

# Paeoniflorin Ameliorates Chronic Hypoxia/SU5416-Induced Pulmonary Arterial Hypertension by Inhibiting Endothelial-to-Mesenchymal Transition

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**Background:** Endothelial cells dysfunction is one of the hallmark pathogenic features of pulmonary arterial hypertension (PAH). Paeoniflorin (PF) is a monoterpene glycoside with endothelial protection, vasodilation, antifibrotic, anti-inflammatory and antioxidative properties. However, the effects of PF on PAH remain unknown.

**Methods:** Here, we investigated the efficacy of PF in the SU5416/hypoxia (SuHx) rat model of PAH. Human pulmonary arterial endothelial cells (HPAECs) were exposed to 1% O<sub>2</sub> with or without PF treatment.

**Results:** Hemodynamics analysis showed that prophylactic treatment with PF (300 mg/kg i.g. daily for 21 days) significantly inhibited chronic hypoxia/SU5416-induced elevations of right ventricular systolic pressure (RVSP) and right ventricular hypertrophy index in rats. Meanwhile, PF significantly reduced pulmonary vascular remodeling, as well as alleviated collagen deposition in lungs and right ventricles in SuHx rats. Additionally, PF inhibited SuHx-induced down-regulation of endothelial marker (vascular endothelial cadherin) and up-regulation of mesenchymal markers (fibronectin and vimentin) in lung, suggesting that PF could inhibit SuHx-induced endothelial-to-mesenchymal transition (EndMT) in lung. Further in vitro studies confirmed that PF treatment suppressed hypoxia-induced EndMT in HPAECs, which was abolished by the knockdown of bone morphogenetic protein receptor type 2 (BMPR2) in HPAECs.

**Conclusion:** Taken together, our findings suggest that PF ameliorates BMPR2 down-regulation-mediated EndMT and thereafter alleviates SuHx-induced PAH in rats.

**Keywords:** paeoniflorin, pulmonary arterial hypertension, endothelial-to-mesenchymal transition, BMPR2, hypoxia

## Introduction

Pulmonary arterial hypertension (PAH) is a lethal disorder characterized by pulmonary arterial obstruction and sustained elevation of pulmonary arterial pressure, eventually resulting in right ventricle hypertrophy and failure.<sup>1</sup> The key pathological feature of PAH is pulmonary vascular remodeling, comprising the dysfunction of pulmonary arterial endothelial cells, excessive proliferation and apoptosis resistance of smooth muscle cells and abnormal activation of adventitial fibroblasts.<sup>2</sup> Despite major advances achieved in the management of PAH in the past decade, current approaches mainly target pulmonary vasoconstriction rather than proliferative vascular remodeling, and the long-term clinical outcome improvement for this devastating disease is limited.<sup>3</sup> Thus, novel therapeutic approaches are urgently required for better treatment of PAH.

Endothelial cell dysfunction plays a key role in the development of PAH through altered production of endothelial vasoactive mediators, impaired vasoconstriction, unbalanced endothelial cell proliferation and apoptosis, and aberrant endothelial-to-mesenchymal transition (EndMT).<sup>2</sup> Generally, EndMT is a complex biological process in which endothelial cells (ECs) lose their specific phenotype and gradually transdifferentiate into cells with a mesenchymal phenotype characterized by loss of cell-cell junctions and polarity, and the acquisition of cellular motility and invasive properties. During this process, ECs lose specific endothelial markers such as CD31, vascular endothelial cadherin (VE-cadherin) and progressively express mesenchymal markers such as fibronectin, vimentin and  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA). EndMT has been shown to play a vital role not only in embryonic developmental processes but also in the pathogenesis of fibrotic lung disease.<sup>4</sup> Recently, EndMT has emerged as a critical player in the pulmonary vascular remodeling in human PAH and experimental models of PAH.<sup>5,6</sup>

Paeoniflorin (PF), a monoterpene glucoside extracted from the root of *Paeonia lactiflora*, possesses many pharmacological activities, such as anti-inflammatory, antioxidative, analgesic, anticancer and immunoregulatory effects.<sup>7–10</sup> PF is widely used in clinical practice for the treatment of rheumatoid arthritis and systemic lupus erythematosus.<sup>11</sup> A recent study demonstrated that PF exerts a direct vasculoprotective effect through antioxidative and anti-inflammatory effects in fluctuant hyperglycemia-induced vascular endothelial injuries.<sup>8</sup> Moreover, it has been documented that PF could protect against lipopolysaccharide, oxidized low-density lipoprotein or radiation-induced endothelial cell dysfunction.<sup>12–14</sup> Recently, PF was reported to prevent hypoxia-induced epithelial-mesenchymal transition (EMT) in human breast cancer cells.<sup>15</sup> However, the effects of PF on pulmonary vascular remodeling and EndMT process in PAH are still unknown. Therefore, we explored the effects of PF on pulmonary hemodynamics, pulmonary artery thickness, and right ventricular remodeling in the SU5416/hypoxia (SuHx) rat model of PAH, and investigated its possible molecular mechanism.

## Materials and Methods

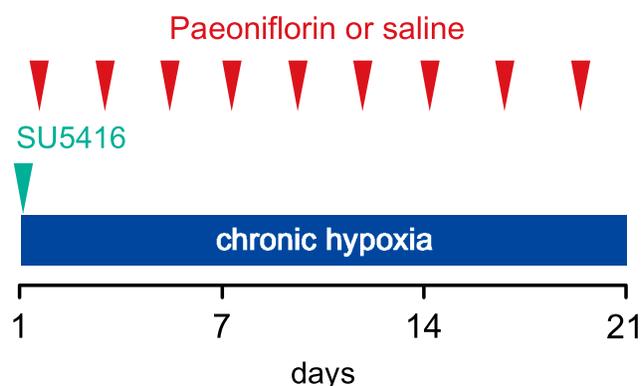
### Animals

All animal procedures were conducted following the guidelines published by the National Institutes of Health Guide for

the Care and Use of Laboratory Animals (NIH publication No. 85–23, revised 1996). All study protocols were approved by the Institutional Animal Care and Use Committee of Nanjing Medical University (NJMU/IACUC-1809008). Male Sprague Dawley rats (Nanjing Medical University Experimental Animal Center, Nanjing, China) weighing 200–220 g were randomly assigned into 4 groups: Control, Control+PF (300 mg/kg), SuHx, SuHx+PF (300 mg/kg) (n=6 in each). In order to investigate the preventive efficacy of PF in PAH, the SuHx rat model of PAH was induced by a single subcutaneous injection of SU5416 at 20 mg/kg (MedChemexpress, USA, a vascular endothelial growth factor receptor inhibitor) in a solution (composed of 0.5% (w/v) carboxymethylcellulose sodium, 0.9% (w/v) sodium chloride, 0.4% (v/v) polysorbate 80, 0.9% (v/v) benzyl alcohol in deionized water) followed by 3 weeks chronic hypoxia (10% O<sub>2</sub>) using a ventilated chamber as published previously.<sup>16,17</sup> Control rats were injected only the vehicle. The experimental rats received saline as the vehicle control or paeoniflorin (Nantong Jingwei biological science and Technology Co, Ltd, Nantong, China) at the dose of 300 mg/kg by intragastric administration once daily for 3 weeks from the first day (Figure 1). At the end of the hypoxia exposure period, rats were sacrificed for the following experimental measurements.

