Int J Clin Exp Pathol 2018;11(7):3398-3404
www.ijcep.com /ISSN:1936-2625/IJCEP0075805

Original Article

Slit3 regulates migration of endothelial progenitor cells by activation of the RhoA/Rho kinase pathway

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Received March 11, 2018; Accepted April 15, 2018; Epub July 1, 2018; Published July 15, 2018

Abstract: Nerves and blood vessels are in close proximity, indicating possible biomolecular interactions. Slit/Robo signaling pathways play critical roles in cell proliferation and motility. Endothelial progenitor cells (EPCs) participate in angiogenesis and vascular homeostasis. EPC migration induced by Slit3 has not been fully characterized. Thus, the expression of Slit and Robo in EPCs was examined, and the chemotactic functions of Slit3 and the Slit/Robo signaling pathway regulatory mechanisms were explored. We observed that EPCs express mainly the Robo4 receptor, and its ligand Slit3 plays roles in regulation of EPCs migration through activating the RhoA/Rho related kinases. Regulation of Slit3/-Robo4 signaling in EPCs may provide a new therapeutic target for ischemic disease.

Keywords: Cell migration, protein kinases/phosphatases, stem cells, neurotransmitters/biogenic amines

Introduction

Endothelial progenitor cells (EPCs) are thought to preserve endothelial integrity, function, and neovascularisation, as well as participate in angiogenesis [1, 2]. Adult EPCs can form new blood vessels by means of vasculogenesis [3, 4]. Therefore, EPC-based therapeutic approaches can improve vascular perfusion and offer clinical benefits [5-7]. A study has identified the vital role of EPCs in repairing vascular injury and neovascularization [3].

The Roundabout (Robo) family of receptors and their ligands, known as Slit proteins, were discovered in the 1990s. Slit and Robo have also been found to be key players in axon guidance [8]. Several other studies have demonstrated that the Slit/Robo pathway is widely expressed in many cell lines such as kidney, skeletal muscle, and heart [9, 10], and one of its roles is to regulate cell migration [11].

EPC migration induced by Slit3 has not been fully characterized. Herein, the expression of Slit and Robo in EPCs was examined, and the chemotactic functions of Slit3 and the Slit/Robo signaling pathway regulation mechanisms were explored.

Materials and methods

Isolation and culture of EPCs

All the experiments were approved by the Animal Care and Use Committee of Gansu Provincial Hospital, China. Male Sprague-Dawley rats with body weights between 100-150 g were used. Experimental animals were purchased from the animal center of Lanzhou University in China.

The mononuclear cells were isolated from rat bone marrow, cultured and induced using the EGM-2 Bullet Kit system. EPCs of less than five passages were used. Endothelialcell lineage was determined by Ulex europeus agglutinin I (UEA-I, 10 μg/ml, Sigma-Aldrich, MO, USA) binding and 1,1'-dioctadecyl-3,3,3',3'-tetramethylindo-carbocyanine perchlorate labelled LDL (DiI-acLDL, 10 μg/ml, Biomedical Technologies Inc., MA, USA) uptake. Cells with double positive staining were differentiated EPCs [12, 13].

RT-PCR and western blotting

RT-PCR was performed to determine the expression of Slit1-3 and Robo1-4 in EPCs as previously described [12, 14]. Total RNA was extracted from EPCs after 3-4 passages using

