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Crosstalk between BMP signaling and KCNK3 in phenotypic switching of pulmonary vascular smooth muscle cells

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Pulmonary arterial hypertension (PAH) is a progressive and devastating disease whose pathogenesis is associated with a phenotypic switch of pulmonary arterial vascular smooth muscle cells (PASMCs). Bone morphogenetic protein (BMP) signaling and potassium two pore domain channel subfamily K member 3 (KCNK3) play crucial roles in PAH pathogenesis. However, the relationship between BMP signaling and KCNK3 expression in the PASMC phenotypic switching process has not been studied. In this study, we explored the effect of BMPs on KCNK3 expression and the role of KCNK3 in the BMP-mediated PASMC phenotypic switch. Expression levels of BMP receptor 2 (BMPR2) and KCNK3 were downregulated in PASMCs of rats with PAH compared to those in normal controls, implying a possible association between BMP/BMPR2 signaling and KCNK3 expression in the pulmonary vasculature. Treatment with BMP2, BMP4, and BMP7 significantly increased KCNK3 expression in primary human PASMCs (HPASMCs). *BMPR2* knockdown and treatment with Smad1/5 signaling inhibitor substantially abrogated the BMP-induced increase in KCNK3 expression, suggesting that KCNK3 expression in HPASMCs is regulated by the canonical BMP-BMPR2-Smad1/5 signaling pathway. Furthermore, *KCNK3* knockdown and treatment with a KCNK3 channel blocker completely blocked BMP-mediated anti-proliferation and expression of contractile marker genes in HPAMSCs, suggesting that the expression and functional activity of KCNK3 are required for BMP-mediated acquisition of the quiescent PASMC phenotype. Overall, our findings show a crosstalk between BMP signaling and KCNK3 in regulating the PASMC phenotype, wherein BMPs upregulate KCNK3 expression and KCNK3 then mediates BMP-induced phenotypic switching of PASMCs. Our results indicate that the dysfunction and/or downregulation of BMPR2 and KCNK3 observed in PAH work together to induce aberrant changes in the PASMC phenotype, providing insights into the complex molecular pathogenesis of PAH. [BMB Reports 2022; 55(11): 565-570]

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INTRODUCTION

Pulmonary arterial hypertension (PAH) is a progressive and fatal disease clinically defined by abnormally high mean pulmonary arterial pressure ($>$ 25 mmHg at rest or $>$ 30 mmHg during exercise), leading to heart failure and ultimately death, if not treated. The increase in mean pulmonary arterial pressure is a consequence of pulmonary vascular remodeling that involves a phenotypic switch of pulmonary arterial smooth muscle cells (PASMCs) from a quiescent state to a synthetic state (1). While PASMCs in healthy vasculature do not proliferate and express high levels of contractile marker genes, those in PAH have elevated proliferation rates and reduced expression of contractile marker genes in response to genetic and environmental cues. This phenotype switching leads to hypertrophy and PASMC overgrowth, increasing pulmonary vascular resistance and elevating pulmonary arterial pressure.

A comprehensive understanding of the molecular etiology of PAH has been achieved by identifying genetic mutations in genes such as bone morphogenetic protein receptor 2 (*BMPR2*) and *KCNK3* and defining their individual roles in PAH pathogenesis. Loss of function mutations in *BMPR2*, the most common genetic factor associated with PAH, have been identified in $>$ 70% of familial PAH and 15% of sporadic PAH (2). The expression levels and signaling activity of BMPR2 are substantially reduced in the pulmonary vasculature of patients with PAH who carry no *BMPR2* mutations. BMPR2 is a serine/threonine kinase receptor of bone morphogenetic proteins (BMPs). In normal vasculature, BMP/BMPR2 signaling inhibits cell proliferation and upregulates contractile marker genes, and thus contribute to the maintenance of the quiescent and contractile phenotype of vascular smooth muscle cells (VSMCs) (3, 4). Impaired BMP/BMPR2 signaling can cause aberrant changes in the PASMC phenotype into proliferative or synthetic states, demonstrating a crucial role for the BMP/BMPR2 pathway in

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the development and progression of PAH. *KCNK3*, a potassium two pore domain channel subfamily K member 3 encoding TWIK-related acid-sensitive K^+ channel (TASK)-1, has recently been identified as a new predisposing gene for PAH (5). Loss of function mutations in *KCNK3* were first identified in six patients with PAH and since then, several additional *KCNK3* mutations have been observed in 3.2% of patients with familial PHA and 1.3% of patients with sporadic PAH (5, 6). Moreover, expression levels and functional activity of KCNK3 were substantially reduced even in patients with PAH who did not carry *KCNK3* mutations. Indeed, decrease in the expression and/or activity of KCNK3 promoted cell proliferation, resistance to apoptosis, and vasoconstriction in PASMCs. These findings demonstrate that KCNK3, originally known for its role in regulating resting membrane potential, is directly involved in the pulmonary vascular remodeling process of PAH.