### Hemodynamics

At the end of the 21-days exposure period, the rats were anesthetized with 45mg/kg of pentobarbital sodium (Sigma-Aldrich, St. Louis, MO, USA) by intraperitoneal injection. A polyethylene catheter was inserted through the right jugular vein into the right ventricle (RV) to measure the right



**Figure 1** Male SD rats received a single subcutaneous injection of SU5416 (20 mg/kg) or vehicle and were placed in hypoxia for 3 weeks (10% O<sub>2</sub>). These rats received daily doses of 300 mg/kg of paeoniflorin (PF) or saline by gavage once daily for 3 weeks from the first day. Rats were sacrificed at 21 days after the SU5416 administration to evaluate the preventive efficacy of PF in PAH.

ventricular systolic pressure (RVSP). The signals were continuously recorded using the Power Lab data acquisition system (ADI Instruments). Then, the thorax was opened and the lung tissues were flushed with cold saline through the pulmonary artery. The left lung was immediately removed and frozen for protein isolation. The right lung and RV heart tissue were excised for histological assessment. The RV hypertrophy was presented as the weight ratio of the RV to the left ventricle and interventricular septum (RV/LV+S).

## Morphological Measurements

The isolated lung and RV heart tissue were fixed in 4% paraformaldehyde, embedded in paraffin and sectioned at 5- $\mu$ m thickness. Then, lung sections were stained with hematoxylin-eosin (H&E) and examined by a light microscope (DM2500, Leica, Wetzlar, Germany) using a standard protocol. Morphometric analysis was performed in the pulmonary arteries (external diameter: 25–100  $\mu$ m) at a magnification of 400 $\times$ . Pulmonary arterial wall thickness was calculated as medial wall thickness index (%) = (external diameter – internal diameter)/external diameter  $\times$  100. For quantitative analysis, 20 randomly selected vessels from each rat were analyzed, and the average was calculated. Right ventricular hypertrophy was measured by the cardiomyocyte cross-sectional area (CSA) in HE-stained sections. To evaluate the collagen deposition in lung and RV heart tissue, Masson's trichrome and Picrosirius red staining were performed. The degree of fibrosis in the lung and RV heart tissue was calculated by measuring the percentage of the collagen-positive area using Image-Pro Plus software.

## Culture of Human Pulmonary Artery Endothelial Cells (HPAECs)

HPAECs were obtained from ScienCell Research Laboratories (ScienCell, Carlsbad, CA), maintained in endothelial cell medium (ScienCell) with 5% fetal bovine serum, 1% endothelial cell growth supplement, and 1% penicillin/streptomycin solution at 5% CO<sub>2</sub> and 37 °C. Cells between passages 3 and 8 were used for further experiments.

## Endothelial-Mesenchymal Transition of HPAECs in vitro

Cells were cultured at approximately 80% confluency. To determine the effects of paeoniflorin on EndMT, the cells were cultured under normoxia or hypoxia (1% O<sub>2</sub>) with or without paeoniflorin (10  $\mu$ M, Sigma-Aldrich, St. Louis MO, USA) treatment for 0 h, 6 h, 12 h, 24 h, 48 h, and

72 h. EndMT was assessed by loss of the endothelial marker (VE-cadherin) and gain of the mesenchymal markers ( $\alpha$ -SMA, fibronectin and vimentin) and EndMT-related transcription factor (snail and twist) using immunoblot analysis and immunofluorescence.

## RNA Interference

For small interfering RNA (siRNA) transfection, HPAECs were grown in 24-well plates until the confluence of cells reached approximately 50%. siRNA against human bone morphogenetic protein receptor type II (BMPRII) (forward, 5'-GCAGCAAGCACAAAUCAAAATT-3'; reverse, 5'-UUUGAUUUGUGCUUGCUGCTT-3') and scrambled control (NC) siRNA were purchased from GenePharma (Shanghai, China). Cells were transfected with BMPRII siRNA (50 nM) or scrambled siRNA (50 nM) using the siRNA transfection reagent siRNA-mate (GenePharma), following the manufacturer's protocol. The knockdown efficiency of siRNAs was performed 24 h after transfection by Western blotting. After 24 h of transfection, the cells were cultured under normoxia or hypoxia with or without paeoniflorin for another 48 h, followed by lysis buffer for Western blot analysis.

## Immunofluorescence Staining

HPAECs were fixed in 4% paraformaldehyde for 30 min, permeabilized with PBS containing 0.1% Triton-X100, then blocked with 2% BSA (Beyotime, Jiangsu, China) at room temperature. To access the EndMT process, samples were further incubated with anti-VE-cadherin antibody (Abcam, Cambridge, MA) and anti-vimentin (Abcam, Cambridge, MA) antibody overnight at 4°C. Subsequently, cells were incubated with secondary antibody (donkey anti-mouse IgG, Alexa Fluor 555; and donkey anti-rat IgG, Alexa Fluor 488) for 1 h at room temperature. Nuclei were visualized by 4, 6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich). Fluorescence was observed with the Leica 2500 microscope. All samples were examined, and at least five images from each were acquired.

