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Original Paper

SphK1/S1P Mediates PDGF-Induced Pulmonary Arterial Smooth Muscle Cell Proliferation via miR-21/BMPRII/Id1 Signaling Pathway

Fangwei Li[a,b] Jian Wang[a] Yanting Zhu[a] Lu Liu[a] Wei Feng[a] Wenhua Shi[a] Qingting Wang[a] Qianqian Zhang[a] Limin Chai[a] Manxiang Li[a]

[a]Department of Respiratory Medicine, the First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, [b]Department of Respiratory Medicine, Lanzhou University Second Hospital, Lanzhou, China

Key Words

SphK1 • S1P • PDGF • Pulmonary Arterial Smooth Muscle Cell • Proliferation • miR-21 • BMPRII • Id1

Abstract

Background/Aims: The underlying molecular mechanisms involved in sphingosine kinase 1 (SphK1)/sphingosine 1-phosphate (S1P) mediation of platelet-derived growth factor (PDGF)-induced pulmonary arterial smooth muscle cell (PASMC) proliferation are still unclear, and the present study aims to address this issue. ***Methods:*** Small interfering RNA (siRNA) and microRNA inhibitor transfection was performed to block the expression of SphK1, bone morphogenetic protein receptor II (BMPRII) and microRNA-21 (miR-21). Gene expression levels of SphK1, BMPRII and inhibitor of DNA binding 1 (Id1) were detected by immunoblotting, miR-21 expression level was examined with qRT-PCR, and S1P production was measured by ELISA. Additionally, PASMC proliferation was determined by BrdU incorporation assay. ***Results:*** Our results indicated that PDGF increased the expression of SphK1 protein and S1P production, up-regulated miR-21 expression, reduced BMPRII and Id1 expression, and promoted PASMCs proliferation. Pre-silencing of SphK1 with siRNA reversed PDGF-induced S1P production, miR-21 up-regulation, BMPRII and Id1 down-regulation, as well as PASMC proliferation. Pre-inhibition of miR-21 also blocked BMPRII and Id1 down-regulation as well as PASMC proliferation caused by PDGF. Knockdown of BMPRII down-regulated Id1 expression in PASMCs. We further found that inhibition of PI3K/Akt and ERK signaling pathways, particularly ERK cascade, suppressed PDGF-induced above changes. ***Conclusion:*** Our study indicates that SphK1/S1P pathway plays an important role in PDGF-induced PASMC proliferation via miR-

Dr. Manxiang Li

Department of Respiratory Medicine, the First Affiliated Hospital of Xi'an Jiaotong University, No. 277, West Yanta Road, Xi'an 710061, Shaanxi (China)
Tel. +86-029-85324053, E-Mail manxiangli@hotmail.com

21/BMPRII/Id1 axis and targeting against SphK1/S1P axis might be a novel strategy in the prevention and treatment of pulmonary arterial hypertension (PAH).

Introduction

Pulmonary arterial hypertension (PAH) is a hemodynamic state defined as the resting mean pulmonary arterial pressure equal to or more than 25 mmHg, leading to right heart failure and ultimate death [1, 2]. The common pathological mechanisms underlying the development of PAH include persistent pulmonary vasoconstriction, vascular remodeling, and thrombosis in situ [3, 4]. Pulmonary arterial remodeling characterized by thickening of all layers of vascular wall plays an important role in the pathological progress of PAH [5]. Abnormal pulmonary arterial smooth muscle cell (PASMC) proliferation in situ and migration to intima is critical to the pathogenesis of pulmonary arterial remodeling [6]. Therefore, exploring the molecular mechanisms responsible for PASMC proliferation and searching for new targets are critical for the prevention and treatment of PAH.

Sphingosine 1-phosphate (S1P) is a bioactive sphingolipid metabolite promoting cell survival and proliferation, contrary to the effects of other two sphingolipid metabolites named ceramide and sphingosine inducing cell growth arrest and apoptosis [7]. These sphingolipids with opposing functions are interconvertible inside of cells, suggesting that a finely tuned balance between them determines cell fate [8]. Sphingosine kinases (SphKs) are a family of ubiquitous lipid kinases responsible for the conversion of sphingosine to S1P via phosphorylation, and they have been shown to play an active role in proliferation and survival of a variety of tumor cells as well as several nonmalignant cells [9-12]. The SphKs family includes two distinct isoforms of SphK1 and SphK2, of which SphK1 is the predominant form in the lung, kidney, blood, and spleen [13]. Studies have shown that SphK1/S1P axis plays a crucial role in tumorigenesis, hormonal therapy and chemotherapy resistance [14].

Recent study has confirmed that the expression of SphK1 and S1P production are significantly increased in the lungs from PAH patients and rodent models and SphK1/S1P axis promotes PASMC proliferation, suggesting that SphK1/S1P axis is a novel pathway and a potential therapeutic target in PAH [15]. SphK1/S1P axis is activated by multiple growth factors, including platelet-derived growth factor (PDGF), which is well accepted as a mitogen inducing PASMC proliferation and further promoting pulmonary vascular remodeling [16]. To determine whether SphK1/S1P medicates PDGF-induced PASMC proliferation and its underlying mechanisms, the levels of sphK1 protein and S1P were assessed in primary cultured PASMCs stimulated with PDGF, and the levels of microRNA-21 (miR-21), bone morphogenetic protein receptor II (BMPRII) protein, inhibitor of DNA binding 1 (Id1) protein as well as cell proliferation were also examined.

Materials and Methods

Cell preparation and culture

PASMCs were prepared from 4- to 5-week-old male Sprague-Dawley rats using previous methods [17]. All animal procedures were carried out in accordance with the Laboratory Animal Care Committee of Xi'an Jiaotong University. Briefly, pulmonary arteries were rapidly removed from sacrificed rats, washed in phosphate-buffered saline (PBS) under sterile conditions. Next, the adventitia and endothelium of pulmonary arteries were stripped off carefully and the remaining smooth muscle layer was minced (~1 mm^2) and placed into a culture flask with Dulbecco's Modified Eagle Medium (DMEM)/High glucose (Gibco, Grand Isle, NY, USA) supplemented with 10% fetal bovine serum (FBS, Sijiqing, HangZhou, China), 100 U/ml penicillin, and 100 ug/ml streptomycin (complete DMEM). Then PASMCs were cultured in an atmosphere of 95% air and 5% CO_2 at 37 °C. Cells were passaged using 0.25% trypsin (Invitrogen, Carlsbad, CA, USA) when reaching 80% confluence and PASMCs between passages 4 and 6 were used for all studies. The purity of PASMCs was determined by immunostaining with an antibody against α-smooth muscle actin (α-SMA, Sigma-Aldrich,

St. Louis, MO, USA) [17]. Serum starvation was performed by culturing cells in 1% FBS-DMEM overnight to induce cell cycle synchronization before each experiment. We used PDGF (Peprotech, Rocky Hill, NJ, USA; distillated water as vehicle) to stimulate cell proliferation and used LY294002 (Sigma, DMSO as vehicle) and PD98059 (CST, Beverly, MA, USA; DMSO as vehicle) to inhibit the activity of phosphatidylinositol-3-kinase (PI3K) and extracellular signal-regulated kinase 1/2 (ERK1/2), respectively.