Although these studies clearly identified the individual roles of BMP signaling and KCNK3 during the establishment and progression of PAH, little attention has been given to the possible crosstalk between BMP signaling and KCNK3 in PAH pathogenesis. In the present study, we aimed to explore how BMP/BMPR2 signaling and KCNK3 work together to regulate the PASMC phenotypic switch. We found that canonical BMP-BMPR2-Smad1/5 signaling upregulates KCNK3 in PASMCs and that BMP-induced expression of KCNK3 is required for BMP-mediated maintenance of the contractile and quiescent PASMC phenotype, suggesting a connection between BMP signaling and KCNK3 in the regulation of PASMC phenotypic switching.

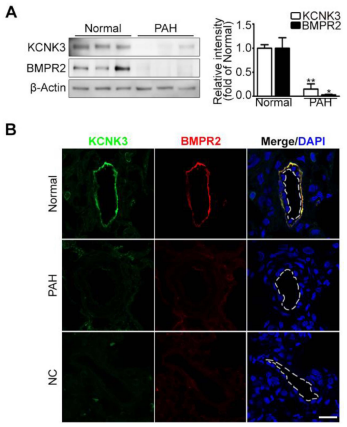

Fig. 1. KCNK3 and BMPR2 expression is simultaneously reduced in PASMCs of rats with PAH. (A) Levels of KCNK3 and BMPR2 proteins in lung tissues of rats with MCT-induced PAH and normal controls were quantified using western blotting assay and expressed relative to levels in normal controls. Data represent mean ± SEM (*P < 0.05, **P < 0.01, n = 3 rats per group). (B) Representative immunofluorescence images of KCNK3 (green) and BMPR2 (red) in the small pulmonary arterioles (white dotted lined circles) of normal rats or rats with PAH. Nuclei were stained with DAPI (blue). Negative controls (NC) were treated with non-relevant primary IgGs. Scale bars = 20 μm.

RESULTS

KCNK3 and BMPR2 expression is simultaneously reduced in PASMCs of rats with PAH

We first measured KCNK3 and BMPR2 expression levels in lung tissues from PAH animal models and normal controls. We found that KCNK3 and BMPR2 protein levels were substantially lower in lung tissues of rats with MCT-induced PAH compared with those in the normal controls (Fig. 1A). We then conducted immunohistochemical staining to identify correlations between KCNK3 and BMPR2 expression patterns in pulmonary vasculature. While KCNK3 and BMPR2 were highly co-expressed in the α-SMA^+ PASMC layer of normal pulmonary arterioles, their expression was simultaneously downregulated in the thickened medial layer of pulmonary arterioles associated with PAH (Fig. 1B, Supplementary Fig. 1). These data imply that impaired BMP/BMPR2 signaling due to reduced BMPR2 expression may be associated with downregulation of KCNK3 in pulmonary vasculature of the PAH animal model.