## Western Blotting Analysis

Total proteins were extracted from the lung tissues and HPAECs with RIPA Lysis and Extraction buffer (Thermo Scientific, Rockford) containing protease inhibitors, phosphatase inhibitors, and PMSF. The protein concentrations were measured using BCA protein assay kit (Beyotime, Jiangsu, China). An equal amount of protein from each sample was subjected to electrophoresis on 10% SDS-PAGE gels and

transferred to nitrocellulose/polyvinylidene difluoride membranes (Millipore, Bedford, MA) in a Bio-Rad Trans-Blot system. The membranes were blocked in TBST containing 5% powdered non-fat dry milk for 1 h at room temperature and then probed with primary antibodies, followed by incubation with the corresponding secondary antibodies. Antibodies against VE-cadherin,  $\alpha$ -SMA, vimentin, fibronectin, p-SMAD1/5/SMAD1/5 and BMPR2 were obtained from Abcam (Cambridge, MA).  $\beta$ -actin, snail, twist antibody was obtained from Proteintech (Proteintech Group, CHI, USA). Protein signal was visualized by a WesternBright ECL (Advansta, CA). The band intensity was measured by Quantity One software (Bio-Rad, USA).

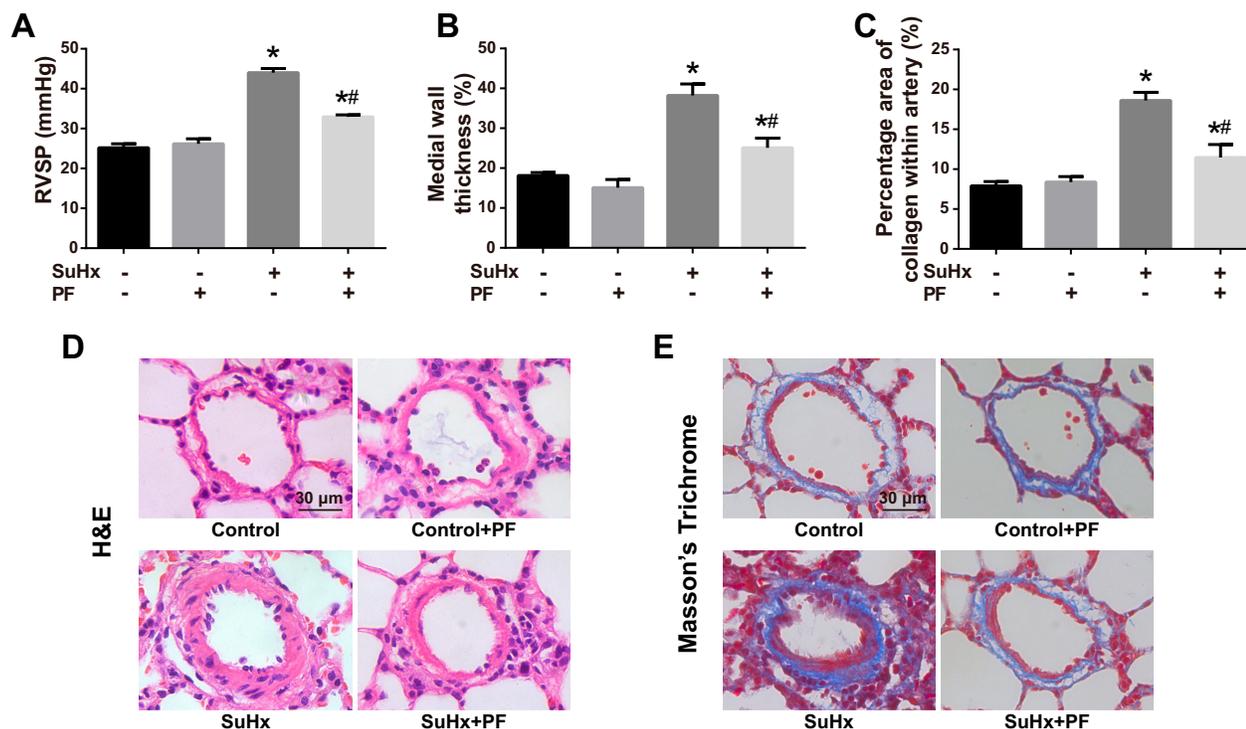
## Statistical Analysis

All data were presented as the mean  $\pm$  standard error of the mean (SEM). One-way or two-way ANOVA followed by Fisher's LSD post-hoc test was used to compare the data of multiple groups.  $P < 0.05$  was considered statistically significant.

## Results

### Effects of PF on RVSP and Pulmonary Vascular Remodeling in the SuHx Rat Model of PAH

Hemodynamic data showed that rats in the SuHx group developed pulmonary hypertension as indicated by a significant increase in right ventricular systolic pressure (RVSP) on the 21th day, which was prevented by PF (Figure 2A). Pulmonary vascular remodeling is a key pathological feature of pulmonary hypertension, which is characterized by the thickening of all three layers of the blood vessel wall and exaggerated collagen deposition and perivascular infiltration of inflammatory cells. As is shown in Figure 2B–E, treated with PF (300 mg/kg) significantly decreased the thickness of pulmonary artery medial wall and the degree of collagen deposition in the distal pulmonary arteries induced by SU5416/hypoxia. Together, these results demonstrate that PF could prevent the development of experimental PAH and inhibited pulmonary vascular remodeling.



**Figure 2** Effects of PF on hemodynamic parameter and pulmonary vascular remodeling in the SU5416/hypoxia (SuHx) rat model of pulmonary arterial hypertension (PAH). (A) Assessment of the right ventricular systolic pressure (RVSP) in rats ( $n = 6$ ). Lung sections were stained with hematoxylin-eosin (H&E) solution for histopathological evaluation or with Masson's trichrome for collagen assessment. (B) The degree of pulmonary vascular remodeling was evaluated by the pulmonary arterial medial wall thickness ( $n = 6$ ). (C) The degree of pulmonary vascular adventitial fibrosis was evaluated by the percentage of the collagen-positive area ( $n = 6$ ). (D) Representative images of H&E staining in lung sections. (E) Representative images of Masson's trichrome staining in lung sections. Data were indicated as the mean  $\pm$  SEM. \* $P < 0.05$  vs untreated control; # $P < 0.05$  vs untreated SuHx group.

