches were measured using Chemi Imager 5500 V2.03 software.

Western blot assay

HBMECs were lysed and the supernatant extracts were quantified for protein using the BCA protein assay kit (Beyotime Institute of Biotechnology, Jiangsu, China). Total cell lysates containing 40 µg of protein

were fractionated using SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, USA). After blocking with 5% non-fat dry milk in TBST for two hours, membranes were incubated with primary antibodies (anti-GAPDH antibody diluted at 1:1000; anti-Robo4, anti-VEGFR2, anti-PI3k, anti-AKT and anti-FAK antibodies diluted at 1:500; anti-p-VEGFR2, anti-p-PI3k, anti-p-AKT, anti-p-FAK antibodies diluted at 1:250) at 4°C overnight. After being washed three times with PBS-Tween (20 mM Tris, 137 mM NaCl, 0.1% Tween-20, pH=7.6), membranes were incubated with the corresponding HRP-conjugated secondary antibody (diluted at 1:5000) at room temperature for two hours. Protein bands were visualized by ECL (Santa Cruz Biotechnology, USA) and detected using the ECL Detection System (Thermo Scientific, USA). Then the Protein bands were scanned using Chemi Imager 5500 V2.03 software, and integrated light density values (IDVs) were calculated by Fluor Chen 2.0 software and normalized with those of GAPDH.

Quantitative real-time PCR analysis

Total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Reverse transcription was carried out with High Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA, USA). Quantitative real-time PCR was performed using TaqMan® Universal Master Mix II (Applied Biosystems, Foster City, CA, USA) by the following gene expression assays (Taqman®, Applied Biosystems): the probe for Robo4 (Hs00219408_m1), slit2(Hs01061407_m1), VEGFA(Hs00900055_m1) and GAPDH (Hs03929097_g1). PCR amplification condition was introduced as follows: 50°C for two minutes, 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds, 60°C for one minute. Relative mRNA abundances of Robo4 to endogenous control GAPDH were calculated by the standard "ΔΔ Ct method". All data were measured repeatedly via three independent experiments and thus provided the median values as final outputs.

Statistical analysis

Statistical analysis was performed using SPSS 18.0 statistical software. One-way ANOVA followed by Bonferroni post-test was used to analyze the difference between two groups. Data were described as mean ± standard deviation (SD). $P < 0.05$ was considered to be statistically significant.

Results

Glioma conditioned medium stimulated endothelial cell proliferation, migration and tube formation in vitro

To test the effects of glioma conditioned medium on endothelial cell proliferation, CCK-8 assays were performed. As shown in Fig. 1A, the viability of ECs was significantly promoted in glioma conditioned medium group compared with the endothelial cell medium group ($P < 0.05$).

Transwell migration assays were performed to test the migration ability of ECs. As shown in Fig 1B, the number of migrating cells was increased in glioma conditioned medium group compared with the endothelial cell medium group ($P < 0.05$).

Results of tube formation assay were shown in Fig 1C. Our data showed that relative tubule length and number of branches were significantly increased in glioma conditioned medium group compared with the endothelial cell medium group ($P < 0.05$).

Glioma conditioned medium significantly down-regulated Robo4 expression in endothelial cells

Quantitative real-time PCR analysis and western blot analysis were applied to detect Robo4 expression (Fig. 2A). The expression of Robo4 was significantly down-regulated in glioma conditioned medium group compared with the endothelial cell medium group ($P < 0.05$).

Establishment of endothelial cell lines with stable Robo4 over-expression or knockdown

The HBMECs were respectively transfected with Robo4 over-expression plasmid (Robo4 (+) groups), over-expression control plasmid (Robo4 (+) NC group), Robo4 silencing

Fig. 1. Glioma conditioned medium stimulated endothelial cell proliferation, migration and tube formation *in vitro*. (A) CCK-8 analysis of the viability of ECs cultured in endothelial cell medium or glioma conditioned medium. (B) Transwell migration analysis of migration ability of ECs cultured in endothelial cell medium or glioma conditioned medium. (magnification, $\times 200$; scale bar, $100\mu\text{m}$). (C) Tube formation analysis of relative tubule length and number of branches of ECs cultured in endothelial cell medium or glioma conditioned medium. (Magnification, $\times 100$; scale bar, $100\mu\text{m}$). Data represent mean $\pm$ SD ($n=5$, each). $*P<0.05$ vs. endothelial cell medium group.