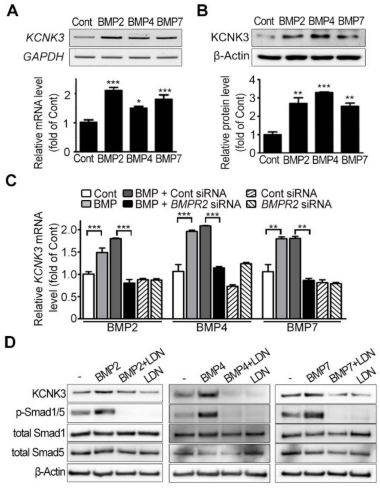

Fig. 2. BMPs enhance KCNK3 expression in HPASMCs via the BMPR2-Smad1/5 signaling pathway. (A, B) BMP2, BMP4, and BMP7 increased KCNK3 expression in HPASMCs. Cells were treated with BMPs (30 ng/ml) in growth media for 48 h and harvested for RT-PCR and western blot analyses. mRNA (A) and protein (B) levels of KCNK3 were analyzed relative to those in untreated controls (Cont) (triplicate experiments). (C) SiRNA-mediated *BMPR2* knockdown blocked BMP-induced increase in KCNK3 expression in HPASMCs. SiRNA-transfected and non-transfected cells were treated with or without BMPs (30 ng/ml) in growth media for 48 h and harvested for real-time RT-qPCR. *KCNK3* mRNA levels were measured relative to those in untreated Cont (n = 4). (D) Inhibition of Smad signaling blocked BMP-induced KCNK3 expression in HPASMCs. Cells were pre-treated with LDN193189 (LDN, 2.5 μM) and incubated with BMPs (30 ng/ml) in growth media for 48 h and analyzed using western blotting assay (triplicate experiments). Data are presented as mean ± SEM (*P < 0.05, **P < 0.01, ***P < 0.001 vs. Cont).

BMPs enhances KCNK3 expression in HPASMCs via BMPR2-Smad1/5 signaling pathway

Assuming a potential association between BMP/BMPR2 signaling and KCNK3 expression in PASMCs, we investigated whether BMPs can regulate KCNK3 expression in HPASMCs. Previous studies have shown that BMP2, BMP4, and BMP7, regulate cell proliferation, apoptosis, and phenotypic changes in PASMCs (7-9). We found that BMP2, BMP4, and BMP7 treatment significantly increased KCNK3 expression in HPASMCs, as observed using RT-qPCR and western blot analyses (Fig. 2A, B). To investigate whether the BMP-induced increase in KCNK3 expression is mediated by BMPR2, we silenced *BMPR2* expression using siRNA (Supplementary Fig. 2). Knockdown of *BMPR2* expression substantially abrogated the BMP-induced increase in *KCNK3* expression in HPASMCs. However, no change was detected in BMP-induced KCNK3 expression in the control siRNA group (Fig. 2C). BMPs bind to BMPR2, and BMPR2 then associates with activin receptor-like kinase (ALK), activating the downstream Smad1/5 signaling pathway (10). Blocking BMP-induced phosphorylation of Smad1/5 using LDN193189, an ALK inhibitor, markedly abrogated the BMP-induced increase in KCNK3 expression (Fig. 2D). These findings indicated that BMPs upregulate KCNK3 expression in HPASMCs via the BMPR2-Smad1/5 signaling pathway.

KCNK3 expression is required for BMP-induced switching of HPASMC phenotypes

Next, we explored whether KCNK3, whose expression is upregulated by BMPs, might be involved in the BMP-induced switching of PASMC phenotypes. HPASMCs cultured in growth media containing growth factors and fetal bovine serum exhibit proliferative or synthetic phenotype characterized by high cell proliferation rates and decreased expression of VSMC contractile marker genes (11). In contrast, BMPs enhance the expression of contractile marker genes and suppress cell proliferation, inhibiting VSMCs from changing into proliferative or synthetic phenotypes in response to growth factors produced following vascular injury (7-9). We found that BMP2, BMP4, and BMP7 treatment substantially inhibited cell proliferation, as illustrated using the anti-Ki67 staining assay (Fig. 3A, B). This anti-proliferative effect of BMPs was abrogated by siRNA-mediated knockdown of *KCNK3* (Supplementary Fig. 3, Fig. 3A, B). In addition, treatment with BMPs substantially increased the percentage of VSMC contractile marker (SM-MHC)-positive cells in HPASMCs, but not in *KCNK3* siRNA-treated HPASMCs (Fig. 3C, D). RT-PCR data also confirmed that the BMP-induced increase in the expression of VSMC contractile marker genes (*ACTA2*, *TAGLN*) in HPASMCs was abolished by siRNA-mediated knockdown of *KCNK3* (Fig. 3E). These findings indicate that KCNK3 expression is required for BMP-induced maintenance of the quiescent and contractile HPASMC phenotype.