## Effect of PF on Right Ventricular Hypertrophy and Fibrosis in the SuHx Rat Model of PAH

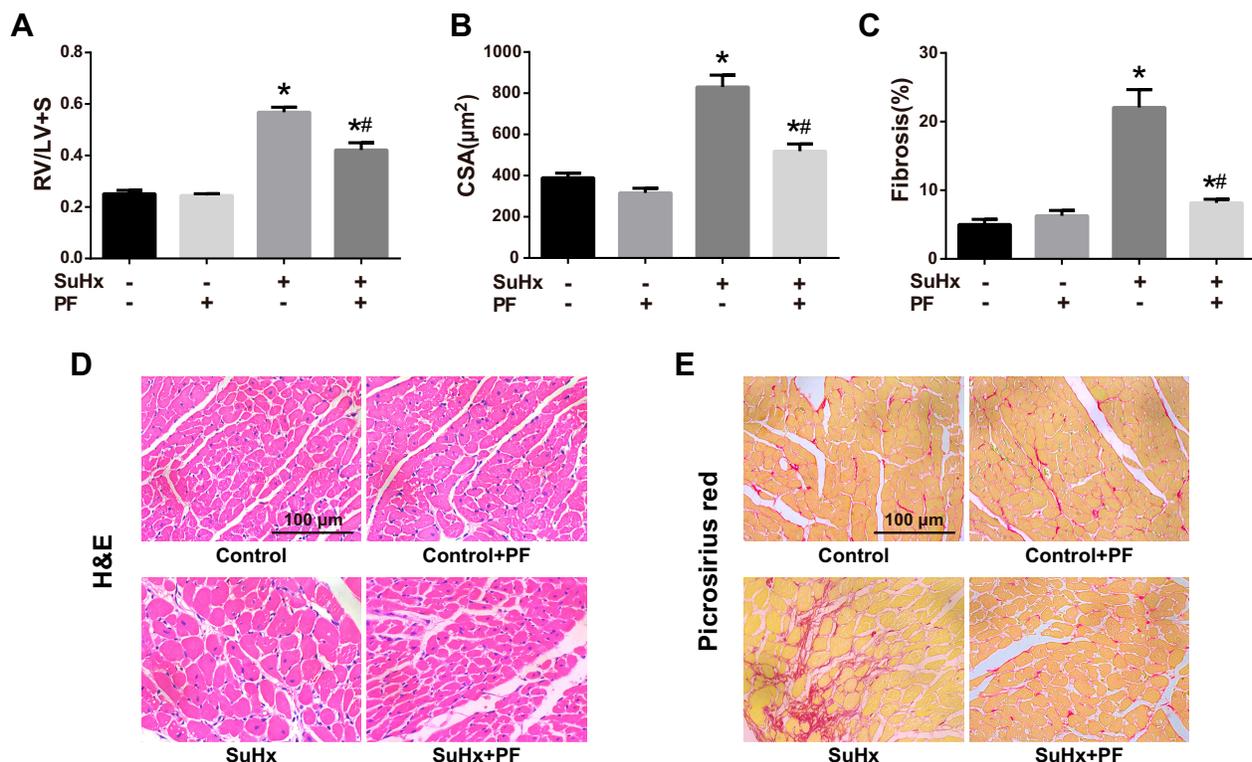
We next evaluate the efficacy of PF on right ventricular changes in experimental PAH in vivo. As shown in Figure 3A, RV/LV+S in the SuHx group showed a significant elevation compared to the control groups, which was alleviated by PF treatment. CSA of RV cardiomyocytes was calculated to evaluate the effect of PF on RV remodeling. Statistical analyses indicated that PF effectively attenuated SU5416/hypoxia-induced increment in CSA (Figure 3B). Representative images of H&E staining are shown in Figure 3D. Similar changes were observed in the interstitial RV fibrosis. As revealed by picrosirius red staining (Figure 3C and E), a significant elevation of interstitial fibrosis was observed in the right ventricle of SuHx rats, which was markedly decreased by the treatment of PF. Thus, the above observations suggest that PF attenuated right ventricular hypertrophy and fibrosis in the SuHx model in rats.

## Paeoniflorin Decreased EndMT in Lung in the SuHx Rat Model of PAH

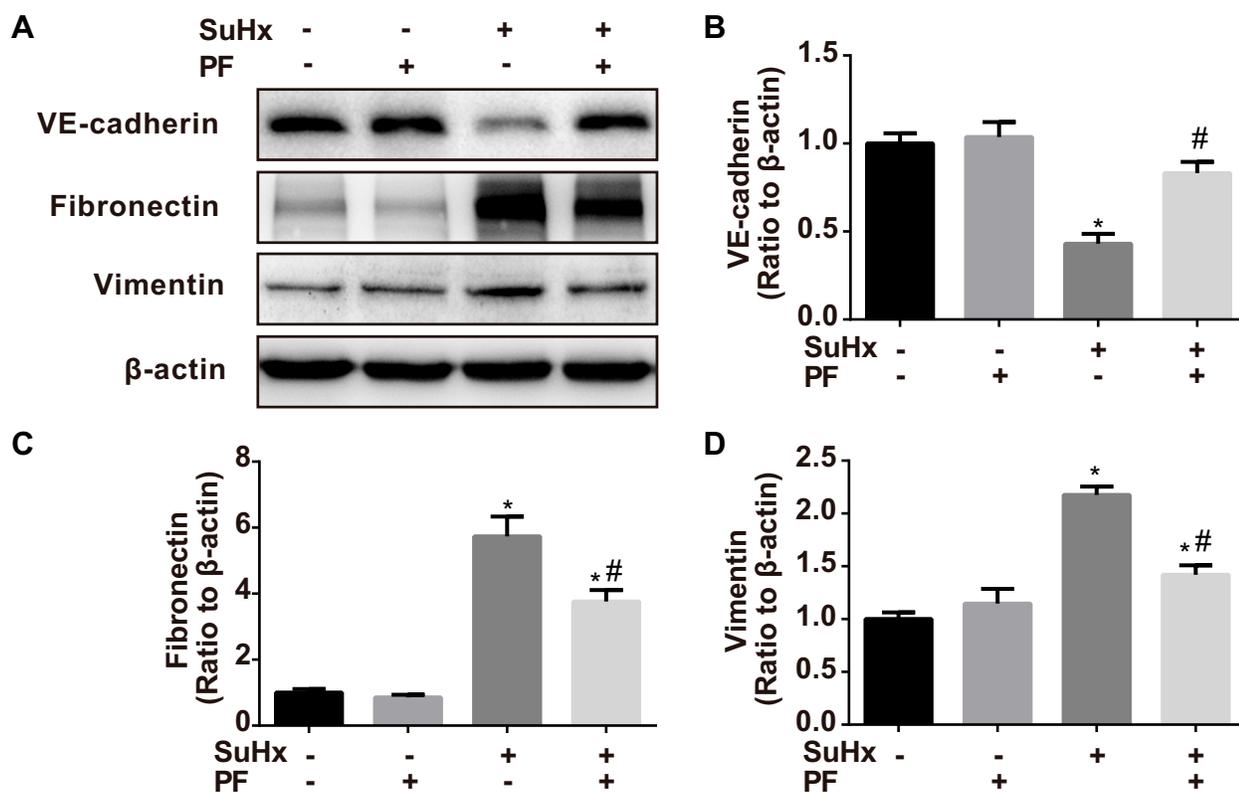
EndMT is a critical process in the progression of pulmonary hypertension and pulmonary vascular remodeling. The expression of EndMT-related markers was measured to evaluate the effects of PF on EndMT in vivo (Figure 4A). Our data indicated that SU5416/hypoxia treatment significantly decreased the expression of the endothelial marker VE-cadherin and increased the expression of mesenchymal markers including fibronectin and vimentin. Pretreated with PF restored the down-regulation of VE-cadherin and partially inhibited up-regulation of the mesenchymal markers induced by SU5416/hypoxia (Figure 4B–D). These results suggest that PF could inhibit EndMT in the SuHx rat model of PAH.