PASMC transfection

Lipofectamine™ 2000 (Invitrogen) was used for PASMC transfection according to the manufacturer's protocols. Briefly, PASMCs were seeded into a 6-well plate and cultured until reaching 30-40% confluence. The nucleotides of SphK1 siRNA, miR-21 inhibitor and BMPRII siRNA were used at a final concentration of 100 nM, 80 nM and 100 nM in DMEM, respectively (without serum or antibiotics). After 6 h, the complex of siRNA (or inhibitor) and Lipofectamine 2000 was replaced with complete DMEM with 10% FBS (without antibiotics) for 48 h in a 37 °C, 5% CO_2 humidified incubator. The silencing efficiency was determined by immunoblotting.

BrdU incorporation assay

To examine PASMC proliferation, the rate of BrdU incorporation was determined according to the instructions of the BrdU ELISA Kit (Maibio, Shanghai, China). Briefly, aliquots of 5×10^3 cells were spread in a 96-well plate and serum deprived (1% FBS in DMEM) overnight. BrdU was added to label the cells at 37°C during the final 2 h of treatment. Then the labeling medium was replaced with FixDenat solution for 30 min and subsequently with anti-BrdU monoclonal antibody conjugated to peroxidase for 90 min at room temperature. After that, substrate solution was added to each well, and the absorbance at 370 nm was determined with a microplate reader (Bio-Rad, Richmond, CA, USA).

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from cells with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Thermo, Waltham, MA, USA). U6 small nuclear RNA (snRNA) was used as endogenous control for miR-21. qRT-PCR was carried out using the Maxima SYBR Green/ROX qPCR Master Mix (Thermo) on a CFX RT-PCR system (Bio-Rad) according to the manufacturer's protocol. Expression of detected gene was quantified by measuring cycle threshold (Ct) values and normalized using $2^{-\Delta\Delta Ct}$ method relative to U6. Primers specific for miR-21 and U6 snRNA were purchased from Sangon Biotech (Shanghai, China) and the following primer sets were used:

U6 RT, CGCACTGGATACGACGGCATTCT;
U6 forward, CTCGCTTCGGCAGCACA;
U6 reverse, AACGCTTCACGAATTTGCGT;
miR-21 RT, GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCAACA;
miR-21 forward, GCCCGCTAGCTTATCAGACTGATG;
miR-21 reverse, GTGCAGGGTCCGAGGT.

Enzyme-linked immunosorbent assay (ELISA)

The S1P levels in cell culture supernates and cell lysates were measured using a rat S1P ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Briefly, conditioned medium was collected and centrifuged at 3, 000 g for 20 min at 4°C and the supernates were collected. Subsequently cells were washed twice and scraped repeatedly for 30 min with ice-cold PBS, and cell lysates were then centrifuged at 3, 000g for 20 min at 4°C. The concentration of each sample was calculated automatically by the instrument software according to the measured optical density values. The lower detection limit of the kit was 1.0 nM, with intra- and inter-assay coefficients of variations less than 15%. Duplicate samples were assayed, and all results were reported as the mean values.

Immunoblotting

Cells were washed twice with PBS, and then lysed in RIPA lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, 1 mM NaF and proteinase inhibitors). Lysates were centrifuged at 10, 000 g for 20 min at 4°C, and the supernatant was collected as total protein. BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA) was used to measure the amounts of protein in each extract. Subsequently, protein was separated on a SDS-PAGE gel under denaturing conditions and then transferred to polyvinylidene fluoride (PVDF, Bio-Rad) membrane using wet transfer. The PVDF membrane was then blocked by 5% non-fat milk for 2 h. After washing, the blots were incubated at 4°C overnight with the primary antibodies against p-Akt (CST; 1:1000 dilution), t-Akt (CST; 1:1000 dilution), p-ERK1/2 (CST; 1:1000 dilution), t-ERK1/2 (CST; 1:1000 dilution), SphK1 (CST; 1:1000 dilution), BMPRII (Bioworld, Louis Park, MN, USA; 1:1000 dilution), Id1 (Bioworld; 1:1000 dilution) and β-actin (Santa Cruz, Dallas, TEX, USA; 1:500 dilution). After that, horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma-Aldrich; 1:5000 dilution) was used as the secondary antibodies (Sigma, 1:5000 dilution). Finally, immunoreactive bands were visualized with Super Signal West Pico Chemiluminescent Substrate (Pierce Biotechnology) and quantified by Quality One software (Bio-Rad).

Statistics

Statistical analysis was carried out using the SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). All data were expressed as mean ± standard deviation. Group comparisons were performed using one-way analysis of variance (ANOVA) followed by Dunnett post hoc test (between the control group and treatment groups) or S-N-K (Student-Neuman-Keuls) post hoc test (among different groups). $P < 0.05$ was considered to be statistically significant.

Results

PDGF increases the expression of SphK1 and S1P production

Recent study has found that the expression of SphK1 and S1P production are elevated in the lungs from PAH patients and rodent models [15]. To explore whether PDGF increases the expression of SphK1 and S1P production in PASMCs, cells were treated with 10 ng/ml PDGF for 24 h, then SphK1 protein level was determined using immunoblotting and S1P expression level was tested by ELISA. As shown in Fig. 1A, PDGF caused a 2.43-fold increase