Table 1. Sequences of the primers used in RT-PCR

Name	Product size (bp)	GenBank accession no.	Sequence
Slit1	170	AF144627	F: 5' CCCGAGGTGTATTTGGAGG 3'
			R: 5' CCGTAGGGAGGTGAAGGTG 3'
Slit2	272	AF144628	F: 5' CACCTTCTCAGCCCTCA 3'
			R: 5' CACAGCGACACTTCTCA 3'
Slit3	271	AF210320	F: 5' GGCTACAAGGGTCGTAACTG 3'
			R: 5' CAACCACCTCCTCGCACA 3'
Robo1	424	AF304130	F: 5' TGCGGCAGGAAGATTACG 3'
			R: 5' ATGGTGGGCTCTGGGTGT 3'
Robo2	307	AF337035	F: 5' AGCCAGCCACCCTAAAC 3'
			R: 5' TCTCCAGCAGCGACCAC 3'
Robo3	117	AF337036	F: 5' GCAGCAAGCCAGATGAA 3'
			R: 5' GGGTCTGTCGGAAATCA 3'
Robo4	431	AY277635	F: 5' AAGAAGTGACCCTAAGACCTGG 3'
			R: 5' AGCTTTCCGTCGCCTGTA 3'
β-actin 200	200	LT575466	F: 5' CCACGAGACCACCTACAA 3'
			R: 5' TCCAGACGGAGTATTTGC 3'

TRIzol reagent per the manufacturer's direction instructions (Invitrogen, Carlsbad, CA, U.S.A.). The primers were designed using Primer Designer 5.0. Primers for RT-PCR and cDNA amplification products of Slit1-3 and Robo1-4 are listed in **Table 1**.

Western blot analyses were performed as previously described [12, 14]. Briefly, total protein was extracted from EPCs using 1% SDS lysis buffer (P0013G, Beyotime, Jiangsu, China). The following antibodies were used: goat anti-Robo1 (1:200; sc-16611), mouse anti-Robo2 (1:100; sc-376177), goat anti-Robo3 (1:200; sc-46495), goat anti-Robo4 (1:200; sc-46500), goat anti-Slit1 (1:200; sc31590), rabbit anti-Slit2 (1:200; sc-28945), rabbit anti-Slit3 (1:200; sc-28946), goat anti-rabbit IgG-HRP (1:2,000; sc-2004), donkey anti-goat IgG-HRP (1:2,000; sc-2033), goat anti-mouse IgG-HRP (1:3,000; sc-2005) (all antibodies listed above are from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), and mouse anti-GAPDH (1:1,000; Beyotime, Jiangsu, China). GAPDH was used for normalization.

Motility assays

Motility assays were performed in transwell cell culture chambers using 5 μm pore size, 6.5 mm diameter polycarbonate membranes [15]. As chemoattractants, different concentrations of Slit3 (10, 20, 40, 80 ng/ml, sc31597; Santa Cruz Biotechnology, CA, USA) and Slit2 (10, 20, 40, 80 ng/ml, 5444-SL, Bio-Techne, R&D Systems) dissolved in low-melting agarose (100 μl) were placed in the lower chamber and 0.5×10^6 EPCs suspended in EBM-2 BulletKit system (CC-3202; Lonza Walkersville, MD, USA) and 0.2% bovine serum albumin (100 μl) loaded in the upper chamber. Cells were pretreated with either anti-Robo1 antibody (10 μM, Abcam, ab7279; Cambridge, UK), anti-Robo4 antibody (10 μM, Abcam, ab103674; Cambridge, UK) or Y-27632 (10 μM, STEMCELL Technologies, British Columbia, CAN) for 30 min. Chambers were incubated at 37°C with 5% CO_2 in a humidified incubator for 3 h, then the cells migrated to the bottom of the porous membrane were stained using crystal violet. Six random fields were counted in triplicate and repeated three times under a phase-contrast microscope at a 100 × magnification. Results are shown as the migration index, which represents the ratio between cells that migrated toward the chemo-attractants and cells that migrated to the vehicle [16].

Rho GTPase activity analysis

EPCs were incubated with or without Y-27632 (10 μM, 30 min), then treated with Slit3 (80 ng/ml, 30 min). With polyclonal antibodies directed to the anti-RhoA antibody (Abbiotec, Cat. No.: 251549; San Diego, CA, USA), total cell lysates were analyzed by immunoblot analysis. The RhoA activity was measured using the pull-down assay, and total RhoA and precipitated GTP-bound RhoA were measured by Western blot.