Fig. 2. Glioma conditioned medium significantly down-regulated Robo4 expression in endothelial cells. (A) The mRNA expression levels of Robo4 were determined by qPCR. Western blot analysis showing the Robo4 protein expression levels in ECs cultured in endothelial cell medium or glioma conditioned medium, using GAPDH as an endogenous control. Representative protein expression and their integrated light density values (IDVs) of Robo4 are shown. Data represent mean $\pm$ SD ($n=3$, each). $*P<0.05$ vs. endothelial cell medium

group. (B) Establishment of human brain microvascular endothelial cell line with Robo4 stable over-expression or knockdown. Western blot analysis showed the Robo4 protein expression levels in control, Robo4 (+) NC, Robo4 (+), Robo4 (-) NC and Robo4 (-) groups, using GAPDH as an endogenous control. The IDV of Robo4 protein levels are shown. Data represent mean $\pm$ SD (n = 3, each). * P <0.05 vs. Robo4 (+) NC group. # P <0.05 vs. Robo4 (-) NC group.

Fig. 3. Different kinds of glioma conditioned medium significantly down-regulated Robo4 expression in endothelial cells. (A) The mRNA of Robo4 in ECs cultured in endothelial cell medium or three glioma conditioned mediums were shown. (B) The protein expression of Robo4 in ECs cultured in endothelial cell medium or three glioma conditioned mediums were shown, using GAPDH as an endogenous control. Representative protein expression and their integrated light density values (IDVs) of Robo4 are shown. (C) Different kinds of glioma conditioned medium stimulated endothelial cell proliferation, migration and tube formation *in vitro*. Data represent mean $\pm$ SD (n=3, each). * P <0.05 vs. endothelial cell medium group. # P <0.05 vs. A172 glioma conditioned medium group. & P <0.05 vs. U118 glioma conditioned medium group. ECM: endothelial cell medium; A172CM: A172 glioma conditioned medium; U118CM: U118 glioma conditioned medium; U87CM: U87 glioma conditioned medium.

plasmid (Robo4 (-) groups) and silencing control plasmid (Robo4 (-) NC groups) to construct stably transfected cell lines. Western blot analysis was applied to detect Robo4 protein expression and to validate the efficiency of Robo4 over-expression and knockdown (Fig 2B). No significant difference in Robo4 expression was found among the control, Robo4 (+) NC and Robo4 (-) NC groups. The protein expression of Robo4 in Robo4(+) group was 2.62 times higher than that in the Robo4(+)NC group (P < 0.05), while it was 72.15% lower in the Robo4(-) group than that in the Robo4(-) NC group (P < 0.05).

Different kinds of glioma conditioned medium significantly down-regulated Robo4 expression in endothelial cells

Robo4 expression in different kinds of glioma conditioned mediums groups were detected (Fig. 3A, B). The protein expression of Robo4 in each glioma conditioned mediums group was down-regulated than that in endothelial cell medium group (P < 0.05). But no significant difference in Robo4 expression was found between U118 MG glioma conditioned medium group and A172 glioma conditioned medium group. The expression level of Robo4 was lowest in U87MG glioma conditioned medium group (P <0.05). Therefore, U87MG

Fig. 4. Effect of Robo4 on glioma-induced endothelial cell proliferation, migration, and tube formation *in vitro*. Endothelial Cell viability was measured by CCK-8 proliferation assay, and results were expressed as percent viability, from left to right, the lanes are in control, Robo4(+) NC, Robo4(+), Robo4(-) NC and Robo4(-), respectively. Migration of endothelial cell was measured by transwell migration assay, and results were expressed as the number of migrated cells per field (magnification, $\times 200$; scale bar, $100\mu\text{m}$). Tube formation of ECs was measured, and results were expressed as relative tubule length and number of branches (magnification, $\times 100$; scale bar, $100\mu\text{m}$). Data represent means $\pm$ SD ($n = 5$, each). * $P < 0.05$ vs. Robo (+) NC group, # $P < 0.05$ vs. Robo (-) NC group.

glioma conditioned medium were selected as the optimum glioma conditioned medium in the subsequent experiments.