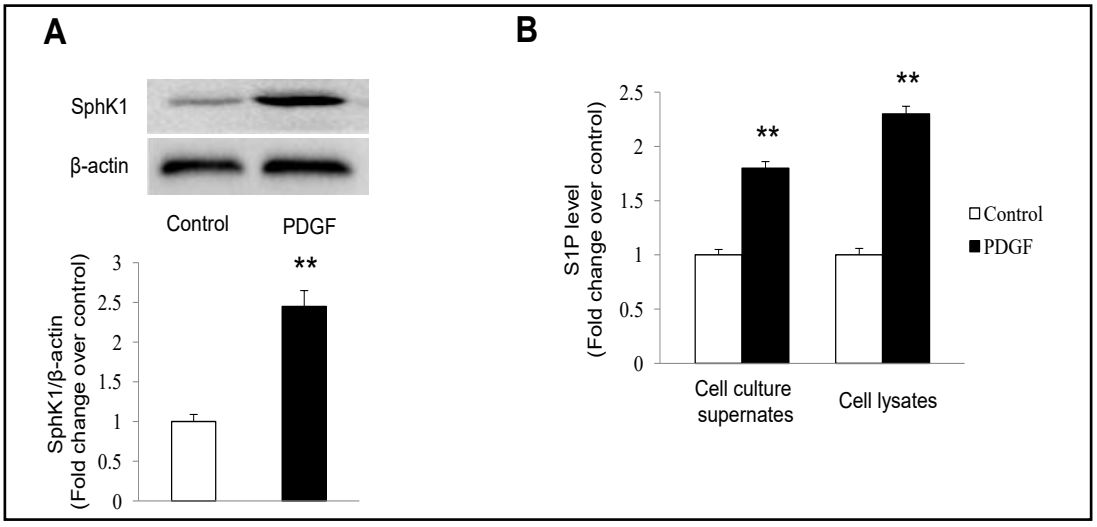

Fig. 1. PDGF increases the expression of SphK1 and S1P production. PASMCs were intervened with 10 ng/ml PDGF for 24 h. (A) SphK1 protein level was examined by immunoblotting (n = 4 each group). (B) S1P levels both in cell culture supernates and cell lysates were determined by ELISA (n = 4 each group). **P<0.01 vs. control.

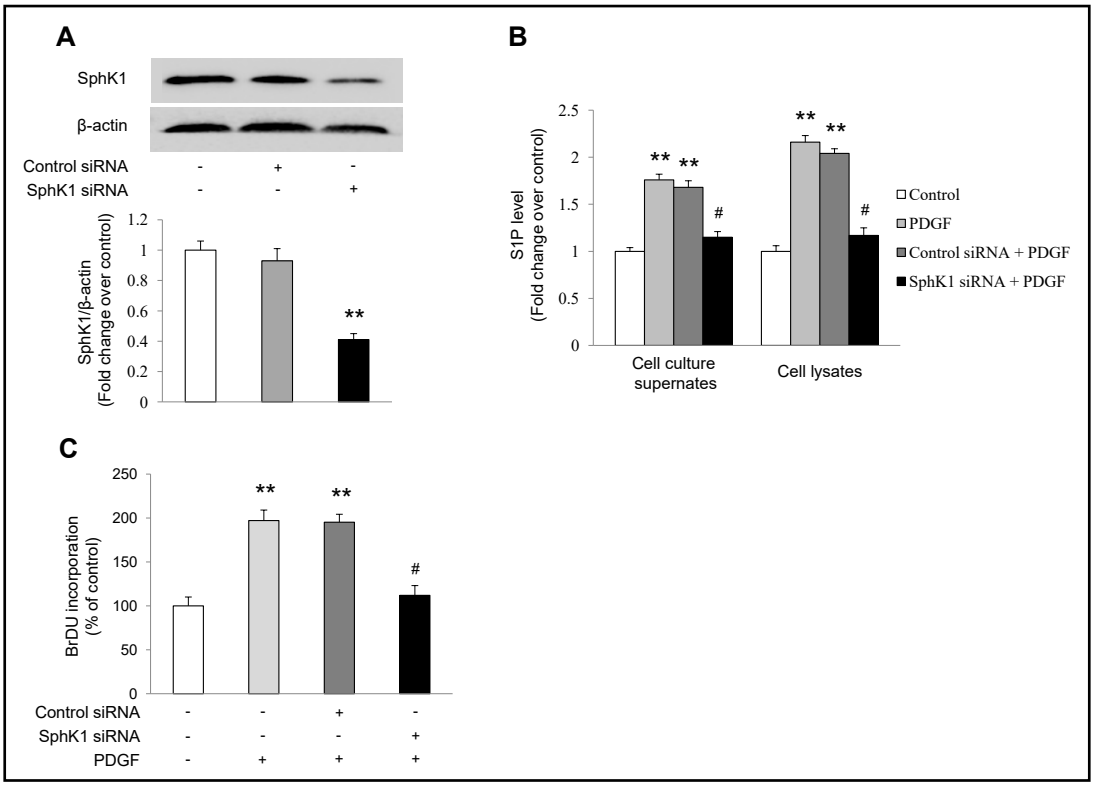

Fig. 2. SphK1/S1P mediates PDGF-induced PASMC proliferation. (A) PASMCs were transfected with SphK1 sequence-specific siRNA or non-targeting siRNA for 48 h. SphK1 protein was measured using immunoblotting (n = 4 each group). (B, C) Cells were stimulated with 10 ng/ml PDGF for 24 h with or without pre-treatment of non-targeting siRNA or SphK1 specific siRNA for 24 h. (B) S1P levels both in cell culture supernates and cell lysates were determined by ELISA (n = 4 each group). (C) BrdU incorporation rate was measured for cell proliferation (n = 4 each group). **P<0.01 vs. control, #P<0.05 vs. PDGF-treated cells.

in SphK1 protein level compared with the control group ($P < 0.01$). Fig. 1B indicates that PDGF increased S1P levels both in cell culture supernates and cell lysates, which raised to 1.82-fold and 2.30-fold, respectively, compared with the control group (both $P < 0.01$). These results suggest that PDGF up-regulates the expression of SphK1 and induces S1P production in PASMCs.

SphK1/S1P mediates PDGF-induced PASMC proliferation

To verify whether induction of SphK1/S1P axis played a role in PDGF-stimulated PASMC proliferation, cells were treated with 10 ng/ml PDGF for 24 h with or without prior silencing of SphK1 for 24 h. As shown in Fig. 2A, SphK1-specific siRNA transfection reduced SphK1 protein to 41% of the control group ($P < 0.01$), whereas non-targeting siRNA did not affect SphK1 protein level. Fig. 2B shows that PDGF stimulation increased S1P level to 1.76-fold over control ($P < 0.01$) in cell culture supernates and 2.16-fold over control ($P < 0.01$) in cell lysates, respectively, whereas pre-silencing of SphK1 reversed PDGF-induced up-regulation of S1P in both cell culture supernates and cell lysates, which was reduced to 1.15-fold and 1.17-fold over control, respectively (both $P < 0.01$ vs. PDGF-treated cells). Fig. 2C indicates that PDGF triggered a 1.97-fold increase in cell proliferation compared with control cells ($P < 0.01$), whereas prior knockdown of SphK1 attenuated PASMC proliferation stimulated by PDGF, which decreased to a 1.12-fold increase over control group ($P < 0.01$ vs. PDGF-treated cells). Non-targeting siRNA did not affect PDGF-induced S1P expression and PASMC proliferation. These results indicate that SphK1/S1P axis mediates PDGF-induced PASMC proliferation.