## Hypoxia Induced EndMT in HPAECs

To induce the EndMT of HPAECs in vitro, cells were exposed to hypoxia for 0 h, 6 h, 12 h, 24 h, 48 h, and 72 h, respectively. As shown in Figure 5A, hypoxia induced



**Figure 3** Effect of PF on right ventricular hypertrophy and fibrosis in the SuHx rat model of PAH. (A) Assessment of right ventricular hypertrophy (the ratio of right ventricular (RV) weight to left ventricular plus septal weight in rats ( $n = 6$ )). RV remodeling was demonstrated by H&E staining, and RV fibrosis was demonstrated by picrosirius red staining. (B) RV remodeling was evaluated by cross-sectional area (CSA) of RV cardiomyocytes ( $n = 6$ ). (C) Interstitial RV fibrosis was evaluated by the percentage of the collagen-positive area ( $n = 6$ ). (D) Representative images of H&E staining in RV sections. (E) Representative images of picrosirius red staining in RV sections. Data were indicated as the mean  $\pm$  SEM. \* $P < 0.05$  vs untreated control; # $P < 0.05$  vs untreated SuHx group.



**Figure 4** Effect of PF on endothelial-to-mesenchymal transition (EndMT) in the SuHx rat model of PAH. (A) Representative Western blots of VE-cadherin, fibronectin, and vimentin were shown. (B–D) Quantitative analysis of VE-cadherin, fibronectin, and vimentin was shown. Data were indicated as the mean  $\pm$ SEM.  $n = 3$ –4 independent experiments, respectively. \* $P < 0.05$  vs untreated control; # $P < 0.05$  vs untreated SuHx group.

a gradually upregulation of mesenchymal markers ( $\alpha$ -SMA, fibronectin and vimentin) and EndMT-related transcription factor (snail and twist), but a downregulation of the endothelial marker (VE-cadherin), indicating hypoxia induced EndMT in HPAECs in a time-dependent manner. To validate this result, the expressions of EndMT-related markers were determined by immunofluorescence staining. After hypoxia for 48 h, the expression of vimentin was raised in the cytoplasm and the expression of VE-cadherin was decreased on cellular membrane compared to normoxia (Figure 5B). Taken together, these data demonstrated that hypoxia induced EndMT in HPAECs.

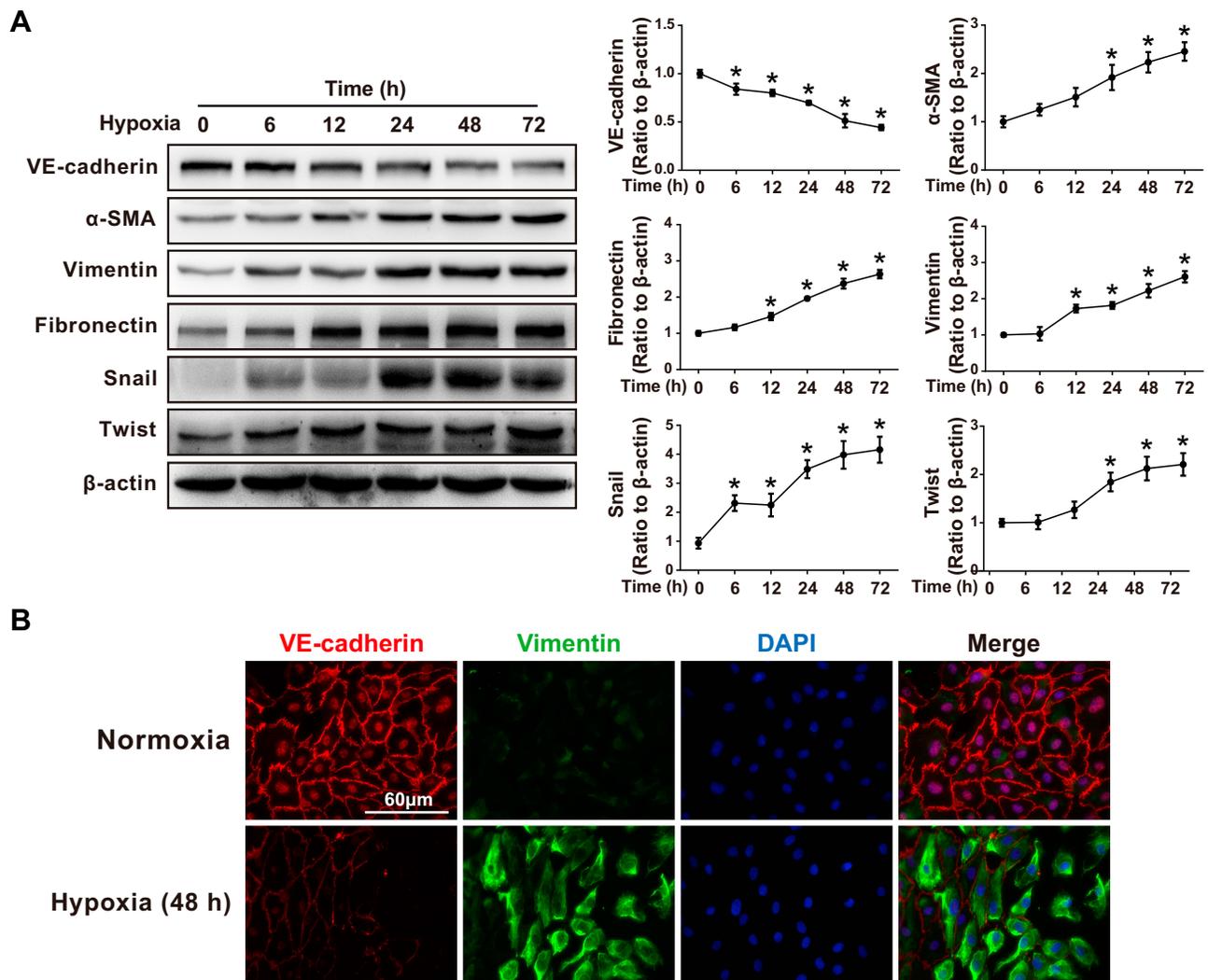
### PF Prevented Hypoxia-Induced EndMT in HPAECs