KCNK3 antagonist inhibits BMP-induced switching of HPASMC phenotypes

As *KCNK3* encodes a major K^+ channel regulating the resting membrane potential in PASMCs, we investigated whether the functional activity of KCNK3 as an outward rectifier K^+ channel might be important in BMP-induced switching of PASMC phenotypes. Immunocytochemistry data revealed that BMP2, BMP4, and BMP7 treatment significantly reduced the number

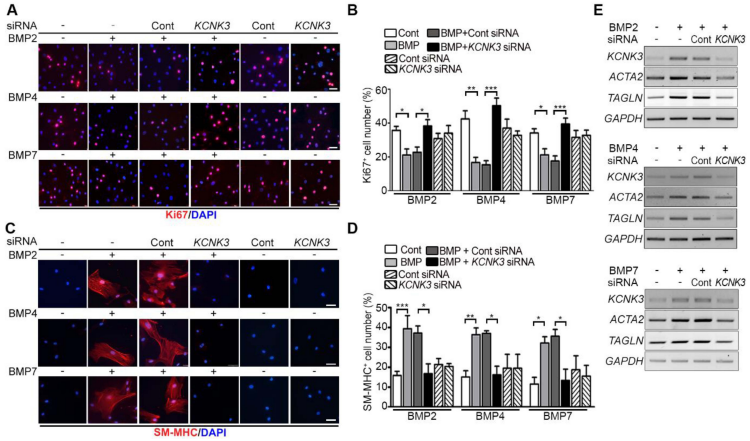

Fig. 3. KCNK3 expression is required for BMP-induced switching of HPASMC phenotypes. (A, B) SiRNA-mediated *KCNK3* knockdown blocked the anti-proliferative effect of BMPs on HPASMCs. SiRNA-transfected and non-transfected cells were cultured with or without BMPs (100 ng/ml) in growth media for 72 h and then stained with anti-Ki67 IgG. The percentages of $Ki67^+$ cells were measured (n = 6). (C-E) SiRNA-mediated *KCNK3* knockdown blocked the BMP-induced expression of differentiation marker genes in HPASMCs. SiRNA-transfected and non-transfected cells were cultured with or without BMPs (30 ng/ml) in growth media for 48 h and then stained with anti-SM-MHC IgG (red). The percentages of SM-MHC^+ cells were measured (n = 4). In (E), mRNA levels of *KCNK3* and SMC differentiation marker genes (*ACTA2*, *TAGLN*) were analyzed using RT-PCR and expressed relative to those in untreated Cont (n = 4). In (A) and (B), nuclei are shown in blue (DAPI). Scale bars = 50 μm. All data are presented as mean ± SEM (*P < 0.05, **P < 0.01, ***P < 0.001).

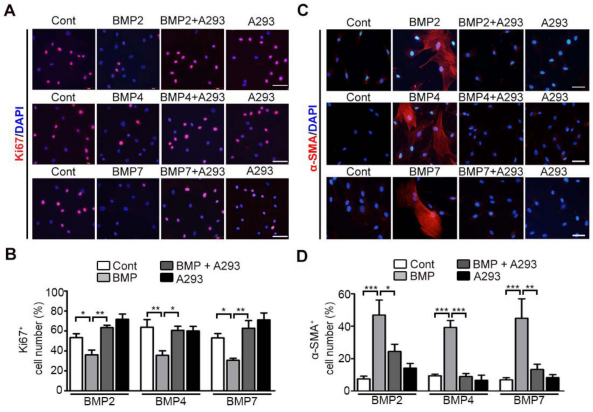

Fig. 4. KCNK3 antagonist inhibits BMP-induced switching of HPASMC phenotypes. (A, B) Treatment with a KCNK3 antagonist, A293, blocked the anti-proliferative effect of BMPs on HPASMCs. Cells were cultured in growth medium containing BMPs (100 ng/ml) or A293 (200 nM) for 72 h and then stained with anti-Ki67 IgG. (A) Representative images of Ki67 (red) staining and (B) Quantitative analysis of the percentage of $Ki67^+$ cells in HPASMCs (n = 6). (C, D) Treatment with a KCNK3 antagonist, A293, blocked the BMP-induced the expression of SMC differentiation marker genes in HPASMCs. SiRNA-transfected and non-transfected cells were cultured in growth media with or without BMPs (30 ng/ml) for 48 h and then stained with anti-α-SMA IgG (red). The percentages of $\alpha\text{-SMA}^+$ cells were assessed (n = 4). Nuclei in (A) and (C) are shown in blue (DAPI). Scale bars = 50 μm. Data are presented as mean ± SEM (*P < 0.05, **P < 0.01, ***P < 0.001).

of $Ki67^+$ proliferating cells in HPASMCs (Fig. 4A, B). However, inhibiting the functional activity of KCNK3 using a selective KCNK3 channel blocker, A293, completely abrogated the BMP-induced decrease in cell proliferation. Moreover, A293 substantially abolished the BMP-induced increase in the number of VSMC contractile marker (α-SMA)-positive HPASMCs (Fig. 4C, D). These data indicate that the K^+ channel activity of KCNK3 is required for BMP-mediated maintenance of the quiescent and contractile phenotype of HPASMCs.