SphK1/S1P mediates PDGF-induced miR-21 up-regulation, as well as BMPRII and Id1 down-regulation

To determine whether PDGF regulates miR-21, BMPRII and Id1 expression by activating SphK1/S1P pathway in PASMCs, cells were treated with 10 ng/ml PDGF for 24 h with or without prior silencing of SphK1 for 24 h. Fig. 3A indicates that PDGF dramatically up-regulated miR-21 level, which increased to 2.64-fold compared with control group (P < 0.01), while prior knockdown of SphK1 reduced miR-21 level to 1.16-fold over control in the presence of PDGF (P < 0.01 vs. PDGF-treated cells). As shown in Fig. 3B, PDGF decreased BMPRII protein level to 0.37-fold over control (P < 0.01), whereas pre-silencing of SphK1 counteracted PDGF-induced down-regulation of BMPRII protein, which was raised to 0.91-fold over control (P < 0.01 vs. PDGF-treated cells). Fig. 3C indicates that PDGF down-regulated Id1 protein level, which decreased to 0.51-fold over control (P < 0.01), while prior knockdown of SphK1 increased Id1 protein level to 0.89-fold over control in the presence of PDGF (P < 0.01 vs. PDGF-treated cells). These results suggest that SphK1/S1P axis is particularly responsible for PDGF-induced miR-21 up-regulation as well as BMPRII and Id1 down-regulation.

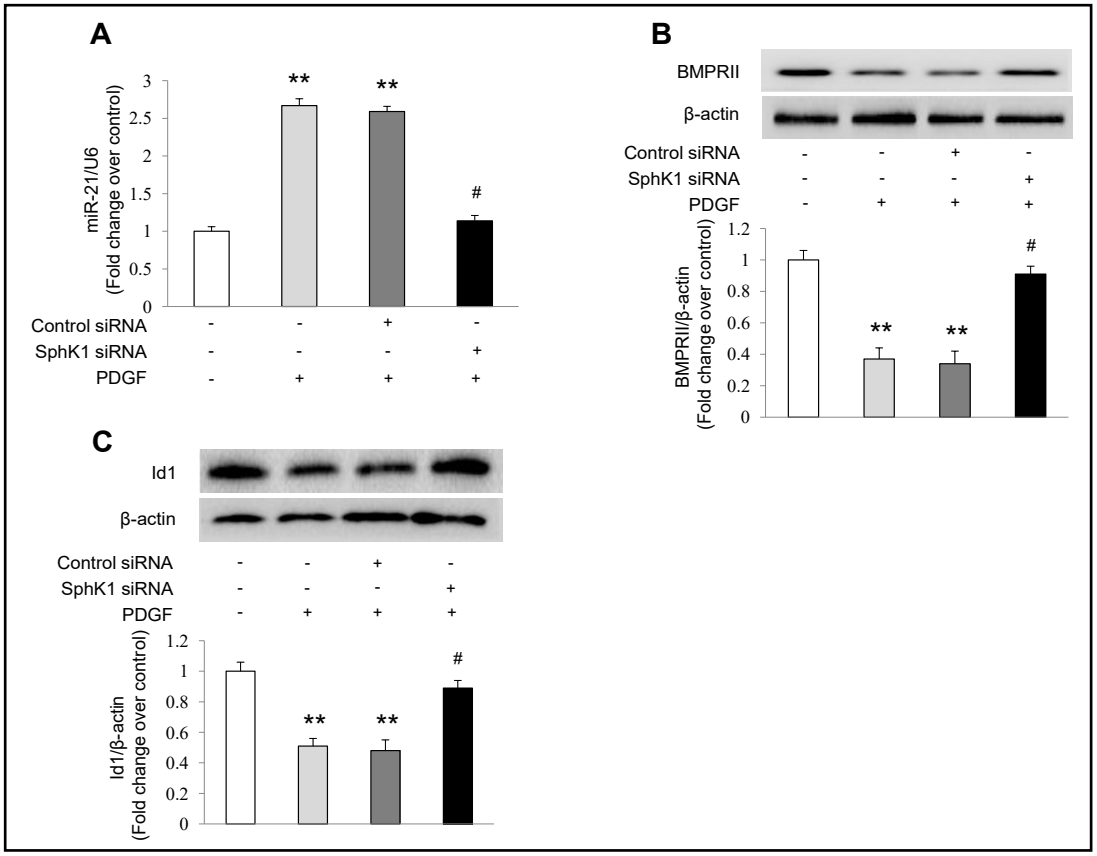

Fig. 3. SphK1/S1P mediates PDGF-induced miR-21 up-regulation, as well as BMPRII and Id1 down-regulation. PASMCs were stimulated with 10 ng/ml PDGF for 24 h with or without pre-treatment of non-targeting siRNA or SphK1 specific siRNA for 24 h. (A) miR-21 level was detected by qRT-PCR (n = 4 each group). (B) BMPRII protein level was examined by immunoblotting (n = 4 each group). (C) Id1 protein level was determined by immunoblotting (n = 4 each group). **P<0.01 vs. control, #P<0.01 vs. PDGF-treated cells.

miR-21 mediates PDGF-induced BMPRII and Id1 down-regulation, as well as PASMC proliferation

To examine whether miR-21 was involved in BMPRII and Id1 down-regulation as well as PASMC proliferation caused by PDGF, cells were treated with 10 ng/ml PDGF for 24 h with or without prior inhibition of miR-21 for 24 h. Fig. 4A indicates that miR-21 inhibitor transfection reduced miR-21 level to 0.48-fold over control ($P < 0.01$), whereas negative control inhibitor transfection did not change miR-21 level. Fig. 4B shows that PDGF significantly reduced BMPRII protein level ($P < 0.01$ vs. control), whereas prior inhibition of miR-21 blocked PDGF-induced BMPRII protein reduction, which increased from 0.41-fold over control to 1.07-fold over control ($P < 0.01$ vs. PDGF-treated cells). Fig. 4C demonstrates that PDGF decreased Id1 protein level to 0.48-fold over control ($P < 0.01$), while prior inhibition of miR-21 increased Id1 protein level to 1.03-fold over control ($P < 0.01$ vs. PDGF-treated cells). As shown in Fig. 4D, prior inhibition of miR-21 also blocked PDGF-stimulated PASMC proliferation, which decreased from a 1.97-fold increase over control to a 0.98-fold increase over control ($P < 0.01$ vs. PDGF-treated cells). These findings indicate that up-regulation of miR-21 specifically mediates PDGF-induced BMPRII and Id1 down-regulation as well as PASMC proliferation.