Different kinds of glioma conditioned medium stimulated endothelial cell proliferation, migration and tube formation in vitro

To test the effect of different glioma conditioned medium on endothelial cell proliferation, CCK-8 assays were performed. As shown in supplementary Fig 3C, the viability of ECs was significantly promoted in each glioma conditioned medium group compared with the endothelial cell medium group ($P < 0.05$). No significant difference in the viability of ECs was found among A172 glioma conditioned medium, U118 MG glioma conditioned medium and U87 MG glioma conditioned medium group ($P > 0.05$).

Transwell migration assays were performed to test migration ability of ECs. As shown in Fig. 3C, the number of migrating cells was increased in each glioma conditioned medium group compared with the endothelial cell medium group ($P < 0.05$). No significant difference in the number of migrating cells was found among A172 glioma conditioned medium, U118 MG glioma conditioned medium and U87 MG glioma conditioned medium group ($P > 0.05$).

Result of tube formation assay was shown in Fig. 3C. Our data showed that relative tubule length and number of branches were significantly increased in each glioma conditioned medium group compared with the endothelial cell medium group ($P < 0.05$). No significant difference in relative tubule length and number of branches was found among A172 glioma conditioned medium, U118 MG glioma conditioned medium and U87 MG glioma conditioned medium group ($P > 0.05$).

Fig. 5. Slit2 was lowly expressed in human glioma tissues. (A–B) Expression levels of slit2 were determined by qPCR in control brain tissues, glioma tissues with pathological grades from I–II to III–IV. Data represent mean $\pm$ SD (n=3, each). * $P < 0.05$ vs. control brain tissues group. (C, D) Expression levels of slit2 were determined by qPCR in glioma cell lines and endothelial cell. (E, F) Expression levels of VEGFA were determined by qPCR in glioma cell lines and endothelial cell. Data were presented as mean $\pm$ SD from three independent experiments. ECM: endothelial cell medium; A172CM: A172 glioma conditioned medium; U118CM: U118 glioma conditioned medium; U87CM: U87 glioma conditioned medium.

Over-expression of Robo4 suppressed glioma-induced endothelial cell proliferation, migration and tube formation in vitro

The effects of Robo4 on the endothelial cell proliferation were checked by CCK-8 assays. As shown in Fig. 4A, there was no significant difference in the viability of ECs among groups of endothelial cell medium. Under the treatment of glioma conditioned medium, no significant difference in the viability of endothelial cells was identified among the control, Robo4 (+) NC and Robo4 (-) NC groups. Viability of ECs in Robo4 (+) group was significantly lower than that in Robo4 (+) NC group ($P < 0.05$). Viability of endothelial cells in Robo4 (-) group was significantly higher than that in Robo4 (-) NC group ($P < 0.05$).

As shown in Fig. 4B, there was no significant difference in the number of migrating cells among the groups of endothelial cell medium. Under glioma conditioned medium, no significant difference in the number of migrating cells was shown among the control, Robo4 (+) NC and Robo4 (-) NC groups. The numbers of migrating cells were significantly decreased in Robo4 (+) group compared with the Robo4 (+) NC group ($P < 0.05$). However, the numbers of migrating cells were increased in the Robo4 (-) group compared with the Robo4 (-) NC group ($P < 0.05$). These results prove that Robo4 could suppress glioma-induced endothelial cell migration.

Result of tube formation Assay was shown in Fig. 4C. Our data showed that there was no significant difference in the relative tubule length and the number of branches among each group using endothelial cell medium. Under glioma conditioned medium, no significant difference in the relative tubule length and number of branches was found among the control, Robo4 (+) NC and Robo4 (-) NC groups. Relative tubule length and numbers of branches significantly decreased in Robo4 (+) group compared with the Robo4 (+) NC group ($P < 0.05$). However, Relative tubule length and numbers of branches were increased in Robo4 (-) group