Data analysis

All data are expressed as mean ± SEM. The significance of differences between groups was

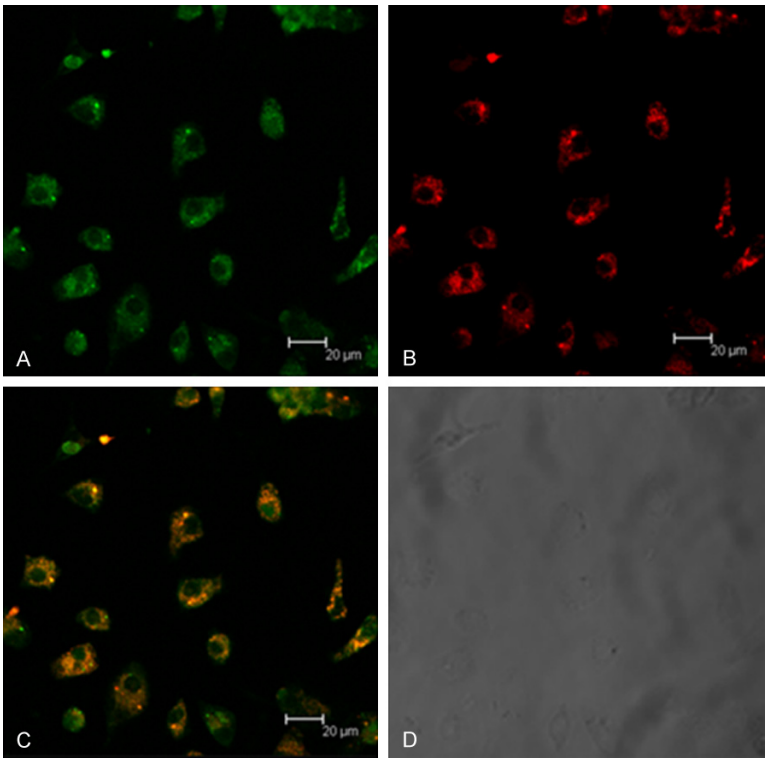

Figure 1. Characterization of EPCs. A. Ulex europaeus lectin binding cells (green). B. DiI-Ac-LDL uptake cells (red). C. Double positive cells (yellow, fluorescence microscopic images × 400). D. No stain.

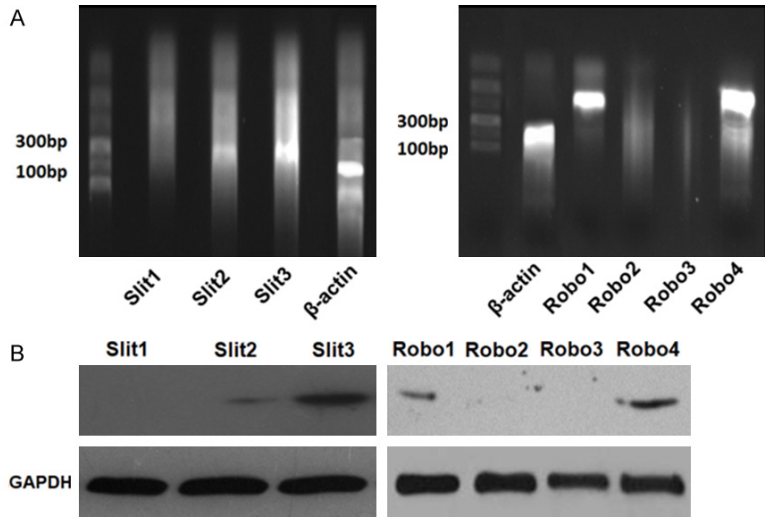

Figure 2. Expression of Slit and Robo in EPCs. At the molecular (A) and protein (B) levels, Slit2 was expressed at low levels and Slit3 as highly expressed in EPCs. There was no expression of Slit1, Robo2, or Robo4.

evaluated with one-way analysis of variance (ANOVA) and Student-Newman-Keuls post-test. $P < 0.05$ was considered statistically significant.

Results

Characterization of EPCs

Laser Scanning Confocal Fluorescence Microscopy (LSCFM) analysis results showed that the adherent cells were double positive for both DiI-acLDL uptake and FITC-UEA-1 binding (**Figure 1**).