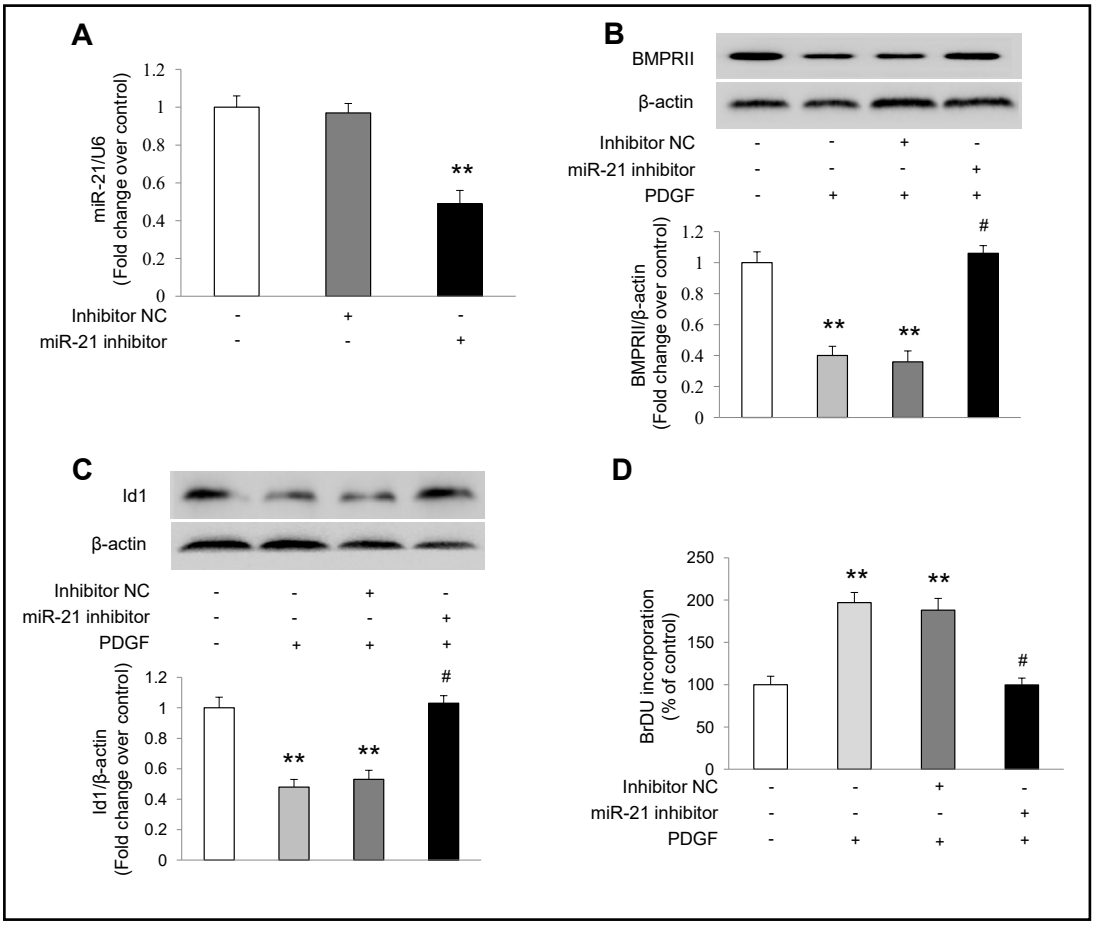

Fig. 4. miR-21 mediates PDGF-induced BMPRII and Id1 down-regulation as well as PASMC proliferation. (A) PASMCs were transfected with miR-21 inhibitor or inhibitor NC for 48 h. miR-21 level was measured using qRT-PCR (n = 4 each group). (B-D) Cells were intervened with 10 ng/ml PDGF for 24 h with or without pre-treatment of inhibitor NC or miR-21 inhibitor for 24 h. (B) BMPRII protein level was examined by immunoblotting (n = 4 each group). (C) Id1 protein level was determined by immunoblotting (n = 4 each group). (D) BrdU incorporation rate was measured for cell proliferation (n = 4 each group). Inhibitor NC: a negative control inhibitor. **P<0.01 vs. control, #P<0.01 vs. PDGF-treated cells.

Id1 is a downstream target of BMPRII

To determine whether Id1 was a downstream target of BMPRII in PASMCs, BMPRII expression was silenced with specific siRNA for 48 h. As shown in Fig. 5A, BMPRII siRNA transfection reduced BMPRII protein to 39% of the control group ($P < 0.01$), whereas non-targeting siRNA did not affect BMPRII protein level. Fig. 5B indicates that prior loss BMPRII reduced Id1 protein to 48% of the control ($P < 0.01$), whereas non-targeting siRNA did not affect Id1 protein level. These results suggest that Id1 lies downstream of BMPRII and reduction of BMPRII protein down-regulates Id1 expression in PASMCs.

PDGF up-regulates SphK1 and S1P expression via activation of PI3K/Akt and ERK1/2 signaling pathways

PI3K/Akt and ERK1/2 are two main intracellular signaling pathways activated by PDGF in PASMC proliferation [18]. Therefore, we determined the involvement of these two pathway in PDGF regulation of SphK1/S1P. Cells were treated with 10 ng/ml PDGF for 10 min and phosphorylation levels of Akt and ERK1/2 were examined using immunoblotting. As depicted in Fig. 6A, PDGF caused a 2.17-fold increase in Akt phosphorylation compared with control group ($P < 0.01$). Fig. 6B indicates that PDGF increased ERK1/2 phosphorylation to 1.89-fold compared with control group ($P < 0.01$).