Fig. 6. Role of Slit2 in Robo4-regulated glioma angiogenesis *in vitro*. (A) Slit2 and VEGF levels were analyzed in the endothelial cell medium or glioma conditioned medium by ELISA. Data represent mean $\pm$ SD (n=5, each). &&P<0.01 vs. endothelial cell medium group, &P<0.05 vs. endothelial cell medium group. ECs cultured in endothelial cell medium were pretreated with exogenous Slit2N, VEGF-165, or both of them for 24 hours; endothelial cell viability (B), migration (C) and Tube formation (D) are showed. Data represent mean $\pm$ SD (n = 5, each). *P<0.05 vs. corresponding groups were not pre-treated with PBS, #P<0.05 vs. corresponding groups were pre-treated with VEGF-165 group. (E) The mRNA of Robo4 in ECs cultured in endothelial cell medium and glioma conditioned medium were determined by qRT-PCR. (F) The protein expression of Robo4 in ECs cultured in endothelial cell medium or glioma conditioned medium Data were presented as mean $\pm$ SD from three independent experiments. *P<0.05 vs. ECM group. &P<0.05 vs. GCM group. ECM: endothelial cell medium; GCM: glioma conditioned medium; GCM + IgG: glioma conditioned medium preincubated with 1ug/ml normal rabbit IgG; GCM + anti-VEGFA: glioma conditioned medium preincubated with 1ug/ml anti-VEGFA-neutralizing antibody.

compared with the Robo4 (-) NC group ($P<0.05$). These results suggest that Robo4 could suppress glioma-induced endothelial cell tube formation.

Down-regulated expression of slit2 in grade III-IV glioma tissues

The expression levels of slit2 in control brain, grade I-II and grade III-IV glioma tissues were analyzed by qRT-PCR (Fig. 5A, B). No significant difference in slit2 expression was found in control brain and grade I-II glioma tissues groups ($P>0.05$). Slit2 expression levels

Fig. 7. Over-expression of Robo4 down-regulated the phosphorylation of VEGFR2, PI3K, AKT and FAK. (A) Western blot analysis of p-VEGFR2 / t-VEGFR2 (A), p-PI3K / t-PI3K (B), p-AKT / t-AKT (C) and p-FAK / t-FAK (D) levels among the control, Robo4(+) NC, Robo4(+), Robo4(-) NC and Robo4(-) groups, using GAPDH as an endogenous control. The IDVs of VEGFR2 / t-VEGFR2, p-PI3K / t-PI3K, p-AKT / t-AKT and p-FAK / t-FAK levels are shown. Data represent mean $\pm$ SD (n = 4, each). * P < 0.05 vs. Robo4 (+) NC group. # P < 0.05 vs. Robo4 (-) NC group.

were significantly down-regulated in grade III-IV glioma tissues compared with the average expression levels in control brain tissues (P < 0.05).

Robo4 inhibited glioma-induced endothelial cell proliferation, migration and tube formation via binding to its ligand Slit2

Secretions of Slit2 and VEGF were measured by ELISA assay (Fig 6A). Results demonstrated that secretions of Slit2 and VEGF were significantly promoted in the glioma conditioned medium group compared with the endothelial cell medium group.

ECs cultured in endothelial cell medium were pretreated with exogenous Slit2N, VEGF-165, or both of them for 24 hours, and then CCK-8 cell proliferation assay was performed to measure the cell viability (Fig. 6B). Results showed that there was no significant difference in the viability of endothelial cells between Robo4 (-) + Slit2N and Robo4 (-) + PBS groups (P > 0.05). Viability of endothelial cells in the control, Robo4 (+) NC, Robo4 (+) and Robo4 (-) groups was significantly reduced compared with the corresponding non-treated groups (P < 0.05). Besides, the combined treatment of Slit2N and VEGF-165 showed no significant

Fig. 8. VEGFR2 mediated PI3K/AKT and FAK signaling pathways were involved in the Robo4-regulated glioma angiogenesis *in vitro*. After ECs in glioma conditioned medium were pretreated with VEGFR2 inhibitor SU-1498(10uM), AKT inhibitor LY294002(10uM) and FAK inhibitor 14(5uM) for 24 hours, ECs proliferation (A), migration (B) and Tube formation (C) are showed. Data represent mean $\pm$ SD (n = 5, each). * $P < 0.05$ vs. Robo4 (-) NC group, # $P < 0.05$ vs. Robo4 (-) group.

difference compared with single VEGF-165 in the Robo4 (-) group ($P > 0.05$). Viability of endothelial cells in the control, Robo4 (+) NC, Robo4 (+) and Robo4 (-) groups pretreated with both Slit2N and VEGF-165 decreased significantly compared with the corresponding groups pretreated only with VEGF-165 ($P < 0.05$).