Expression of Slit1-3 and the receptors Robo1-4

As shown in **Figure 2**, at molecular levels (A) and protein levels (B), Slit2 was expressed at low levels and Slit3 was highly expressed in EPCs. EPCs express high levels of Robo4 and low levels of Robo1. There was no expression of Slit1, Robo2, or Robo4 detected in EPCs. These findings suggest that Slit3 may bind to and activate the Robo1 receptor and/or the Robo4 receptor to regulate EPCs function.

Slit3 regulates migration of EPCs

As shown in **Figure 3**, Slit3 regulates EPCs' migration with an atypical concentration-dependent manner ($P < 0.05$ vs. control group). However, Slit2 cannot regulate EPC migration. Compared to controls, our cell count assay revealed an approximately 1.2-, 1.6-, 2.6-, and 3.2-fold increase in Slit3-induced migration of EPCs at concentrations of 10 ng/ml, 20 ng/ml, 40 ng/ml, and 80 ng/ml, respectively. There was no significant difference in cell migration at various Slit2 concentrations, indi-

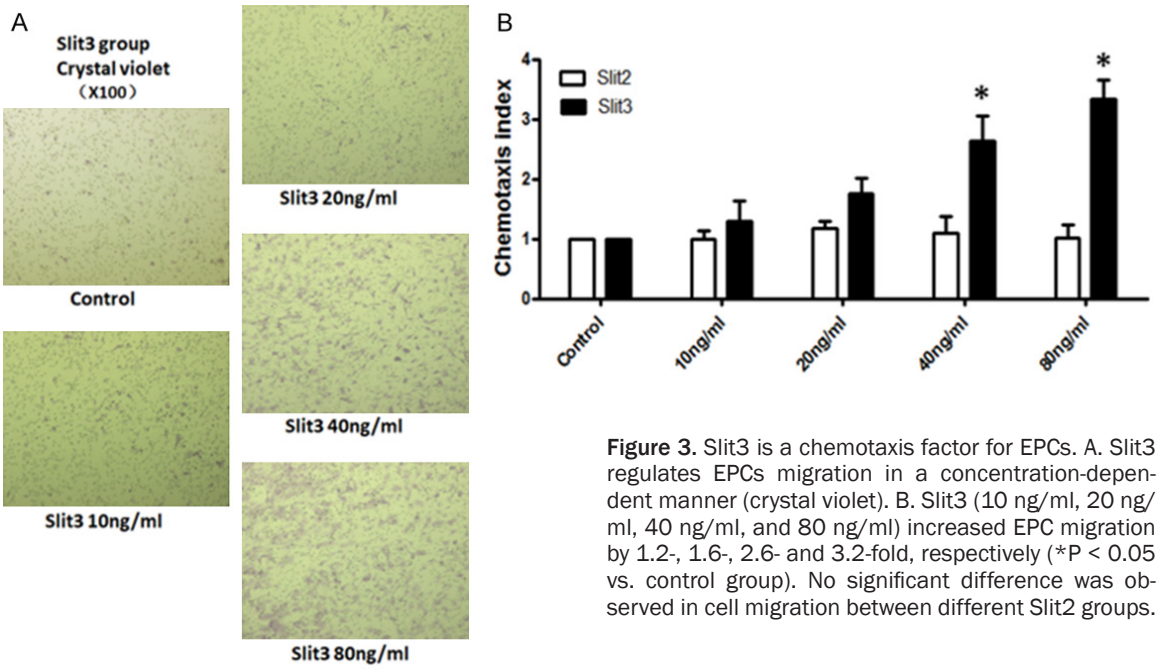

Figure 3. Slit3 is a chemotaxis factor for EPCs. A. Slit3 regulates EPCs migration in a concentration-dependent manner (crystal violet). B. Slit3 (10 ng/ml, 20 ng/ml, 40 ng/ml, and 80 ng/ml) increased EPC migration by 1.2-, 1.6-, 2.6- and 3.2-fold, respectively (*P < 0.05 vs. control group). No significant difference was observed in cell migration between different Slit2 groups.