We next explored whether PDGF up-regulates SphK1 and S1P expression in PASMCs via activation of PI3K/Akt or ERK1/2 signaling pathways. Cells were stimulated with 10 ng/ml PDGF for 24 h with or without prior treatment of 25 μM LY294002 or 25 μM PD98059 for 30 min. Fig. 6C shows that PDGF increased SphK1 protein level to 2.21-fold over control ($P < 0.01$), whereas LY294002 or PD98059 reduced PDGF-induced up-regulation of SphK1 protein, which declined to 1.67-fold and 1.33-fold over control, respectively ($P < 0.05$ vs. PDGF-treated cells). Fig. 6D indicates that PDGF increased S1P level to 1.70-fold and 2.41-fold over control (both $P < 0.01$), respectively, in cell culture supernates and cell lysates. Pre-inhibition with LY294002 reduced PDGF-induced elevation of S1P, which was decreased to 1.35-fold and 1.75-fold over control, respectively, in cell culture supernates and cell lysates (both $P < 0.05$ vs. PDGF-treated cells). In addition, pre-treatment with PD98059 also attenuated PDGF-induced increase of S1P, which reduced to 1.20-fold and 1.34-fold over control, respectively, in cell culture supernates and cell lysates (both $P < 0.05$ vs. PDGF-treated cells). These findings suggest that PDGF up-regulates SphK1 and S1P expression via activation of both PI3K/Akt and ERK1/2 signaling pathways, while activation of the ERK1/2 signaling pathway is a major cascade.

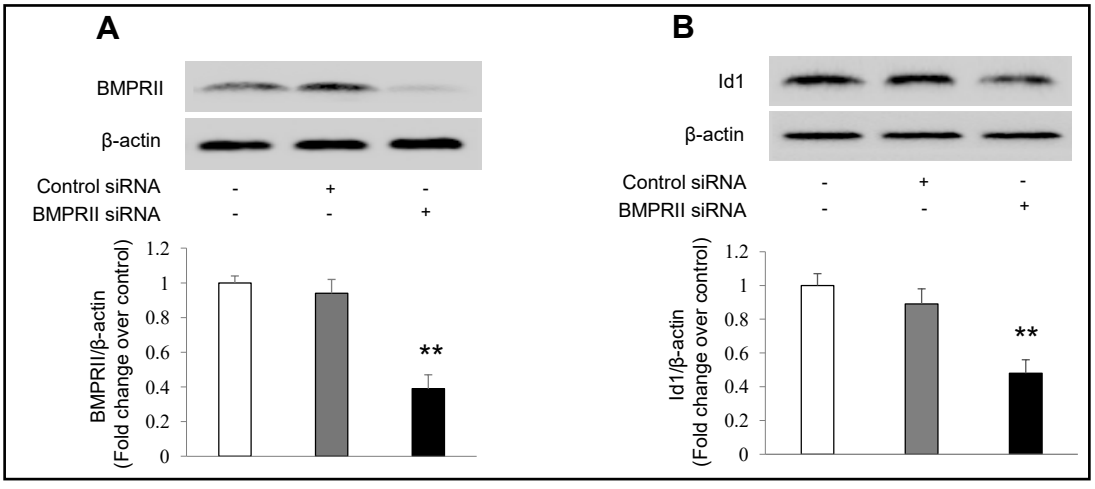

Fig. 5. Id1 is a downstream target of BMPRII. PASMCs were transfected with non-targeting siRNA or BMPRII-specific siRNA for 48 h. (A) BMPRII protein level was examined by immunoblotting (n = 4 each group). (B) Id1 protein level was determined by immunoblotting (n = 4 each group). **P<0.01 vs. control.

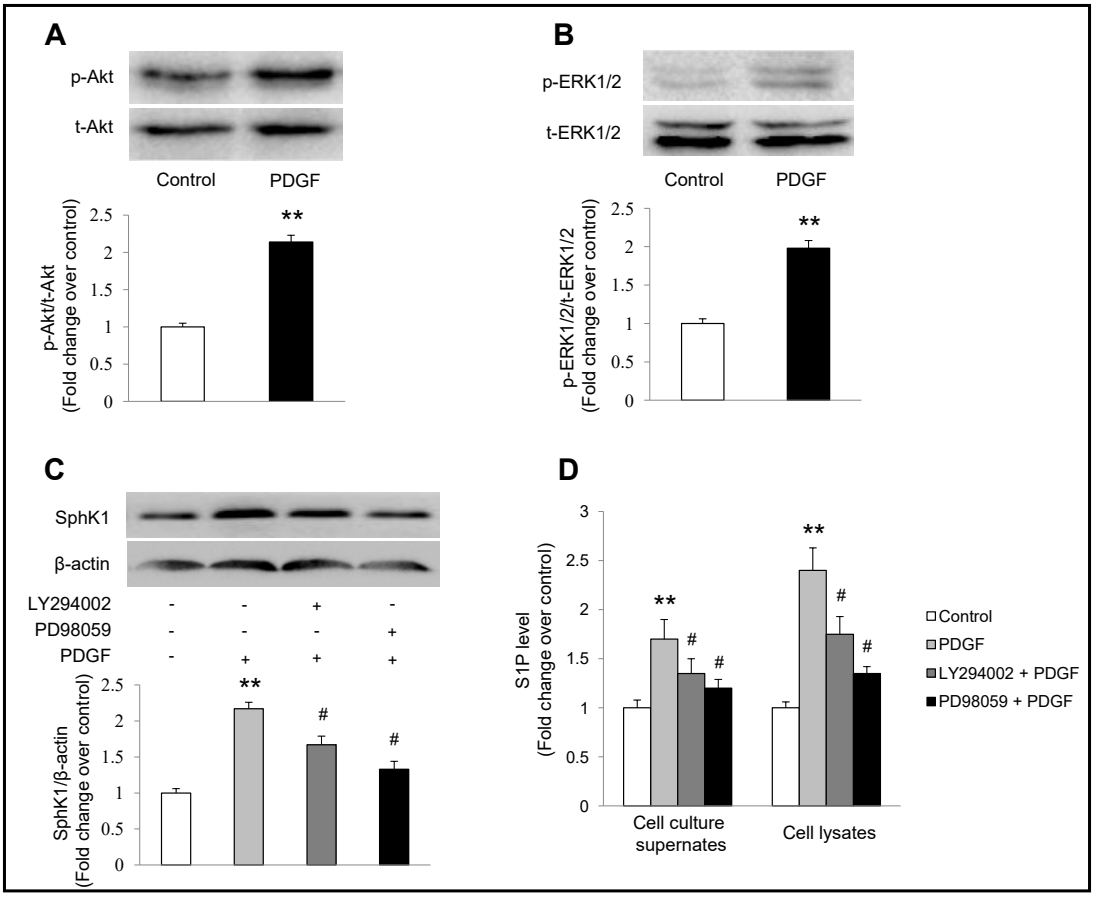

Fig. 6. PDGF up-regulates SphK1 and S1P expression via activation of PI3K/Akt and ERK1/2 signaling pathways. (A, B) PASMCs were treated with 10 ng/ml PDGF for 10 min. (A) Phosphorylation level of Akt was determined using immunoblotting (n = 4 each group). (B) Phosphorylation level of ERK1/2 was examined by immunoblotting (n = 4 each group). (C, D) Cells were stimulated with 10 ng/ml PDGF for 24 h with or without prior treatment of 25 μM LY294002 or 25 μM PD98059 for 30 min. (C) SphK1 protein level was detected using immunoblotting (n = 4 each group). (D) S1P levels both in cell culture supernates and cell lysates were determined by ELISA (n = 4 each group). **P<0.01 vs. control, #P<0.05 vs. PDGF-treated cells.