As shown in Fig. 6C, there was no significant difference in the number of migrating cells between Robo4 (-) + Slit2N and Robo4 (-) + PBS groups ($P > 0.05$). The numbers of migrating cells among the control, Robo4 (+) NC, Robo4 (+) and Robo4 (-) groups decreased significantly compared with the corresponding non-treated groups ($P < 0.05$). Similarly to cell viability, combined treatment of Slit2N and VEGF-165 had no obvious effect on cell migration than the single VEGF-165 in the Robo4 (-) group ($P > 0.05$). The number of migrating cells in the control, Robo4 (+) NC, Robo4 (+) and Robo4 (-) groups pretreated with both Slit2N and VEGF-165 was significantly reduced compared with the corresponding groups pretreated only with VEGF-165 ($P < 0.05$).

Result of tube formation assay was shown in Fig 6D. There was no significant difference in the relative tubule length and number of branches between Robo4 (-) + Slit2N and Robo4 (-) + PBS groups ($P > 0.05$). Relative tubule length and the number of branches among the control, Robo4 (+) NC, Robo4 (+) and Robo4 (-) groups were significantly decreased compared with the corresponding non-treated groups ($P < 0.05$). Besides, combined treatment of Slit2N and VEGF-165 showed no significant difference compared with the single treatment of VEGF-165 in the Robo4 (-) group ($P > 0.05$). Relative tubule length and number of branches in the control, Robo4 (+) NC, Robo4 (+) and Robo4 (-) groups pretreated with both Slit2N and VEGF-165 were significantly reduced compared with the corresponding groups pretreated only with VEGF-165 ($P < 0.05$).

Quantitative real-time PCR analysis and western blot analysis were applied to detect Robo4 expression (Fig. 6E, F). After ECs were pretreated with anti-VEGFA-neutralizing antibody (1ug/ml) for 24 hours, the expression of Robo4 was significantly up-regulated in GCM + anti-VEGFA group compared with GCM group ($P < 0.05$), though still down-regulated than those in ECM group ($P < 0.05$).

Over-expression of Robo4 down-regulated the phosphorylation of VEGFR2, PI3K, AKT and FAK

Results about the effect of Robo4 on the activity of VEGFR2 were shown in Fig. 7A. Under the treatment of glioma conditioned medium, VEGFR2 phosphorylation and total expression levels showed no significant difference among the control, Robo4 (+) NC and Robo4 (-) NC groups ($P > 0.05$). In Robo4 (+) and Robo4 (-) groups, the expression of total VEGFR2 did not change compared to the control group ($P > 0.05$). The expression of p-VEGFR2 / t-VEGFR2 was significantly down-regulated in the Robo4 (+) group compared with Robo4 (+) NC group

Fig. 9. Graphical abstract: Robo4 regulated glioma angiogenesis via binding with ligand Slit2 which were secreted by glioma cells. Robo4 blocked the activity of VEGFR2 mediated PI3K/AKT and FAK signaling pathways, and then reduced glioma angiogenesis through suppressing glioma-induced endothelial cell proliferation, migration and tube formation *in vitro*.

($P < 0.05$). However, the expression of p-VEGFR2 / t-VEGFR2 was significantly up-regulated in the Robo4 (-) group compared with Robo4 (-) NC group ($P < 0.05$). The analysis of PI3K (Fig. 7B), AKT (Fig. 7C) and FAK (Fig. 7D) also provided similar results.

VEGFR2-mediated PI3K/AKT and FAK signaling pathways were involved in the Robo4-regulated glioma angiogenesis in vitro

ECs cultured in glioma conditioned medium were pretreated with VEGFR2 inhibitor SU-1498, AKT inhibitor LY294002 and FAK inhibitor 14 for 24 hours, CCK-8 assays were performed. As shown in Fig. 8A, the viability of endothelial cells in the Robo4 (-) + SU-1498, Robo4 (-) + LY294002 and Robo4 (-) + FAK inhibitor 14 groups was significantly decreased compared with the Robo4 (-) group ($P < 0.05$), though still higher than that in the Robo4 (-) NC group ($P < 0.05$).