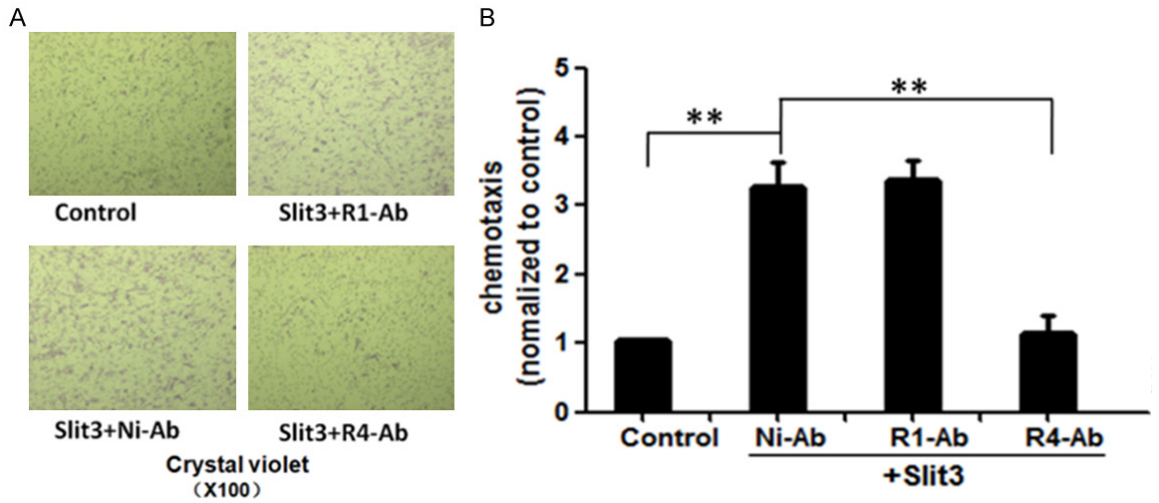

Figure 4. Anti-Robo4 antibody inhibits EPC migration. A. Effects of anti-Robo1 antibody and anti-Robo4 antibody on migration of EPCs induced by Slit3 (crystal violet). B. The anti-Robo1 antibody had no significant effect on migration of EPCs induced by Slit3. In contrary, the anti-Robo4 antibody completely abolished EPC migration (**P < 0.05, Ni-Ab group versus control group or R4-Ab group).

cating that Slit2 cannot regulate EPC migration.

Anti-Robo4 antibody inhibited inhibits EPC migration

As shown in the **Figure 4**, the anti-Robo1 antibody had no significant effect on EPCs migration induced by Slit3. In contrary, the anti-Robo4 antibody completely blocked Slit3-induced migration, confirming that the Slit3-induced EPC migration depends on Robo4, but not Robo1.

Rho/Rho kinase signaling participates in Slit3-induced migration

As shown in **Figure 5**, Slit3 (80 ng/ml) increased the levels of active RhoA-GTP (P < 0.05 versus control group). Y-27632 can abolish the activa-