Inhibition of ERK1/2 signaling pathway reduces PDGF regulation of miR-21/BMPRII/Id1 axis and consequent PASMC proliferation

To further explore the involvement of ERK1/2 pathway in miR-21 up-regulation, BMPRII and Id1 down-regulation, as well as PASMC proliferation caused by PDGF, cells were treated with 10 ng/ml PDGF for 24 h with or without prior incubation of 25 μM PD98059 for 30 min. Fig. 7A indicates that PD98059 reduced miR-21 level from 2.58-fold over control to 1.56-fold over control in the presence of PDGF ($P < 0.05$). Fig. 7B shows that PD98059 blocked PDGF-induced BMPRII protein reduction, which increased from 0.29-fold over control in PDGF-treated cells to 0.56-fold over control in PD98059 and PDGF co-treated cells ($P < 0.05$). Fig. 7C demonstrates PD98059 increased Id1 protein level from 0.32-fold over control to 0.61-fold over control in response to PDGF ($P < 0.05$). As shown in Fig. 7D, PD98059 blocked PDGF-stimulated PASMC proliferation, which decreased from a 2.01-fold increase over control to a 1.25-fold increase over control ($P < 0.05$ vs. PDGF-treated cells). These findings indicate that ERK1/2 signaling pathway majorly mediates PDGF regulation of miR-21/BMPRII/Id1 axis and consequent PASMC proliferation.

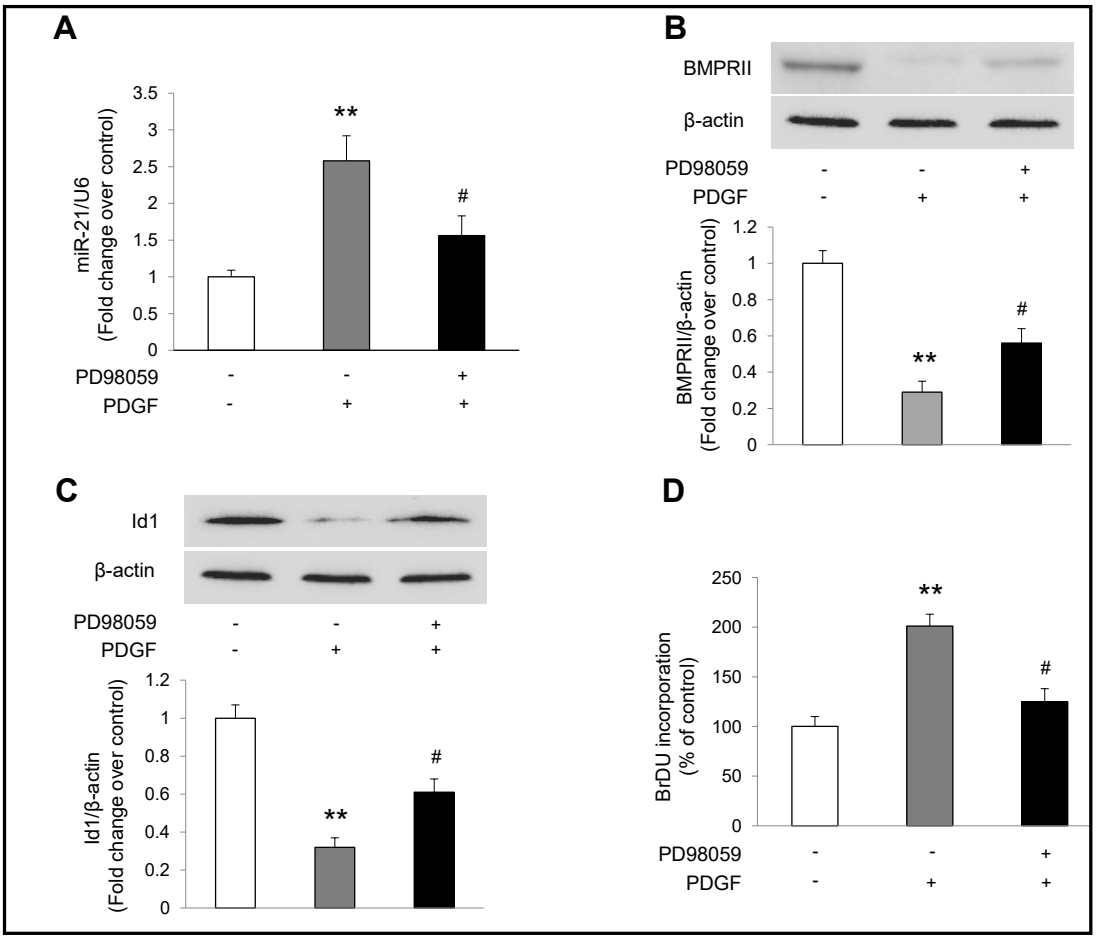

Fig. 7. Inhibition of ERK1/2 signaling pathway reduces PDGF regulation of miR-21/BMPRII/Id1 axis and consequent PASMC proliferation. PASMCs were stimulated with 10 ng/ml PDGF for 24 h with or without prior treatment of 25 μM PD98059 for 30 min. (A) miR-21 level was measured using qRT-PCR (n = 4 each group). (B) BMPRII protein level was examined by immunoblotting (n = 4 each group). (C) Id1 protein level was determined by immunoblotting (n = 4 each group). (D) BrdU incorporation rate was measured for cell proliferation (n = 4 each group). **P<0.01 vs. control, #P<0.05 vs. PDGF-treated cells.

Discussion

In the present study, we have shown that up-regulation of SphK1/S1P axis mediates PDGF-induced PASMC proliferation by increase of miR-21 and consequent inhibition of BMPRII/Id1 pathway (Fig. 8). These results suggest that SphK1/S1P axis is a crucial conductor in PDGF-induced PASMC proliferation, and plays an important role in pulmonary vascular remodeling and might serve as a new target for the prevention and treatment of PAH.