Cell migration was also determined after cells were treated with SU-1498, LY294002 and FAK inhibitor 14 (Fig. 8B). The number of migrating cells in the Robo4 (-) + SU-1498, Robo4 (-) + LY294002 and Robo4 (-) + FAK inhibitor 14 groups was decreased significantly compared with the Robo4 (-) group ($P < 0.05$), though still higher than those in the Robo4 (-) NC group ($P < 0.05$).

Results of tube formation assay, presented in Fig. 8C, showed that the relative tubule length and the number of branches were decreased significantly in the Robo4 (-) + SU-1498, Robo4 (-) + LY294002 and Robo4 (-) + FAK inhibitor 14 groups compared with the Robo4 (-) group ($P < 0.05$), though still higher than those in the Robo4 (-) NC group ($P < 0.05$).

Discussion

In the present study, we have found that glioma conditioned medium stimulated ECs proliferation, migration and tube formation *in vitro*, all of which are critical steps for angiogenesis. Furthermore, the results showed that the Robo4 was significantly down-regulated in the ECs cultured in glioma conditioned medium compared with endothelial cell medium. Robo4 over-expression could remarkably suppress glioma-induced ECs

proliferation, migration and tube formation *in vitro*. The interaction between Robo4 and its ligand Slit2 was responsible for the alteration in glioma angiogenesis. In addition, Robo4 provided the above effects by inhibiting the activity of VEGFR2-mediated PI3K/AKT and FAK signal pathways.

Previous study found that conditioned medium of U373MG and A172 glioma cells with high VEGF concentration could induce endothelial cell migration [28], which was supported by another study showing that stem cell-like glioma cells (SCLGC) conditioned medium significantly promoted endothelial cell migration and tube formation by secreting VEGF [8]. Results in the present study were consistent with the literature.

Robo4 is a new member of axon guidance receptor roundabout family [15]. Various guidance molecule receptors such as Neuropilin-1 [29], EphA2 [30] have been characterized to play critical roles in angiogenesis. It has been suggested recently that Robo4 is a newly identified regulator of vessel formation [15, 24, 26] and its effect might be different under various physiological and pathological states. Robo4 was essential for vascular development in embryonic zebrafish. Knockdown of endogenous Robo4 expression led to disruption of embryonic vascular development [17]. In mouse models of retinal and choroidal vascular disease, Robo4 expression was significantly inhibited by the activation of Src family kinases, and thereby inhibition of VEGF-165 resulted in retinal endothelial cell migration and tube formation [26]. Furthermore, Suchting et al have previously proved that soluble extracellular domain of Robo4 inhibited retinal vessel angiogenesis *in vivo* and suppressed EGF-2 mediated human umbilical vein endothelial cell (HUVEC) proliferation, migration and tube formation *in vitro* [31].

It has been established that Robo4 expression was different between tumor and normal tissues and its expression in primary culture of ovarian cancer epithelial cells was lower than normal ovarian epithelium [32]. Additionally, Robo4 was down-regulated in hepatocellular carcinoma tissues compared to the normal liver tissue [33]. But Grone et al. reported that Robo4 expression had no difference between colorectal cancer tissue and normal colorectal tissue [34]. Hence, whether Robo4 expression in glioma endothelial cells or glioma tissues was also reduced needs to be investigated. In this work, we proved that conditioned medium down-regulated Robo4 expression in endothelial cells. In an attempt to elucidate the effect of Robo4 on glioma vascular endothelium, we further investigated its possible role in regulating glioma-induced proliferation, migration and tube formation of endothelial cells which were critical for angiogenesis. Results indicated that Robo4 over-expression in endothelial cells could remarkably suppress glioma-induced endothelial cell proliferation, migration and tube formation *in vitro*. These results also suggested that Robo4 might play an antiangiogenic role in angiogenesis. However, the molecular mechanisms regarding of the regulation of glioma angiogenesis by Robo4 are still unclear.