DISCUSSION

PASMCs undergo a phenotypic switch during PAH development, displaying high proliferation rates and reduced expression of contractile marker genes, which is one of central events in pulmonary vascular remodeling. Recent studies of patients with PAH have identified BMP/BMPR2 signaling and KCNK3 as key factors regulating the phenotypic switching of PASMCs in PAH. Although the individual roles of BMP/BMPR2 signaling and KCNK3 are well defined, it is not known whether a crosstalk between these two factors occur during PAMSC phenotype switching. In this study, we demonstrated that canonical BMP/BMPR2 signaling induces KCNK3 expression in PASMCs, and KCNK3 mediates BMP-induced phenotypic switching of PASMCs. This crosstalk between BMP/BMPR2 signaling and KCNK3 that is involved in the regulation of PASMC phenotype may provide insights into the complex molecular pathogenesis of PAH.

We found that KCNK3 and BMPR2 expression was simultaneously reduced in PASMCs of rats with PAH, allowing us to postulate that impaired BMP/BMPR2 signaling might be associated with reduced KCNK3 expression in PASMCs. In support of this postulation, BMP2, BMP4, and BMP7 significantly enhanced the expression of KCNK3 in HPASMCs via BMPR2-Smad1/5 signaling pathway, which was confirmed using *BMPR2* siRNA and a Smad1/5 phosphorylation inhibitor. Since BMP response elements (BRE) such as GCCG or GGCGCC in GC-rich regions have been identified in the promoter regions of BMP target genes, we analyzed the 5′ flanking region of human *KCNK3* (12, 13). We identified two putative BREs at locations −178 to −172 and −46 to −42 near *KCNK3*'s transcription start site, indicating that KCNK3 may be a direct target gene activated by canonical BMP-BMPR2-Smad1/5 signaling in HPASMCs. Nevertheless, we cannot rule out the possibility that non-canonical BMP downstream signaling including p38 MAPK pathway may be involved in BMP-induced KCNK3 expression. In keeping with our data on HPASMCs, Antigny *et al*. measured the expression levels of KCNK3 in lung tissues of patients with heritable PAH carrying a *BMPR2* mutation and identified a pronounced decrease in KCNK3 protein expression in patients with PAH compared to that in the control subjects (14). It is therefore conceivable that reduced KCNK3 expression in animal models and patients with PAH can be attributed to impaired BMP/BMPR2 signaling in PASMCs.

We also showed that KCNK3 expression and its ion channel activity are required for BMP-induced PASMC phenotype switching. As TASK-1, encoded by KCNK3, is a major K^+ channel/outward rectifier regulating the resting membrane potential in PASMCs, siRNA-mediated knockdown of *KCNK3* and A293 treatment may cause membrane depolarization in HPASMCs. Indeed, Olschewski *et al*. reported that knockdown of *KCNK3* in HPASMCs resulted in depolarization of the resting membrane potential (15). Lambert *et al*. also showed that the membrane

potential of PASMCs in KCNK3 deficient rats was significantly depolarized (16). Membrane depolarization activates voltage-gated L-type Ca^{2+} channels, enhancing calcium influx and increasing cytoplasmic Ca^{2+} levels ($[Ca^{2+}]_{cyto}$). Ca^{2+} is an important second messenger in VSMCs. Elevated $[Ca^{2+}]_{cyto}$ promotes DNA synthesis and cell proliferation, leading to vascular hypertrophic remodeling. Several studies have shown that membrane depolarization and the subsequent increase in $[Ca^{2+}]_{cyto}$ enhance PASMC proliferation and lead to pulmonary vascular medial hypertrophy (17-19). Furthermore, elevated $[Ca^{2+}]_{cyto}$ is a key determinant in the transition from a contractile to synthetic VSMC phenotype, including in PASMCs (18, 20, 21). Thus, the BMP-mediated increase in KCNK3 expression can maintain $[Ca^{2+}]_{cyto}$ at low levels, restricting vasoconstriction, cell proliferation, and phenotype switching to the synthetic state in PASMCs. Conversely, loss of function mutations and/or downregulation of KCNK3 in PASMCs might limit the anti-proliferative and pro-contractile effect of BMP/BMPR2 signaling in PASMCs and render PASMCs more prone to acquire a synthetic phenotype during PAH development.