As one of the ubiquitous lipid kinases, SphK1 regulates multiple important physiological/pathological processes including cell proliferation and invasion, vascular maturation, as well as angiogenesis by increasing S1P production [7]. SphK1/S1P axis has been shown to play a crucial role in the development of many human diseases, such as cancer, atherosclerosis, inflammation, pulmonary fibrosis and autoimmune disorders [9-12, 19, 20]. Many growth factors modulate SphK1/S1P axis and its downstream targets [16, 21, 22]. Recent studies have shown that induction of SphK1/S1P axis is observed in the lungs from PAH patients and rodent PAH models and contributes to PASMC proliferation [15]. Sysol et al. have demonstrated that PDGF induces SphK1/S1P expression via ERK/ Egr-1 (early growth response protein 1)

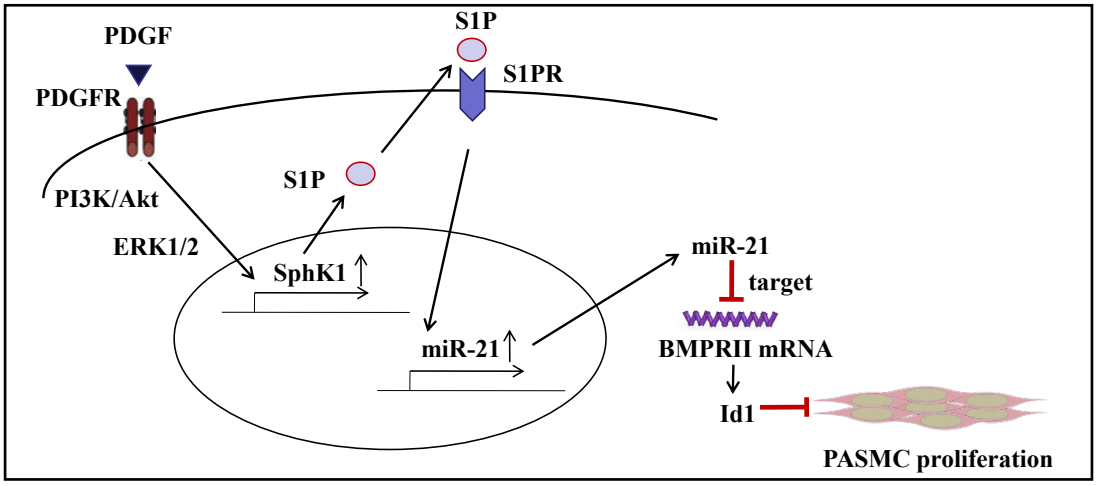

Fig. 8. Proposed mechanism by which SphK1/S1P mediates PDGF-induced PASMC proliferation. This schematic diagram shows that PDGF increases expression of SphK1 via activation of PI3K/Akt and ERK1/2 signaling pathways, and SphK1/S1P mediates PDGF-induced PASMC proliferation by up-regulation of miR-21 and consequent down-regulation of BMPRII and Id1.

pathway to promote PASMC proliferation [16]. Jacquelyn et al. have found that PI3K/Akt signaling pathway plays a key role in PDGF-induced SphK1 expression in human coronary artery smooth muscle cells [23]. Our results indicated that PDGF increased SphK1 expression and S1P level via activating both PI3K/Akt and ERK1/2 signaling pathways with ERK a predominant cascade; knockdown of SphK1 with siRNA blocked PDGF-induced elevation of S1P and PASMC proliferation, suggesting that induction of SphK1/S1P axis mediates PDGF-stimulated PASMC proliferation.

MicroRNAs (miRNAs) are small noncoding RNAs that cause mRNA degradation or translational repression via binding to the 3′ untranslated region (3′ UTR) of the target mRNA [24]. miRNAs are involved in various of fundamental biological processes, including cell apoptosis, differentiation and proliferation, as well as inflammation, angiopoiesis and metabolism [25-28]. Several studies have demonstrated that miR-21 level is elevated in lung tissues from PAH patients and mice PAH models [29], and miR-21 up-regulation contributes to pulmonary vascular remodeling via promoting PASMC proliferation in the pathogenesis of PAH [30, 31]. Liu et al. have found that SphK1/S1P pathway increases miR-21 expression to promote fibrosis in renal tubular epithelial cells [32]. Degagné et al. have further demonstrated that signal transducer and activator of transcription 3 (STAT3) medicates S1P-induced up-regulation of miR-21 in colonic epithelial cells [33]. The present study indicated that PDGF dramatically increased miR-21 level, which in turn induced PASMC proliferation. Our results confirmed that miR-21 lay downstream of SphK1/S1P axis to mediate PDGF stimulation of PASMC proliferation.

BMPRII is a transmembrane serine/threonine kinase receptor belonging to the transforming growth factor beta (TGF-β) superfamily and specifically recognized by bone morphogenetic proteins (BMPs). BMPs are involved in several signaling pathways that regulate cell differentiation, proliferation and apoptosis [34, 35]. Either loss of function due to genetic mutations or reduction in BMPRII expression is sufficient to induce the development of PAH [34, 36]. Id proteins are major downstream targets of BMP signaling, among which Id1 is a critical downstream effector of BMP signaling in PASMCs [37]. Down-regulation of BMPRII protein reduces the expression of Id1 gene, which further promotes PASMC proliferation [37, 38]. Recent study has demonstrated that PDGF inhibits BMPRII/Id1 signaling and thus promotes PASMC proliferation [39]. It has been shown that miR-21 reduces BMPRII protein by directly targeting BMPRII mRNA, promotes PASMC proliferation and further regulates pulmonary vascular remodeling [5, 30, 40]. The results of the present

study indicated that PDGF inhibited BMPRII/Id1 pathway and consequently promoted PASMC proliferation by inducing SphK1/S1P-mediated miR-21 expression.

PAH is a life-threatening syndrome without cure whose cellular and molecular mechanisms are not entirely clear [1]. The present study demonstrates that PDGF stimulates PASMC proliferation by activating SphK1/S1P axis to induce miR-21 up-regulation and the resultant inhibition of BMPRII/Id1 signaling. Giving the fact that enhanced SphK1/S1P signaling is involved in pulmonary vascular remodeling in clinic patients and a variety of animal models of PAH, the strategy of targeting on SphK1/S1P axis might prevent or treat the development of PAH, and this is worthy to test the effect and safety of this approach in clinic patients with PAH.

Disclosure Statement

The authors declare that they have no competing interests.

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