Western blotting analysis showed that 10  $\mu$ M PF reversed hypoxia-induced down-regulation of endothelial marker (VE-cadherin), and partially inhibited the up-regulation of mesenchymal markers (vimentin and fibronectin) (Figure 6A). Further immunofluorescence staining confirmed that PF inhibited hypoxia-induced loss of VE-

cadherin on the cellular membrane and over-expression of vimentin in the cytoplasm in HPAECs (Figure 6B).

### PF Inhibited Hypoxia-Induced EndMT in HPAECs by BMPR2 Up-Regulation

BMPR2 signaling dysfunction is known as one of the key contributors to EndMT in PAH. Our results showed that hypoxia significantly reduced the expression of BMPR2 in HPAECs, which could be partially restored by PF treatment (Figure 7A). In order to investigate the downstream effectors of the BMPR2 signaling pathways to support the hypothesis that the effects of paeoniflorin are partially mediated by BMPR2, we evaluated the phosphorylation levels of SMAD1/5, a downstream signal of BMPR2. Western blotting results showed that hypoxia significantly inhibited the phosphorylation level of SMAD1/5 in HPAECs, while PF treatment partially reversed the decreased phosphorylation levels of SMAD1/5 (Figure 7B). To further explore whether PF could attenuate hypoxia-induced EndMT via BMPR2 upregulation, HPAECs were transfected with BMPR2-siRNA or NC siRNA. The expression of the BMPR2 was



**Figure 5** Hypoxia induce EndMT in HPAECs. HPAECs were exposed to hypoxia for 0 h, 6 h, 12 h, 24 h, 48 h, and 72 h, respectively. The expression of EndMT markers was evaluated by Western blotting and immunofluorescence staining. **(A)** Representative Western blots and quantitative analysis of VE-cadherin,  $\alpha$ -SMA, vimentin, fibronectin, snail and twist were shown.  $n = 3, 4, 3, 3, 5, 3$  independent experiments, respectively. **(B)** Double immunofluorescence staining of HPAECs for VE-cadherin (red) and vimentin (green) was performed ( $n = 3$ ). \* $P < 0.05$  vs normoxia.

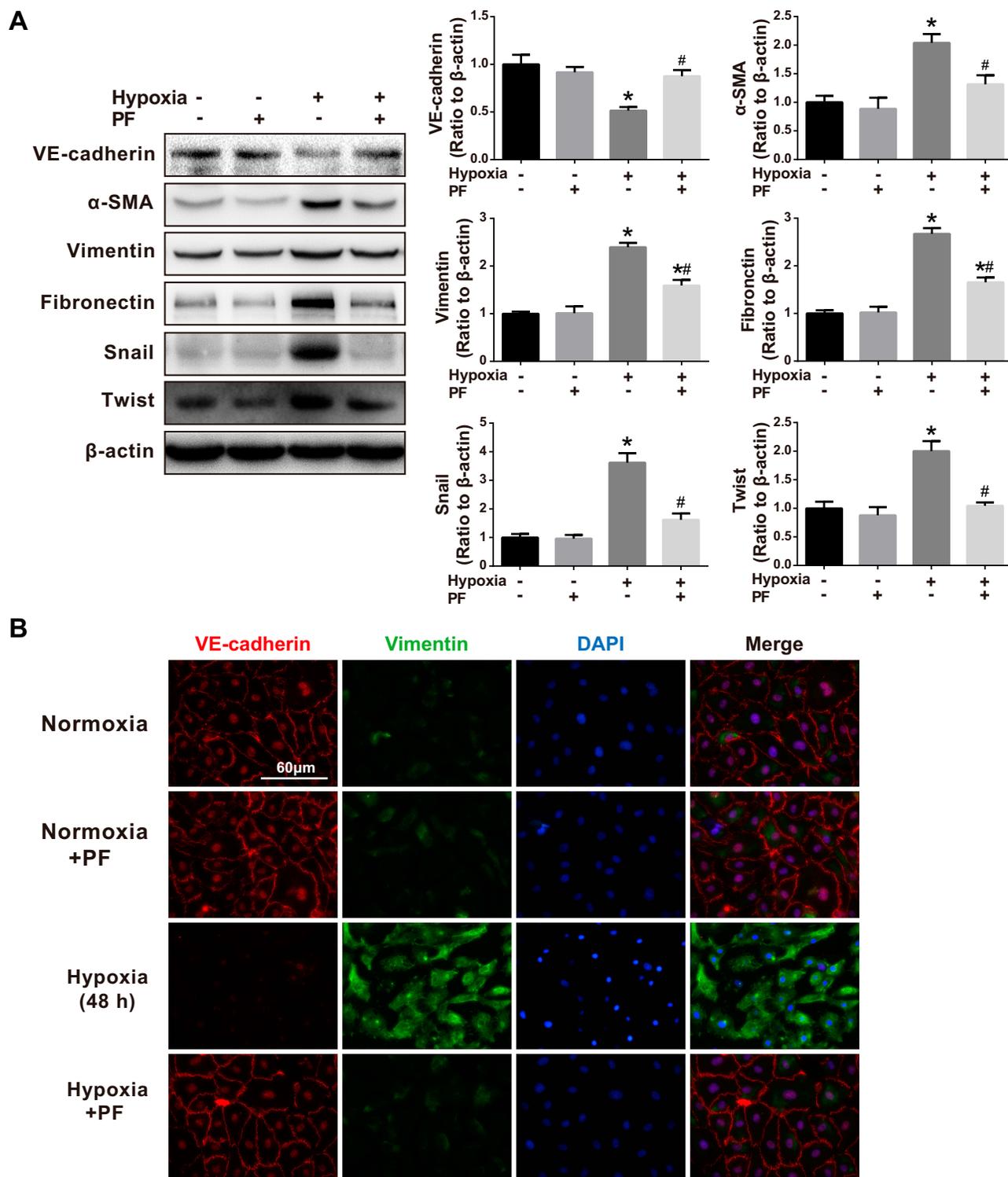
significantly decreased after HPAECs transfected with BMPR2 siRNA for 24 h (Figure 7C). As shown in Figure 7D, BMPR2 knockdown by siRNA reduced the inhibitory effect of PF on hypoxia-induced EndMT. Collectively, these data suggest that BMPR2 is involved in the inhibitory effects of PF in hypoxia-induced EndMT in HPAECs.