In summary, we addressed a crosstalk between BMP signaling and KCNK3 expression during the PASMC phenotype switching process, a hallmark of PAH pathogenesis. We showed that the dysfunction and/or downregulation of BMPR2 and KCNK3 observed in patients with PAH may work together to induce aberrant switching of PASMC phenotypes. Our findings provide novel mechanistic insights into the multifactorial pathogenesis of pulmonary vascular remodeling in PAH.

MATERIALS AND METHODS

Rat model of PAH

All animal experimental protocols were approved by the Institutional Animal Care and Use Committee of Chung-Ang University and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the United States National Institutes of Health. Seven-week-old male Sprague Dawley rats (Orient, Seoul, Korea) received either a single subcutaneous injection of monocrotaline (MCT; 60 mg/kg, Sigma, St. Louis, MO) or saline solution. Five weeks later, the rats were anesthetized using an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (5 mg/kg). The animals were then euthanized by cervical dislocation, and lung tissues were extracted for further analyses.

Cell culture and treatment

Primary human pulmonary artery smooth muscle cells (HPASMCs; ScienCell Research Laboratories, Carlsbad, CA) were seeded and cultured at 37°C in smooth muscle cell growth medium (ScienCell Research Laboratories) from passages five to seven in a humidified atmosphere containing 5% CO_2. Cells were treated with or without recombinant human bone morphogenetic proteins (BMPs; BMP2, BMP4, and BMP7, R&D Systems, Minneapolis, MN), LDN193189 (Cayman Chemical, Ann Arbor, MI), and A293 (Selleck Chemicals, Houston, TX) and cultured in growth media for 48-72 h.

Immunofluorescence staining

Lung tissue sections and HPASMCs were stained with primary immunoglobulins (IgGs) and then incubated with fluorescent secondary IgGs. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA). Tissue sections and cells were visualized using a confocal laser scanning microscope (Carl Zeiss, Jena, Germany). All images presented are representative of more than three independent experiments. The IgGs used in the experiment are listed in Supplementery Table 1.

Western blotting

Tissue and cell lysates were sonicated, centrifuged, and the insoluble fraction were discarded. The concentration of the protein in the supernatant was determined using the bicinchoninic acid protein assay. Protein samples were then boiled in sample buffer and size-fractionated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Membranes were hybridized with the appropriate primary IgG and then incubated with horseradish peroxidase-conjugated secondary IgG. Bound IgGs were detected using an enhanced chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ). Images captured using a western blot imaging system (Vilber, Marne-la-Valleé, France) were analyzed using ImageJ software (National Institute of Health, Bethesda, MD). The IgGs used in the experiment are listed in Supplementery Table 1.

Small interfering RNA (siRNA) transfection

To silence the expression of *BMPR2* and *KCNK3*, HPASMCs were transfected with specific or nonspecific control siRNAs (Dharmacon, Lafayette, CO) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Three days after transfection, gene expression was analyzed using reverse transcription-polymerase chain reaction (RT-PCR). The siRNA sequences used are listed in Supplementery Table 2.

RT-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen). Complementary DNA was synthesized using the Superscript first-strand synthesis kit (Invitrogen), and conventional PCR was performed using Bio-Rad PCR system (Hercules, CA). Real-time quantitative reverse transcription-PCR (RT-qPCR) was performed on the StepOnePlus™ Real-Time PCR System (Applied Biosystems, Carlsbad, CA) using SYBR Green PCR Master Mix (Applied Biosystems), following the manufacturer's instructions. Data were analyzed using the ΔΔCt method. Glyceraldehyde-3-phhosphate dehydrogenase (*GAPDH*) was used as an internal control to semi-quantify and normalize the PCR products. The sense and antisense primer sequences used are listed in Supplementery Table 3.

Statistical analysis

GraphPad prism software (GraphPad Software Inc, San Diego, CA) was used to analyze the data. Statistical significance was evaluated using an unpaired Student's *t*-test or one-way analysis of variance with Bonferroni's *post hoc* multiple comparison test. All data are presented as mean ± standard error of the mean (SEM). Differences were significant at $P < 0.05$. The number of samples is indicated by *n*.

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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