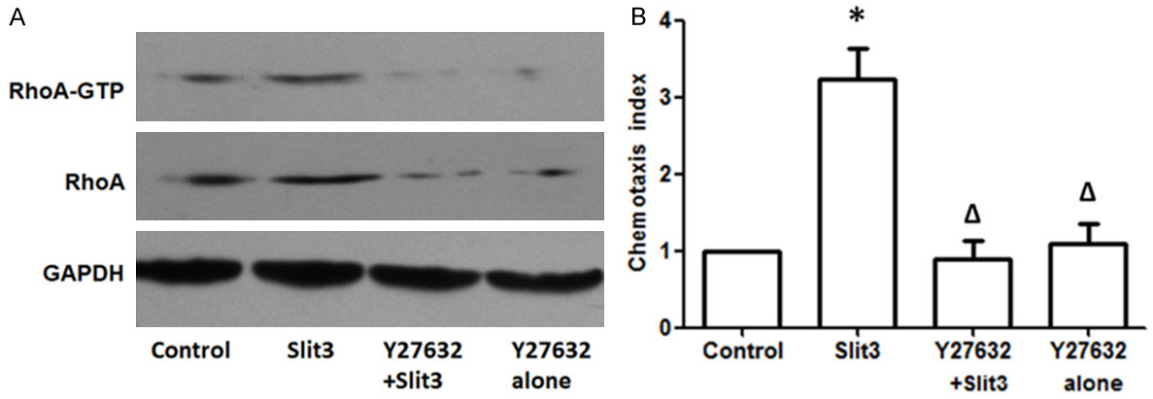

Figure 5. The RhoA pathway participates in EPC migration induced by Slit3. A. Slit3 significantly improved the activity of RhoA-GTP which can be inhibited by Y-27632 Rho-kinase inhibitor (western blot). B. Slit3 (80 ng/ml led to an approximately 3-fold increase in the levels of active RhoA-GTP ($P < 0.05$, control group versus Slit3 group). Y-27632 Rho-kinase inhibitor (10 μM) inhibited migration of EPCs induced by Slit3 (*$P < 0.05$ versus control group, Δ$P < 0.05$ versus Slit3 group).

tion of RhoA-GTP and the migration of EPCs induced by Slit3 ($P < 0.05$ versus Slit3 group).

Discussion

The present study primarily demonstrates that Slit2, Slit3, Robo1, and Robo4 are expressed in rat bone marrow-derived EPCs. Slit3 regulates EPC migration in a concentration-dependent manner. Furthermore, the chemoattractant effect of Slit3 on EPCs is facilitated through the activation of Rho-associated kinase.

Nerves and blood vessels are in close proximity, indicating possible biomolecular interactions. The Slit and Robo family of proteins which function as axon guidance cues and branching factors during development were originally identified in the nervous system [17]. In addition, it is known that Slit/Robo signaling pathways play critical roles in cell proliferation and motility, and also play an essential role in promoting endothelial lumen formation and morphogenesis of the vertebrate early heart tube [18-20]. Earlier studies have demonstrated that Slit3 is widely expressed in several cell lines, including heart, skeletal muscle, and kidney [9, 10, 21, 22]. As a vas-cular-specific receptor, Robo4 is expressed in endothelial cells [23, 24]. Our study showed that EPCs derived from rat bone marrow express mainly Slit3 and Robo4, and that Slit3 may affect the biological functions of the EPCs through the Robo4 receptor. It has been reported that Slit3 is a novel angiogenic factor and that Robo4 plays a role in bone marrow homing and mobilization [25, 26]. In our study, results indicate that Slit3 is a novel chemoattractant for EPCs as one of the ligands of the Robo4 receptor. Therefore, these results suggest that the regulation of Slit3-Robo4 signaling in EPCs under physiological or pathological conditions may provide a new therapeutic target for vascular repair and homeostasis.

Angiogenesis includes endothelial cell proliferation, migration, and tube formation. Current evidence indicates that RhoA/Rho kinase is necessary for the migration of endothelial cells [27, 28]. As a novel angiogenic factor, Slit3 is also involved in endothelial cell migration and angiogenesis. In our study, Slit3 induced EPC migration and activated the RhoA-associated kinase. Y-27632 inhibited migration and blocked the active RhoA-GTP, suggesting that the RhoA/Rho kinase play crucial roles in cell migration. There is the need for further research to explore the underlying mechanisms by which Slit3 activates RhoA in EPCs.

In conclusion, our data show that EPCs express mainly the Robo4 receptor, and its ligand Slit3 plays roles in regulation of EPC migration through activating the RhoA/Rho related kinases. As a novel chemoattractant factor for EPCs, the chemotactic effect of Slit3 may play a pivotal role in promoting angiogenesis. Therefore, additional studies will be required to clarify the precise role of Slit3/Robo4 in EPC homeostasis and functioning.

Acknowledgements

This work was supported by grants from the Provincial Natural Science Foundation of Gansu in China (Grant number: 1506RJZA171) and partly supported by grants from the National Natural Science Foundation of People's Republic of China (Grant number: 81360038).

Disclosure of conflict of interest

None.

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