## Discussion

In the current study, we investigated the effects of PF on the development of pulmonary hypertension induced by SU5416 and hypoxia in rats. PF effectively attenuates SU5416/hypoxia-induced PAH, pulmonary vascular remodeling, right ventricular hypertrophy and fibrosis in rats. Besides, we found that PF attenuates EndMT in lung in the

SuHx rat model of PAH. Furthermore, our data suggest that BMPR2 is involved in the inhibitory effects of PF in hypoxia-induced EndMT of cultured HPAECs.

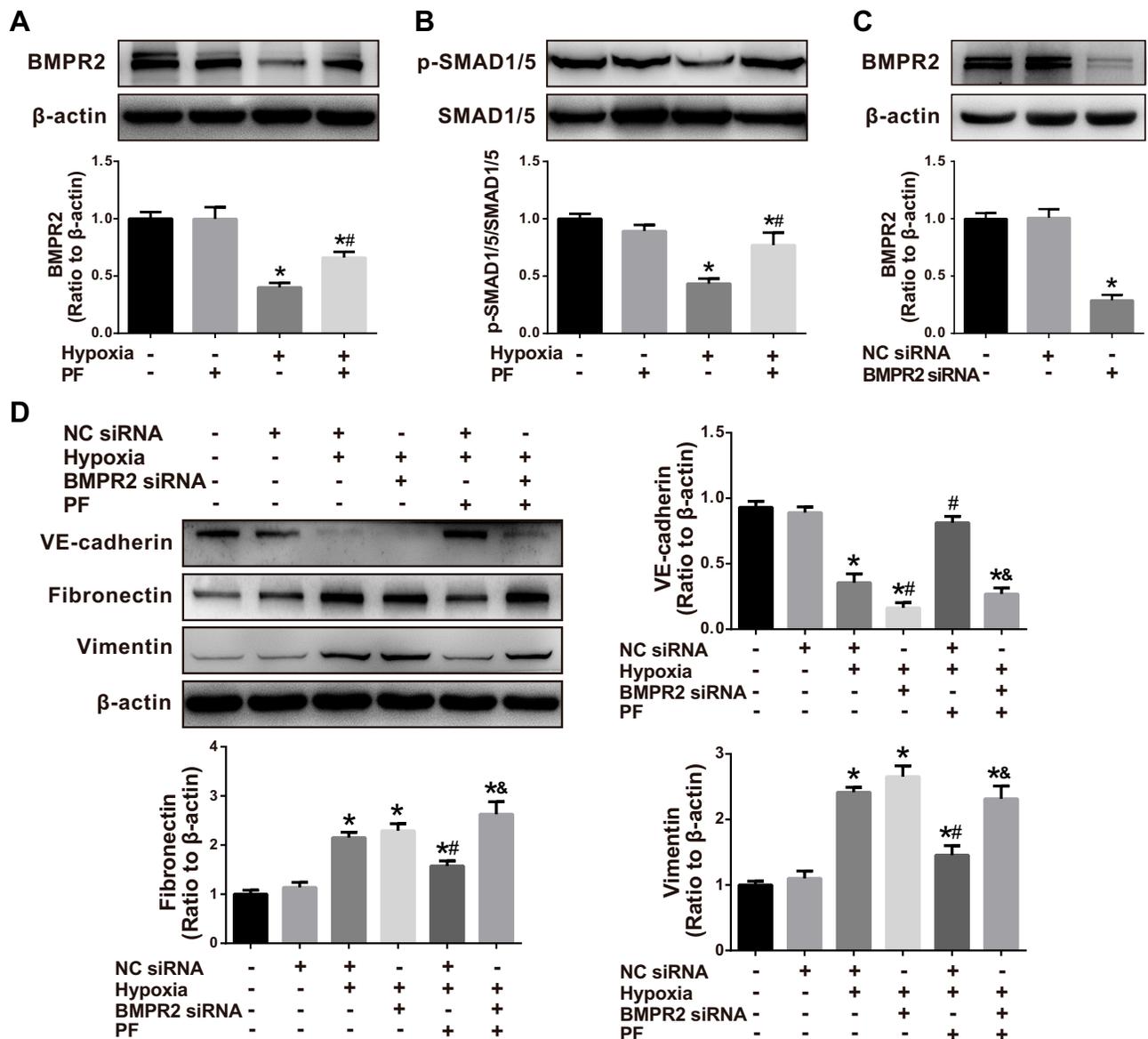
Generally, three animal models have been popular for preclinical testing of new PAH pharmacotherapies: the monocrotaline lung injury model, the chronic hypoxia-induced pulmonary hypertension model and the SuHx model, which leads to severe PAH and closely mimics the vascular changes seen in patients with severe PAH and is now being extensively used.<sup>18</sup> Our data corroborated with previous findings that we observed a significant increase in RVSP, accompanied by pulmonary vascular remodeling and right ventricular hypertrophy in the SuHx rat model. Paeoniflorin, a monoterpene glycoside, has beneficial effects



**Figure 6** Effect of PF on hypoxia-induced EndMT in HPAECs. HPAECs were cultured under normoxia or hypoxia with or without paeoniflorin (10  $\mu$ M) treatment for 48 h. The expression of EndMT markers was evaluated by Western blotting and immunofluorescence staining. **(A)** Representative Western blots and quantitative analysis of VE-cadherin,  $\alpha$ -SMA, vimentin, fibronectin, snail and twist were shown. n = 3, 4, 5, 3, 5, 3 independent experiments, respectively. **(B)** Immunofluorescence staining for VE-cadherin and vimentin was detected (n = 3). \*P < 0.05 vs normoxia; #P < 0.05 vs hypoxia.

on diverse diseases such as rheumatoid arthritis, liver fibrosis, chronic heart failure, and asthma.<sup>19–22</sup> However, its efficacy against the development and progression of PAH has

never been tested. In the present study, we found that paeoniflorin significantly ameliorated SU5416/hypoxia-induced PAH and reduced pulmonary vascular remodeling and right