Slit2 was considered to be the ligand of Robo4 [11, 35]. In this study, we found that both secretions of Slit2 and VEGF were significantly increased in glioma conditioned medium compared with the endothelial cell medium. Furthermore, our data showed that the expression levels of Robo4 were different with the pathological grades of glioma (Fig. 5), which is consistent with the following literature. Slit2, whose gene is located at chromosome 4p15.2, was originally identified as a down-regulated gene in gliomas and might act as a tumor suppressor gene [36]. Slit2 inhibits glioma cell migration and invasion by binding to Robo1 receptor and inactivation of Cdc42-GTP [37, 38]. These results prove that Slit2, which was produced and secreted by glioma cells, could regulate glioma angiogenesis by binding to Robo4. Therefore, we found that the interaction between Robo4 and Slit2 could inhibit VEGF-165-mediated angiogenesis in endothelial medium. These results indicated that Robo4/Slit2 pathway suppressed glioma angiogenesis by blocking VEGF. Glioma cells produce and secrete several angiogenic or antiangiogenic factors such as VEGF and Slit2. These factors act on ECs that express corresponding receptors, resulting in promoted or suppressed glioma angiogenesis.

In the present study, we found that knockdown of Robo4 promoted glioma-induced angiogenesis by promoting the activity of VEGFR2-mediated PI3K/AKT and FAK signal

pathways, and inhibitors against them blocked the promoted glioma angiogenesis induced by Robo4 knockdown. These results suggested that VEGFR2-mediated PI3K/AKT and FAK signal pathways were the key factors in the Robo4-induced regulation of glioma angiogenesis. Recent studies suggested that Robo4 could regulate VEGF downstream signaling. Robo4 binds to UNC5B, inhibits VEGF-induced vessel permeability change and fixes barrier defects in mice by blocking activity of VEGFR2 [24]. Moreover, Robo4 inhibits mammary gland blood vessel growth by restraining VEGF/VEGFR2 signaling [39]. Activation of Robo4 by Slit2 inhibits VEGF-165-induced lung endothelial cells migration, tube formation *in vitro* by blocking the activation of VEGFR2-Src pathway [26]. Besides, our findings indicate that pretreatment of VEGF neutralizing antibody partially rescued the down-regulating of Robo4 by the conditioned medium (Fig. 6E, F). VEGF signaling pathway, known as a molecular switch of tumor angiogenesis [40], plays a critical role in various stages of angiogenesis including endothelial cell proliferation [41], migration [8] and tube formation [42]. The angiogenic response of VEGF is mainly mediated by the activation of VEGFR2 [43]. Therefore, inhibition of VEGFR2 activity as well as VEGF-regulated signaling in endothelial cells could be important in targeting tumor angiogenesis. PI3K is an important regulator of angiogenesis [44]. Phosphorylation at Tyr1175 of VEGFR2 binds and activates the p85 subunit of PI3 kinase [45]. PI3K has been implicated in the formation of EC tubules [46] as well as the proliferation, survival and vascular permeability of EC *in vitro* [47]. AKT is a key downstream signaling factor of PI3K in the VEGFR2-mediated angiogenesis [48]. The activation of AKT stimulates tumor angiogenesis by inducing EC proliferation, migration or promoting matrix metalloproteases secretion [49, 50]. Additionally, downstream signaling of VEGFR2 also converges on the non-receptor tyrosine kinase focal adhesion kinase (FAK), which is an important regulator of endothelial cell survival, migration, and proliferation [51]. Therefore, it is reasonable to infer that VEGFR2-mediated PI3K/AKT and FAK signaling pathways are involved in the regulation of glioma angiogenesis induced by Robo4.

In summary, the present study revealed for the first time that glioma conditioned medium down-regulated the expression of Robo4 in endothelial cells. Over-expression of Robo4 remarkably suppressed glioma-induced endothelial cell proliferation, migration and tube formation *in vitro*, all of which are critical steps for angiogenesis. Moreover, VEGFR2-mediated PI3K/AKT and FAK signal pathways were involved in the process. Based on these findings, Robo4 can be considered to be a potential target in the anti-angiogenic therapy for gliomas in the future.

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