**Figure 7** BMPR2 is critical for the anti-EndMT of PF in HPAECs. **(A)** HPAECs were exposed to hypoxia (1% O<sub>2</sub>) with or without PF treatment, and representative Western blots and quantitative analysis of BMPR2 were shown (n = 3). **(B)** Representative Western blots and quantitative analysis of p-SMAD1/5/SMAD1/5 were shown (n = 4). **(C)** HPAECs were transfected by negative control (NC) siRNA or BMPR2 siRNA and representative Western blots and quantitative analysis of BMPR2 were shown (n = 3). **(D)** HPAECs transfected with BMPR2 siRNA or NC siRNA for 24 h were cultured under normoxia or hypoxia with or without paeoniflorin for 48 h. Representative Western blots and quantitative analysis of BMPR2, VE-cadherin, fibronectin, and vimentin were shown. n = 3, 4, 3 independent experiments, respectively. Data were indicated as the mean  $\pm$  SEM. \*P < 0.05 vs normoxia; #P < 0.05 vs hypoxia; &P < 0.05 vs hypoxia + NC siRNA + PF.

ventricular hypertrophy. A growing body of data from patients with idiopathic pulmonary arterial hypertension suggests that RV fibrosis and pulmonary interstitial fibrosis impair cardiac function, and plays a crucial role in the development of pulmonary hypertension and RV failure.<sup>23</sup> PF have also been reported to attenuate fluctuant hyperglycemia-induced vascular endothelial injuries, and to improve bleomycin-induced pulmonary fibrosis in rats by suppressing collagen synthesis.<sup>8,24</sup> Besides, the anti-remodeling effect of PF on cardiovascular diseases, including acute myocardial

infarction and pressure overload-induced cardiac remodeling, might also contribute to the beneficial effect of PF on PH.<sup>21,25</sup> In the present study, we also found a marked attenuation of the collagen deposition in the pulmonary arterial and RV heart tissue in the PF-treated SuHx rats compared to vehicle-treated SuHx rats. Thus, PF significantly inhibited cardiopulmonary fibrosis, which may represent a novel therapeutic agent for the treatment of PAH.

Vascular cell phenotype switching such as EndMT plays an important role in abnormal pulmonary vascular

remodeling and tissue fibrosis in PAH and it has been reported that 5% of ECs express both endothelial and mesenchymal phenotypes in SU5416/hypoxia-induced PAH.<sup>26,27</sup> Generally, EndMT is characterized by the acquisition of mesenchymal phenotype while the loss of endothelial cell markers in endothelium. EndMT is one source of matrix-generating fibroblasts in pulmonary hypertension, leading to extracellular matrix production and collagen deposition, which contributes to pulmonary vascular remodeling. Previous studies have indicated that PF protected against TGF $\beta$ 1-induced EMT in pulmonary fibrosis.<sup>24</sup> In our study, we found that endothelial marker was down-regulated and mesenchymal markers were significantly up-regulated in SuHx rat lungs. However, these changes were partially reversed by the treatment of PF. Thus, the present results suggest that PF inhibits EndMT in the SuHx rat model of PAH. This result was further confirmed in cultured HPAECs *in vitro*. Notably, hypoxia induced upregulation of mesenchymal markers ( $\alpha$ -SMA, fibronectin and vimentin) and EndMT-related transcription factor (Snail and Twist), but downregulation of the endothelial marker (VE-cadherin) in HPAECs in a time-dependent manner, which was inhibited by PF. Thus, our data from either *in vivo* or *in vitro* studies suggest that PF could suppress EndMT in lung, which subsequently inhibited the development of PAH.

BMPR2 and its related signaling pathway have been recognized as a key regulator of pulmonary vascular homeostasis, as an estimated 80% of familial and 20% of idiopathic PAH patients carrying a heterozygous BMPR2 mutation.<sup>28,29</sup> BMPR2 gene mutations have also been found in patients with pulmonary hypertension caused by other factors (congenital heart disease, appetite suppressant drugs, pulmonary vein occlusion, etc.). Moreover, BMPR2 hypofunction have been shown to contribute to PAH pathogenesis, even though without BMPR2 mutations.<sup>30</sup> A growing number of studies have linked dysfunctional BMPR2 signaling, including BMPR2, p-SMADs and Id1 to the development of EndMT process in PAH, and targeted gene delivery of BMPR2 has a therapeutic effect in the animal models of PAH.<sup>31–33</sup> Moreover, it was reported that TGF- $\beta$ 1 induced EndMT in human pulmonary microvascular ECs is associated with reduced BMPR2 expression, and stimulating BMPR2 signaling partially ameliorated TGF- $\beta$ 1 induced EndMT in ECs.<sup>33</sup> There are multiple causes for the downregulation of BMPR2 signaling, such as RAGE, 17- $\beta$  estradiol.<sup>34,35</sup> Under hypoxic conditions, HIF-1 $\alpha$  induces miR-322, which targets BMPR1a and SMAD5, thereby downregulating BMPR2 signaling.<sup>36,37</sup> There is

strong evidence showing that PF can effectively prevent renal interstitial fibrosis through blocking EMT via BMP-7 recovery.<sup>38</sup> Previous studies also reported that PF attenuated cobalt chloride-induced apoptosis of endothelial cells via HIF-1 $\alpha$  pathway.<sup>39</sup> Our study is consistent with previous reports that hypoxia significantly reduced the expression of BMPR2 and downstream phosphorylation levels of SMAD1/5 in HPAECs, which was partially reversed by PF. Moreover, down-regulating the expression of BMPR2 in HPAECs by siRNA reduced the inhibitory role effect of PF on hypoxia-induced EndMT in HPAECs. Therefore, our results showed that BMPR2 is involved in the inhibitory effects of PF in hypoxia-induced EndMT.

## Conclusion

In summary, our study suggests that PF is a potential therapeutic agent for PAH. Our research results demonstrate that PF alleviates pulmonary vascular remodeling and right ventricular hypertrophy in the SuHx rat model of PAH. The underlying mechanism of PF may involve the inhibition of BMPR2 down-regulation-mediated EndMT. Thus, paeoniflorin might be a promising drug for the treatment of PAH.

## Ethic Approval

The study was approved by the Institutional Animal Care and Use Committee of Nanjing Medical University (NJMU/IACUC-1809008).

## Acknowledgments

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## Author Contributions

All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

## Disclosure

The authors declare that they have no conflicts of interest in this work.

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